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ORIGINAL ARTICLE

Transplantation of Brain-Derived Neurotrophic Factor-Expressing Mesenchymal Stem Cells Improves Lower Urinary Tract Symptoms in a Rat Model

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뇌유래신경영양인자 발현 중간엽 줄기세포의 하부요로증상 개선 효과

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ABSTRACT

This study aimed to explore the effects of brain-derived neurotrophic factor (BDNF), produced by engineered immortalized mesenchymal stem cells (imMSC), on lower urinary tract symptoms (LUTS) in a rat model with neurogenic bladder (NB). Forty-eight Sprague-Dawley (SD) rats were randomly divided into the following groups: Sham control, LUTS, LUTS+imMSC (treated with immortalized MSC), and LUTS+BDNF-eMSC (treated with BDNF-expressing MSC) groups. LUTS was induced by a crush injury to the major pelvic ganglion (MPG). Bladder function was tested under anesthesia, and bladder tissue strips were collected thereafter for contractility test and western blot analysis. Western blot results showed that the expression of both Angiopoietin 1 (Ang 1) and platelet-derived growth factor (PDGF) increased with MSC injection. The effect of treatment with BDNF-eMSC on LUTS was also evaluated, and the results were found to be better than those with imMSC ($P<0.05$). BDNF-eMSC prevented fibrosis in the bladder tissue and significantly reduced caspase-3 levels. In conclusion, high expression of BDNF *in vivo* resulted in recovery of bladder function and contractility, along with the inhibition of apoptosis in a rat model.

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INTRODUCTION

Lower urinary tract symptoms (LUTS), including urinary tract damage, urinary tract infection, storage symptoms, voiding symptoms, and renal failure, may be related to nervous system injury. In a rat model of neurogenic bladder (NB), such symptoms have been shown to be caused by diabetes, cerebrovascular accidents, brain injury, or spinal cord injury [1, 2]. Regenerative medicine, or stem cell therapy, which involves tissue formation and repair to restore the functionality of damaged organs or tissues, have often been employed for tissue regeneration [3-5].

Stem cell therapy has been used to treat various diseases till date [6, 7]. Mesenchymal stem cells (MSCs) are pluripotent stem cells that are capable of

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self-renewal, multiple functions, and multidirectional differentiation. In the field of medicine, stem cell therapy has achieved satisfactory results in recent days [8, 9]. Our previous study had shown MSC treatment to accelerate the recovery of damaged nerves and tissues [10]. Therefore, this treatment was reasonably considered to be effective in treating NB as well. In order to improve the proliferation ability and anti-aging ability of stem cells, several researchers have been using immortalized mesenchymal stem cells (imMSCs), which are stabler than MSCs [10, 11]. In recent past, chemokines had been reported to act as strong chemoattractants for stem cells, and brain-derived neurotrophic factor (BDNF), in particular, has received much attention in tissue engineering research for nerve cell regeneration [12-14].

Following injury, stem cells recruited from the bone marrow navigate to the site of injury, and remain there long enough to participate in nerve tissue repair [4]. Some researchers have found the stable expression of BDNF in progenitor cells to effectively repair damaged nerve tissues [9]. BDNF has also been shown to significantly increase the cross-sectional area of the muscle innervated by the corresponding nerve [12, 14, 15].

In this study, we used a rat model of NB with pelvic ganglion injury and investigated the effects of treatment with engineered MSCs. We hypothesized that when the expression of BDNF in MSCs is increased, the repair of bladder function would improve, thereby improving LUTS in rats with neurogenic bladder.

MATERIALS AND METHODS

1. Cell preparation

Primary bone marrow mesenchymal stem cells (BM-MSCs) were cultured in low glucose-containing Dulbecco's modified Eagle's medium (DMEM; Gibco, US) supplemented with 20% fetal bovine serum (FBS, Gibco, US) and 5 ng/mL basis fibroblast growth factor (bFGF; Cell Signaling Technology, Danvers, US) at 37°C at 5% CO_2, but engineered BM-MSCs were cultured with 10% FBS. To generate engineered BM-MSCs, c-myc, hTERT and tetracycline transactivator (tTA) and BDNF genes were synthesized and transfected with pBD lentiviral vector (SL Bigen, Seongnam, Korea).

2. Experimental animal and study design

Fifty 8-week-old male Sprague-Dawley rats weighing about 270~300 g were purchased from a Korean company (Orient Bio, Seongnam, Korea). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea (CUMC-2016-0218-01). Bilateral pelvic nerve (PN) of all rats were identified under anesthesia. And then rats in 4 experimental groups were administered by a pelvic nerve crash. Rats in normal group were administered by sham surgery. After surgery, rats were housed individually with freely available food and water.

