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Original Article

Investigating the Efficacy of Mesenchymal Stem Cell Therapy for Neurogenic Lower Urinary Tract Dysfunction in Spinal Cord Injury Models: A Focus on c-Fos and Brain-Derived Neurotrophic Factor Expression

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Abstract

Background: Using mesenchymal stem cells (MSCs) is an emergent strategy in the neuro-urology field. This study aimed to investigate whether intra-detrusor injection of MSCs is a possible therapy for spinal cord injury (SCI)-induced neurogenic lower urinary tract dysfunction (NLUTD) by measuring c-Fos as a proto-oncogene and brain-derived neurotrophic factor (BDNF) in the spinal cord and bladder tissue of SCI rats.

Methods: To induce SCI, rats were subjected to T9–10 laminectomy and complete transection of the spinal cord with a surgically sharp blade for a complete SCI model. For hemisection SCI in hemisection SCI (hSCI) groups, the animals underwent laminectomy at the T9 level of vertebrae and left hemisection of the spinal cord, respectively. A total of 42 adult female Wistar rats were divided into 6 groups. The control group received no treatment, while the sham group underwent a surgical procedure without any spinal cord damage. In addition, the complete transection SCI (cSCI) and hSCI groups underwent complete spinal cord transection or hemisection, respectively, and then received a saline injection into their bladder wall four weeks after the injury. Moreover, the cSCI + MSC and hSCI + MSC groups received an injection of bone marrow MSCs (BM-MSCs) into their bladder wall four weeks after the injury.

Results: Endogenous BDNF levels were decreased in the bladder tissue after the induction of hSCI and cSCI compared to the sham and control groups ($P<0.05$). A slight but non-significant increase in the level of BDNF was observed in detrusor muscles. Furthermore, significant changes in the level of c-Fos were detected in the urinary bladder and spinal cord tissues pre-and-post BM-MSC ($P<0.05$). Further, c-Fos decreased in the detrusor muscle in the bladder tissue after BM-MSC administration either in the hSCI or cSCI group ($P<0.05$). A prominent decrease in c-Fos levels was also observed in hSCI rats compared to the cSCI group in spinal and urinary bladder tissues after BM-MSC injection.

Conclusion: The transplantation of BM-MSCs suppressed c-Fos expression but did not change endogenous BDNF levels in the spinal cord. Using stem cell therapy to treat bladder dysfunction is a promising approach.

Keywords: Mesenchymal stem cells, Intra-detrusor, Spinal cord injury, Neurogenic bladder, Rat

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Introduction

Stem cell (SC) therapy has shown promising therapeutic results in regenerative medicine, especially in the restoration of neuro-urology disorders. The beneficial effects of SCs are related to enhanced proliferation rate, angiogenesis induction, immunomodulatory and anti-inflammatory effects, inhibition of fibrotic changes, differentiation into target cells, and secretion of multiple

growth factors and cytokines.[1] Studies in the field of SC therapy for the alleviation of bladder dysfunction have mainly focused on the transplantation of SCs via the systemic and/or local routes. Despite the advantages associated with the use of SCs for neuro-urology disorders, some issues and pitfalls exist in the realm of urinary bladder regeneration. For instance, in addition to ethical issues, cell administration has the potential to induce teratoma and provoke the recipient's immune system activity.[2,3] In the last decades, mesenchymal stem cells (MSCs) have been widely used in different organ injuries due to easy isolation and expansion in culture media.[4-6] Notably, statistics confirm that MSCs are the main cell source applicable to cell-based therapies in many clinical and preclinical settings.[7-15] Paracrine and juxtacrine mechanisms[16-19] are orchestrated to be the therapeutic effects of MSCs. In line with this claim, bone marrow MSCs (BM-MSCs) are at the center of attention largely due to their potent differentiation capacities into a variety of cell types.[20] Recent studies have investigated the ability of BM-MSCs to treat a number of functional voiding pathologies, including bladder outlet obstruction, stress urinary incontinence, and neurogenic lower urinary tract dysfunction (NLUTD).[21] Despite the promising outcomes of MSCs in the management of disorders, such as NLUTD as a major medical problem that has social implications due to quality of life issues, unknown issues remain about the suitable source(s) and route(s) of administration for a particular disease, as well as possible contraindications in their clinical use. Moreover, less attention has been directed to preclinical spinal cord injury (SCI) studies, especially following SC therapy,[22,23] and this application has yet to prove therapeutic effectiveness in the preclinical and long-term clinical settings.[24] Recent studies have explored bladder reconstruction using scaffold-based approaches and the neuro-regenerative capabilities of SCs to facilitate healing after transplantation following SCI. Adipose-derived MSC sheets have been used in an SCI rat model, significantly improving voiding function recovery. Human umbilical cord mesenchymal SCs have also been utilized for bladder repair after acute SCI, contributing to bladder function reconstruction and partially restoring motor function by ameliorating destructive lesions. Neurogenic detrusor overactivity (NDO) frequently arises following SCI, particularly after the spinal shock phase. Neurotrophins, including the nerve growth factor and brain-derived neurotrophic factor (BDNF), are essential for regulating bladder function. According to prior research, BDNF significantly influences both the onset and persistence of NDO. Specifically, BDNF levels in the spinal cord increase over time as NDO develops, and prolonged BDNF sequestration has been shown to enhance bladder function in chronic SCI models. Moreover, sustained BDNF treatment has been linked to the inhibition of NDO onset and a reduction in axonal growth, indicating its pivotal role in post-SCI bladder functionality. Additionally, c-Fos, a proto-oncogene associated with Fos-protein expression in the central nervous system, has been observed to elevate the number and distribution of Fos-immunoreactive cells in specific areas of the L6-S1 spinal cord. While previous studies have suggested the potential for the intrathecal administration of SCs to repair central nervous system structures, there is limited documentation regarding the effectiveness of local MSC administration in restoring neurogenic bladder dysfunction following SCI.[13,25]