3. MSCs treatment

One weeks after NB model established, rats in LUTS+imMSCs group and LUTS+BDNF-eMSCs group were treated with imMSCs or BDNF-eMSCs, through intra-bladder wall injection under anesthesia (1×10^6 MSCs diluted in phosphate-buffered saline). And rats in NB and control group were injected to equal saline. To track the location of engrafted stem cells, we labeled them with a fluorescent dye (Cell Tracker CM-DiI; Molecular Probes, Eugene, OR) before injection according to the manufacturer's protocol.

4. Cystometry

Cystometry experiments were performed in all rats at 4th week after treatment as previously described. In brief, rats were anesthetized using a subcutaneous injection of 1.2 mg/kg of urethane. A suprapubic midline laparotomy was made to expose the bladder, and a 25-gauge needle connected to polyethylene tubing was inserted into the bladder through the bladder dome. The tubing was connected to a pressure transducer and a Harvard syringe pump via a 3-way

stopcock to record intravesical pressure and to infuse saline into the bladder. After emptying the bladder, cystometry was performed using a saline infusion at a rate of 0.04 mL/min. The contraction interval and contraction pressure (maximum bladder pressure during voiding) were recorded using a polygraph (Grass 7D; Grass Instruments, Quincy, MA, US). Nonvoiding contractions (NVCs) was determined during the 4 to 2 minutes before each voiding contraction, and NVCs were defined as contractions of >4 cm H_2O from the baseline pressure during bladder filling. After cystometry, the major pelvic ganglion (MPG), pelvic nerve and bladder were collected for immunofluorescence staining and western blot.

5. Contractility test

For the contractility test, the animals were sacrificed at 4 weeks after the periurethral injection of the porous beads (with or without growth factors), and the proximal urethra, which included the injected porous beads, was dissected, trimmed, weighed, and detubularized in a spiral fashion to produce a 1×10 mm^2 tissue strip. One end of the strip was connected with a 3-0 silk suture to a glass hook, and the other end was tied to an isometric force transducer (FT03; Grass Instruments). The strip was suspended in a 20 mL organ bath containing Tyrode's solution (116 mM NaCl, 5 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, and 11.5 mM glucose) bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C. Before the electrical field stimulation, the strip was equilibrated at 1 g initial tension for 30 min in the organ bath. Then, the strip was stimulated at 32 Hz with 1 ms pulses at 80 volts using an S48 stimulator (Grass Instruments) for 30 s. Contractions of the strip caused by the electrical field stimulation were measured through the isometric force transducer and were recorded on a personal computer by the use of a commercial data acquisition system (PowerLab; AD Instruments). A contractile response, which is expressed by tension per unit weight of strip, was also obtained from the contraction results.

6. Leak point pressure (LPP) measurement

At 4 weeks after the periurethral injection of the porous beads (with or without the growth factors), the LPP to evaluate the physical urethral sphincteric function was measured using a vertical tilt/intravesical pressure clamp model. Before the LPP testing, the spinal cord was transected at the T9 level to avoid spontaneous bladder responses by the increasing intravesical pressures, whereas the rats were under anesthesia of rats. This treatment does not interfere with the spinal continence reflexes of the bladder neck and urethra. The feces in the distal colon and rectum were evacuated by gentle massage via a midline abdominal incision, and a loose suture was secured around the proximal end of the distal colon to prevent any further migration of feces which can affect the LPP. Thereafter, a PE-90 transvesical catheter was inserted into the dome of the bladder and secured with a ligature for bladder filling. The muscle and skin incision was closed with sutures. The rat was then mounted on a tilt table and placed in a vertical position. Intravesical pressure was controlled by the height adjustment of a saline reservoir attached on a metered vertical pole. The reservoir outlet was connected with the transvesical catheter via a 2-way stopcock. The intravesical pressure was increased in 1~3 cm H_2O steps until the visual identification of leakage, and the leak point was determined as the LPP. To guarantee the reliability of the results, all measurements were processed in triplicate.

7. Western blot

The collected tissue was homogenized using ice-cold RIPA buffer (Cell Signaling Technology, Danvers, MA) containing ethylene diamine tetra acetic acid-free protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics GmbH) and particulate mass was removed by centrifugation (15,000 g) for 15 min at 4°C. And supernatants were analyzed by SDS-PAGE. Primary antibodies used included Caspase-3

(diluted 1:300; Cell Signaling Technology, Danvers, US), PDGF (platelet-derived growth factor diluted 1:200; Cell Signaling Technology, Danvers, US), Ang1 (Angiopoietin 1 diluted 1:200; Cell Signaling Technology, Danvers, US), neuronal nitric oxide synthase (eNOS, diluted 1:100; Cell Signaling Technology, Danvers, US) and β-actin (diluted 1:1000; Abcam, Cambridge, UK).