Therefore, our research seeks to fill this gap by examining the effects of BM-MSC transplantation in the context of NDO recovery after SCI.

Materials and Methods

Study Design and Spinal Cord Injury Induction

Overall, 42 female Wistar rats (13-week-old and weighing 220–260 g) were randomly divided into six groups (7 in each) as the control, sham surgery (sham), complete transection SCI (cSCI), hemisection SCI (hSCI), cSCI + BM-MSC, and hSCI + BM-MSC groups. Figure 1 represents the histology of the spinal cord in different study groups.

The control rats did not receive any interventions, while sham rats underwent T9-10 laminectomy without spinal cord damage.

The SCI model was induced according to our previous method. For this purpose, rats from the cCSI group were subjected to T9–10 laminectomy and complete transection of the spinal cord with a surgical sharp blade. In hSCI groups, rats underwent laminectomy at the T9 level of vertebrae and left hemisection of the spinal cord. After SCI induction, the incised layers were sutured by using 3–0 catgut sutures. Then, the urinary bladder was gently compressed to empty contents. All animals were intraperitoneally injected with 3 mL of normal saline just after surgery. To prevent post-operation infection and alleviate neuropathic and postoperative pains, rats received ciprofloxacin (1 mg/kg/intraperitoneally, for 3 days) and acetaminophen syrup through the gavage route (10 mg/kg for 3 days), respectively.[13]

For emptying the bladder, in all spinal cord-injured rats, the manual pressure of the bladder was performed twice daily, and in the non-response cases, the PE10 catheter was used to evacuate the bladder under anesthesia.

Bone Marrow Mesenchymal Stem Cell Isolation and Labeling

To track cells in *in vivo* conditions, rat BM-MSCs were isolated from tibia and femur bones, cultured in Dulbecco's modified Eagle medium with low glucose containing 10% fetal bovine serum and 1–2% Pen-Strep (100 IU/mL penicillin/100 IU/mL streptomycin), and used at the 3rd-5th passage.[26-28] To label MSCs, they were incubated with Dil307 (20 μM of 1,1'-dioctadecyl-tetramethylindocarbocyanine perchlorate; Invitrogen®) for 20–30 minutes at 37 ˚C.

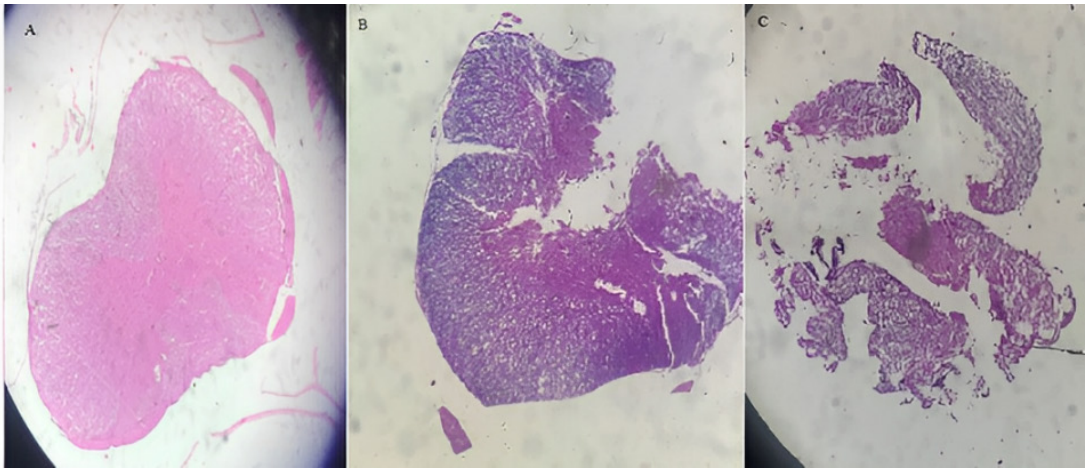

Figure 1. Histology of Spinal Cord in the **(A)** Control Group, **(B)** hSCI, and **(C)** cSCI. *Note*. hSCI: Hemisection spinal cord injury; cSCI: Complete transection spinal cord injury

Bone Marrow Mesenchymal Stem Cell Immunophenotyping by Flow Cytometry Analysis

To characterize rat BM-MSCs, a panel of antibodies was used against CD133, CD44, CD34, and CD45. All antibodies were purchased from eBioscience and utilized according to our previously published protocols.[26] In brief, expanded BM-MSCs were trypsinized using a 0.25% trypsin-ethylenediaminetetraacetic acid solution (Gibco) and incubated with 1% bovine serum albumin (Sigma-Aldrich) to decrease non-specific binding. Then, 100 µL phosphate-buffered saline (PBS) containing 1×10^6 cells was incubated with the recommended doses of antibodies at 4 ˚C for 30 minutes. Appropriate isotype control antibodies were used to exclude background staining. After washing three times with PBS, the cells were analyzed by the BD FACSCalibur system and FlowJo software. This assay was performed in triplicate, and the cells were analyzed at the third passage by flow cytometry.

Viability of Bone Marrow Mesenchymal Stem Cells

Vital dye Trypan blue was utilized to assess cell viability prior to injection as described previously.[13] The number of live cells was counted both before and after transferring into the injection setup to ensure the survival of the cells inside the syringe. The obtained data revealed that nearly 99% were viable.

Bone Marrow-Derived Mesenchymal Stem Cell Transplantation

After four weeks, the rats were candidates to receive intra-detrusor injections of normal saline or BM-MSCs. The study involved rats in two groups with cSCI and hSCI that received intra-detrusor wall injections of BM-MSCs (1×10^6/120 µL) using a 500-µL sterile plastic syringe connected to a 26-gauge sterile needle. In the sham group, the same volume of normal saline was injected into the bladder wall. All injections were performed in six different sites of the bladder wall, confirming the true technique of injection.

In our study, MSC injections were administered at six distinct anatomical sites within the bladder wall, specifically targeting the bladder dome. These sites were selected based on several key considerations that enhance the efficacy of the treatment. By administering injections at six different sites, it was intended to achieve a more uniform distribution of MSCs throughout the bladder wall. This approach minimizes the risk of localized over-concentration while maximizing the overall therapeutic effect. The appearance of a bulge at each injection site served as a visual confirmation of successful injection into the bladder wall. This indicates that the MSCs were delivered to the intended location, ensuring that they are positioned optimally for interaction with the surrounding tissue.

Histological and Molecular Analyses

This study involved rats sacrificed using ketamine and xylazine, and urinary bladder tissues were collected and stored at -80 ˚C. Immunofluorescence staining was performed on samples, which were cut into 5 µm-thick sections using cryostat sectioning. Dil-labeled MSCs were studied in each section, and 4′,6-diamidino-2-phenylindole was used for nuclear staining. The samples were then cut into sections for further analysis.