8. Statistical analysis

All data are presented as mean±standard error (SD) and were analyzed by SPSS version 22.0 software (IBM, Armonk, NY). Student's t-test and one-way ANOVA as appropriate were used to evaluate whether differences among groups were significant. $P<0.05$ was considered statistically significant.

RESULTS

1. BDNF-eMSCs improved bladder function

As we can see from Figure 1, the results of cystometry in LUTS+BDNF-eMSC group was apparently better than other groups. The Figure 2 and 3 showed voiding frequency and contractility in each group, compared with Injury group, the bladders of LUTS+BDNF-eMSC group had better results ($P<0.01$), meanwhile LUTS+ BDNF-eMSC group was better than LUTS+imMSCs group ($P<0.05$). Results from Figures 1~3 proved that imMSCs treatment efficiently improves NB, especially in a high BDNF environment.

2. Ang1 and PDGF expressed in an overexpressed BDNF microenvironment

When we researched the bladder recovery, we found that after stem cells injection Ang1 and PDGF in the increased. Figure 4A shows engrafted stem cells increased Ang1 and PDGF expression in bladder. In the

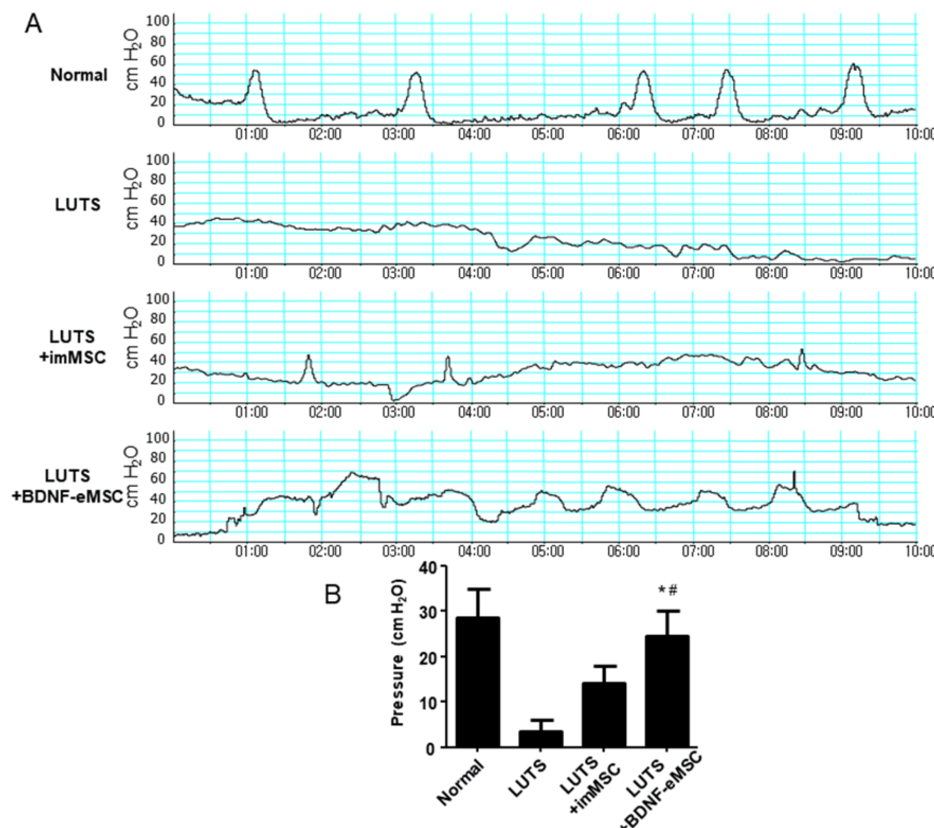

Figure 1. Figure 1. (A) Representative images of cystometry in each group. (B) Mean pressure of the voiding contractions intervals and intermicturition interval compared in during continuous infusion cystometry each group. Each bar shows the mean values (standard deviation). $^{\#}P<0.01$ compared with the Lower Urinary Tract Symptoms (LUTS) group. $^{*}P<0.05$ compared with the Lower Urinary Tract Symptoms (LUTS) + imMSC (immortalized MSCs) group.