Detection of c-Fos and Brain-Derived Neurotrophic Factor

Western blotting was employed to assess the levels of c-Fos and BDNF proteins in both urinary bladder and spinal cord tissues. Tissue samples were lysed and homogenized in 100 µL of radio immunoprecipitation assay lysis buffer, which comprised 150 mM of sodium chloride, 1% of Triton X-100, 0.5% of sodium deoxycholate, 0.1% of sodium dodecyl sulfate, and 50 mM of Tris, along with a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was then centrifuged at 12 000 × g for 15 minutes at 4 ˚C. In addition, 20 µg of protein lysates were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Roche). To block the membranes, 1% bovine serum

albumin (Sigma-Aldrich) was applied for one hour at room temperature. The membranes were incubated overnight with rabbit primary antibodies targeting c-Fos (Cat No. SC-166940; Santa Cruz Biotechnology), BDNF (Cat No. sc-65514; Santa Cruz Biotechnology), and β-actin (Cat No. sc-130657; Santa Cruz Biotechnology) to detect immunoreactive bands. Following three times of washing with PBS, the membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (dilution: 1:5000, Cat No. sc-2004; Santa Cruz Biotechnology) for two hours at room temperature. The membranes were then immersed in enhanced chemiluminescence detection reagents (Amersham, UK) for one minute, air-dried, and exposed to the X-ray film (Kodak, USA). The anti-β-actin antibody (dilution: 1:500, sc-130657; Santa Cruz Biotechnology) served as an internal control.

Statistical Analysis

The obtained data were statistically analyzed by one-way analysis of variance and Tukey post-hoc test using SPSS 21.0 software (SPSS Inc., Chicago, Illinois, USA). The results were expressed as means ± standard deviations (SD), and $P < 0.05$ was considered statistically significant.

Outcome Assessment

In our study, a blinding protocol was implemented during the evaluation of outcomes. Specifically, the assessors who measured the effects of the MSC injections were blinded to the treatment groups, ensuring that their assessments were unbiased and purely based on observed outcomes.

Results

Cell Immuno-phenotyping and Labeling

Based on the data, flow cytometry analysis demonstrated that BM-MSCs, at passage 3, were positively stained with CD133 and CD44 antibodies, coinciding with a high percentage of negative cells toward CD34 and CD45 markers. These features showed BM-MSC purity prior to transplantation (Figure 2A). The incubation of BM-MSCs with Dil stain revealed appropriate cell labeling indicated by a red color appearance (Figure 2B). According to our data, all BM-MSCs were Dil-positive, representing the efficiency of labeling prior to injection into detrusor muscles.

Tracking of Cells at the Site of Injection

Monitoring frozen sections from urinary bladder tissue confirmed the existence of Dil^{+} BM-MSCs in cSCI + BM-MSC and hSCI + BM-MSC rats (Figure 3). These data indicated that the introduction of BM-MSCs into urinary bladder tissue was successful. In addition, the cells were viable after four weeks.

Protein Levels of c-Fos and Brain-Derived Neurotrophic Factor in the Urinary Bladder and Spinal Cord

The protein levels of c-Fos and BDNF in the urinary bladder and spinal cord tissues were monitored in the current experiment. According to our data, endogenous BDNF levels were decreased in bladder tissue after the induction of hSCI and cSCI compared to the sham and control groups ($P < 0.05$, Figure 4A-B). Further, a slight but non-significant increase was found in the level of BDNF in detrusor muscles. Interestingly, BDNF protein levels in all groups were near-to-control levels ($P > 0.05$). In contrast to BDNF changes, significant changes were observed in the level of c-Fos in the urinary bladder and spinal cord tissues pre-and-post BM-MSC ($P < 0.05$, Figure 4C). The data revealed that c-Fos decreased in the detrusor muscle in bladder tissue after BM-MSC administration either in the hSCI or cSCI group ($P < 0.05$, Figure 4D). In addition, a prominent decrease in c-Fos levels was noted in hSCI rats compared to the cSCI group in spinal and urinary bladder tissues after BM-MSC injection. Therefore, it is reasonable to think that cell transplantation into the detrusor muscle could decrease the protein content after hSCI but not cSCI.

Discussion

This study assessed protein levels of c-Fos and BDNF in the urinary bladder and spinal cord tissues using western blotting. The results confirmed that BM-MSCs were successful in introduction into urinary bladder tissue and were viable after four weeks. Endogenous BDNF levels decreased in bladder tissue after the induction of hSCI and cSCI compared to the sham and control groups. However, significant changes in c-Fos levels were observed pre-and-post BM-MSC administration, with a decrease in c-Fos in detrusor muscle and a prominent decrease in hSCI rats.

NLUTD following neurological conditions is accompanied by several morbidities and mortalities.[29,30] The current therapeutics have side effects and lead to incomplete recovery. Hence, new therapeutic methods are necessary.[31] The exact mechanisms underlying BM-MSC-mediated functional improvement are not fully understood yet. Some studies demonstrated functional recovery following BM-MSCs grafting into the injured spinal cord within 2–3 weeks,[32,33] suggesting that neuroprotection rather than regeneration is the underlying mechanism. The objectives of SC therapy in neuro-urological disorders are to repair and replace the damaged neuronal tissue and restore bladder function in the least possible time. Several routes of SC administration were previously described in many experimental or clinical settings.[7-15] Hallmarks of regeneration after SC transplantation include physical discontinuity, axonal growth, reduction of tissue damage and scar formation, and promotion of remyelination.[34-37] Considering the unique SC capacity to differentiate into neurons, oligodendrocytes, and astrocytes, novel clinical SC-based therapies have yet to be identified for central and peripheral nervous system diseases.[38-42]

Apoptosis, a process that takes place in the detrusor muscle, is associated with various voiding disorders. It can be found in both hyperactive and underactive

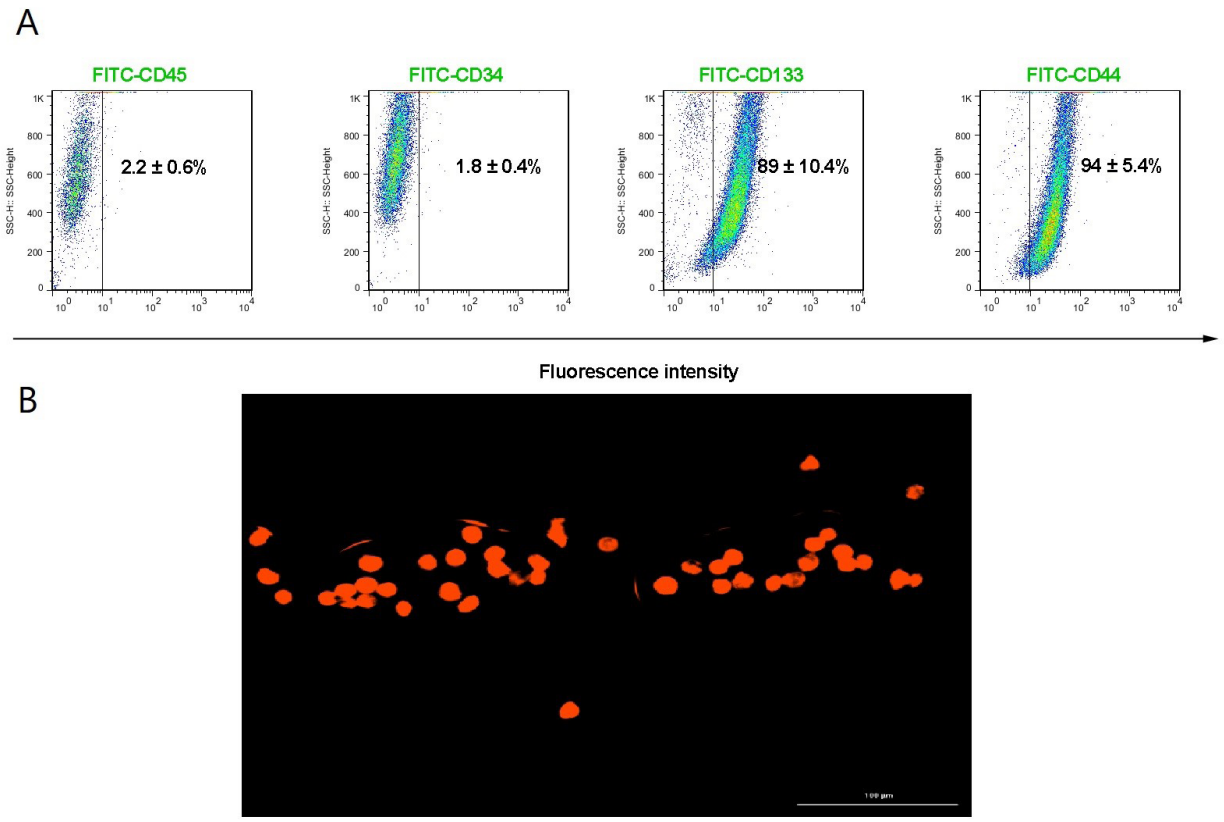

Figure 2. Characterization of Cultured BM-MSCs: **(A)** BM-MSCs Stained With Anti-CD44, CD 45, CD 34, and CD 133 Antibodies and Analyzed by Flow Cytometry and **(B)** Labeled Cells by dil307 (1, 1′-dioctadecyl-*tetramethylindocarbocyanine* Perchlorate, 20 μg/mL Invitrogen®). *Note*. BM-MSC: Bone marrow mesenchymal stem cell

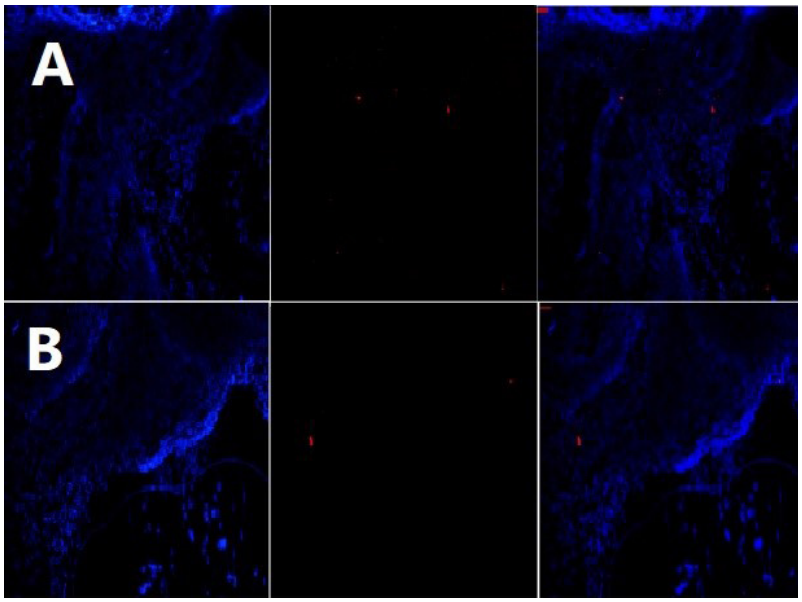

Figure 3. Existence of Dil⁺Cells After Transplantation in Rats Receiving BM-MSCs: **(A)** hSCI + BM-MSC Group and **(B)** cSCI + BM-MSC Group. *Note*. BM-MSC: Bone marrow mesenchymal stem cell; hSCI: Hemisection spinal cord injury; cSCI: Complete transection mesenchymal stem cell

detrusors and may initiate when a voiding issue arises. Detrusor underactivity is linked to a more significant increase in apoptosis compared to heightened DO. The detrusor experiences remodeling in both DO and detrusor underactivity scenarios, which can have either beneficial or detrimental effects. The apoptosis of vascular smooth muscle cells may aid recovery in vessels that have undergone adverse remodeling due to disease or surgical intervention. While apoptosis is typically viewed as a negative factor affecting voiding function, additional research is required to reach conclusive insights.[43,44] It is noteworthy that the induction of SCI initiated apoptosis in the bladder

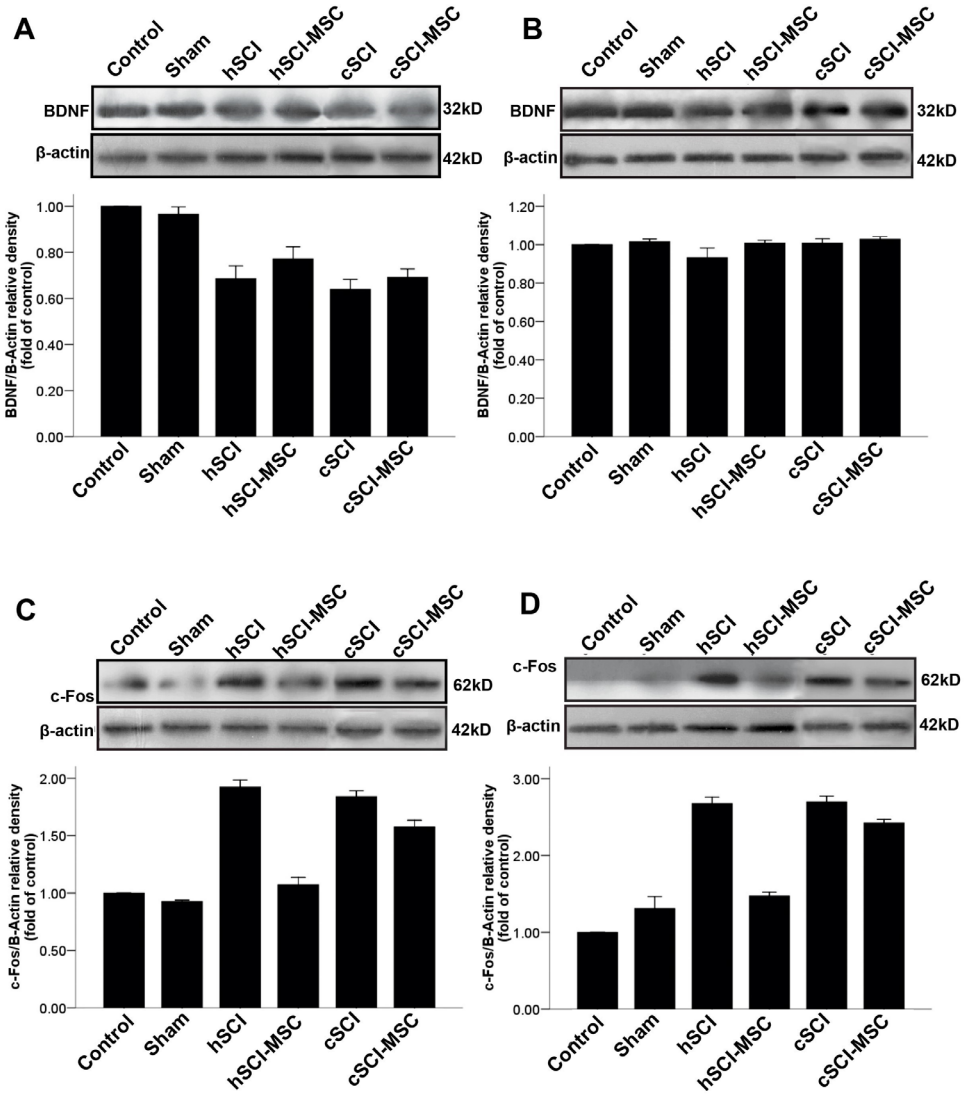

Figure 4. Protein Levels of c-Fos and BDNF: **(A)** Bladder Level of BDNF/B-Actin Relative Density, **(B)** Spinal Cord Level of BDNF/B-Actin Relative Density, **(C)** Bladder Level of c-Fos/B-Actin Relative Density, and **(D)** Spinal Cord Level of c-Fos/B-Actin Relative Density. *Note*. BDNF: Brain-derived neurotrophic factor

tissues, whereas treatment with BM-MSCs suppressed the SCI-induced apoptosis. Based on the same principle, previous studies revealed that DNA fragmentation was increased for hours or days following SCI.[45-47] Extensive research on the mechanism of induction or resistance to apoptosis in the cell was investigated in somatic cells, non-differentiated SCs, and cancer cells.[48] In the study by Cho et al on the contusion model of SCI, the number of TUNEL$^+$cells in the SCI group increased, and treatment significantly decreased these cells, highlighting that apoptosis increased SCI.[49] Therefore, the pivotal function of BM-MSCs in restoring neurogenic bladder function, if not completely, is to decrease the apoptosis rate after cSCI and hSCI. All this information suggests that rat BM-MSCs found in detrusor muscles release active substances and growth factors that may promote neo-angiogenesis and cell proliferation in the bladder through a paracrine mechanism. Neurotrophic factors are crucial for bladder function. Research by Sharma et al indicated that certain growth factors in the central nervous system contribute to neuroprotection. Specifically, the administration of neurotrophins such as BDNF and GDNF can help prevent motor neuron damage. Our findings revealed that levels of BDNF in the bladder decreased following SCI, but the transplantation of MSCs increased these levels, particularly in the hSCI group, although this change was not statistically significant. Namiki et al reported that administering BDNF and NT-3 growth factors improved conditions in the damaged spinal cord and NLUTDNGB. Given the role of GABAergic neurons in mitigating DO induced by SCI in rats, it has been established that GABA receptor agonists can prevent non-voiding contractions and maximum voiding pressure, which are indicators of afferent and efferent inhibition in the urinary pathway.[50,51] The BDNF modifier activity may occur through the property of GABAergic.[52,53] Park et al transplanted human MSCs 9 days after SCI into the lesion site and evaluated the treatment efficacy by measuring the levels of BDNF and NT-3. However, they found no statistically significant differences in these protein levels in the spinal

cord, lumbar, and bladder tissues in comparison to the control group.[32] With regard to our results, BM-MSCs' potential to release trophic factors might play a pivotal role in the promotion of host tissue survival. In line with these statements, future experiments are essential to provide answers related to the discovery of the amount and duration of growth factor production by BM-MSCs in the urinary bladder niche. After intra-detrusor wall injection, our results demonstrated that endogenous BDNF levels in the spinal cord were unchanged. BM-MSC transplantation increased the reduced levels of BDNF in bladder tissue after SCI.

The c-Fos transcription factor, which is produced by the immediate early *c-Fos* genes, functions as a proto-oncogene that encodes the Fos protein in the central nervous system. It is recognized as a marker for the activation of postsynaptic spinal cord neurons associated with the lower urinary tract, including the bladder, urethra, and perineum. In the spinal cord, c-Fos expression serves as a neurotoxic biomarker and has been observed in dorsal spinal neurons following nociceptive stimulation and repeated swim stress. The stimulation of the bladder leads to an increase in c-Fos-immuno-reactive neurons in the periaqueductal gray, primary motor cortex, and spinal cord. After SCI, there is heightened neuronal activity in urinary neuronal systems. The rise in c-Fos expression in the injured bladder represents neuronal activation. In this study, SCI resulted in increased c-Fos expression in both the bladder and spinal cord, while treatment with SCs decreased this expression in both areas. These findings indicate that in addition to inhibiting apoptosis, another mechanism at play is the reduction of neurotoxic biomarkers in the spinal cord following the local administration of BM-MSCs into the detrusor wall.[25,49,54-56] In this study, SCI increased the expression of c-Fos in the bladder and spinal cord, and treatment with SCs reduced its expression in both organs. These data suggested another mechanism rather than apoptosis inhibition, which was the reduction of neurotoxic biomarkers in the spinal cord after the local administration of BM-MSCs into the intra-detrusor wall.

Proliferation is a crucial biological process that significantly contributes to tissue growth and the maintenance of homeostasis. Cho et al found that the administration of SCs enhanced Ki-67 expression, suggesting the effective proliferation of oral mucosal SCs when transplanted into the injured spinal cord of rats with SCI. They confirmed that Ki-67 expression decreased after SCI. The transplantation of BM-MSCs resulted in increased Ki-67 levels in bladder tissue, indicating enhanced cell proliferation. Research has demonstrated that BM-MSCs can readily differentiate into smooth muscle cells, neural cells, and blood vessels within the bladder. It appears that early and timely transplantation of BM-MSCs during the subacute phase (14 days post-injury) yields noticeable therapeutic benefits compared to interventions in acute or chronic phases. Cho et al also reported that SC therapy elevated α-smooth muscle actin expression, indicating the differentiation of SCs into smooth muscle cells in the bladder.[49]

Following SCI, patients often experience spinal shock, which can obscure the typical patterns of bladder function. This period is characterized by a loss of voluntary control and altered reflexes, making it challenging to assess bladder function accurately. By waiting until 4 weeks post-injury, we allow some time for the initial effects of spinal shock to stabilize, providing a clearer picture of the bladder's condition and enabling us to tailor our interventions more effectively. Our primary concern during this period is managing bladder fibrosis, which can develop as a consequence of injury and inflammation. Fibrosis can lead to reduced bladder compliance and impaired function. By administering MSCs at 4 weeks, it is intended to target the fibrotic changes that may have begun to set in. Introducing MSCs at this stage allows us to intervene when the inflammatory response has diminished but before significant fibrosis becomes established. This timing is crucial for maximizing the regenerative potential of the MSCs while minimizing further complications related to bladder function. In summary, our decision to inject MSCs into the bladder wall at 4 weeks post-injury is strategically aligned with the challenges posed by spinal shock and our focus on managing bladder fibrosis. This approach aims to enhance recovery outcomes by addressing the underlying issues on time.

Conclusion

Overall, BM-MSC application in hSCI and cSCI decreased the rate of apoptotic neuronal cell death while increasing proliferation in female Wistar rats. However, therapeutic effects were found only in hSCI groups and coincided with a reduction of c-Fos in the spinal cord and urinary tissues. It opens a new window to future studies indicating that the local transplantation of BM-MSCs acts via central effects beyond the other local capacities. Intra-detrusor wall BM-MSC transplantation may be a novel therapeutic strategy for bladder dysfunction in SCI patients in the chronic phase suffering from NLUTD disorder. It seems that the results of this study can help us develop an alternative modality for alleviating neuro-urology disorders.

Ethics statement

All the experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (Publication No. 85-23, revised 1985) and approved by the local Care and Use of Animal Committee (IR.TBZMED.REC.1395.24). In addition, the study was conducted in accordance with all applicable international, national, and/or institutional guidelines for the care and use of animals.

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Conflict of interests declaration
The authors declare that they have no conflict of interests.

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Data availability statement
The authors confirm that the data supporting the findings of this study are available within the article.

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Writing–review & editing: Sakineh Hajebrahimi, Leila Roshangar.

Consent for publication
Not applicable.

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