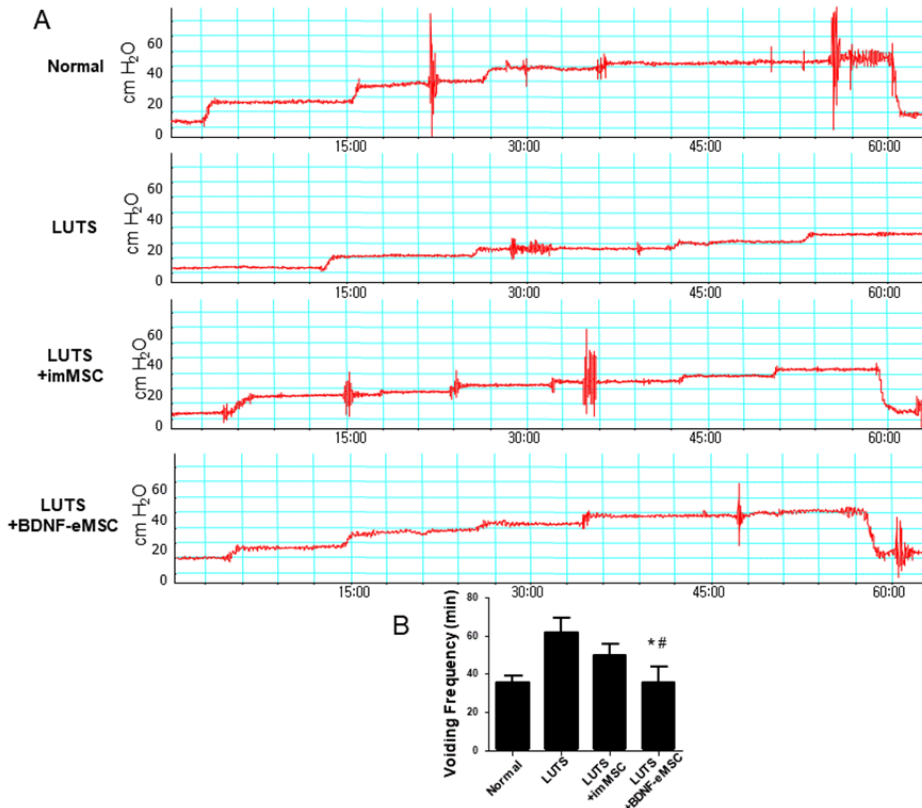

Figure 2. (A) Representative images of voiding frequency of each group. (B) Mean results of voiding frequency. Each bar shows the mean values (standard deviation). #$P<0.01$ compared with the Lower Urinary Tract Symptoms (LUTS) group. *$P<0.05$ compared with the Lower Urinary Tract Symptoms (LUTS)+imMSC (immortalized MSCs) group.

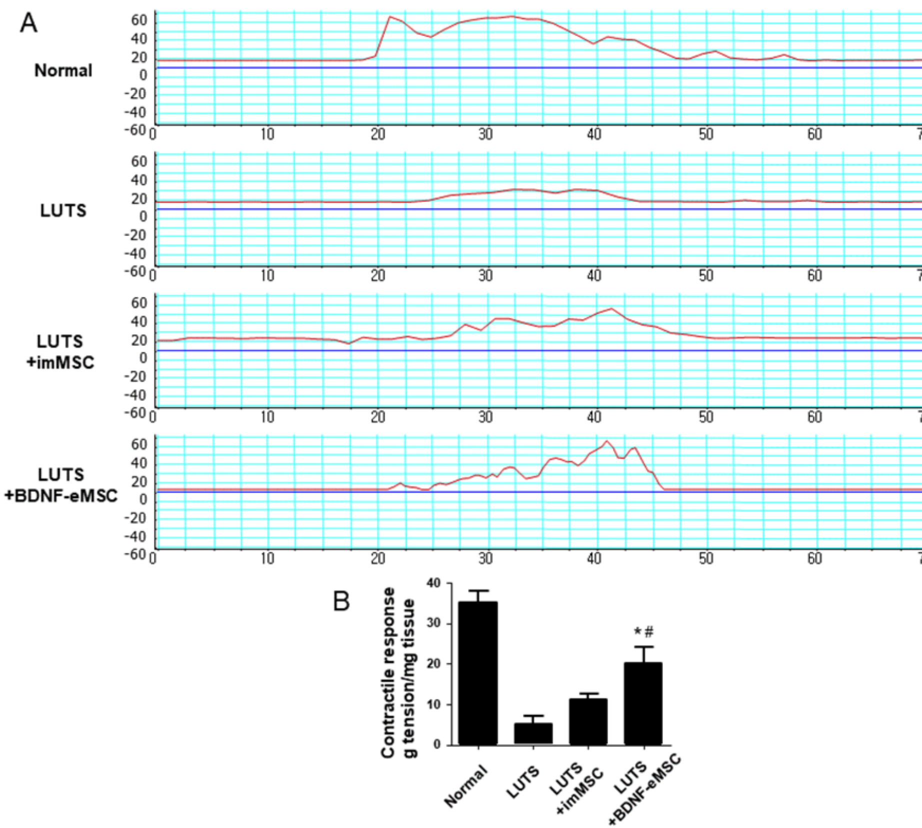

Figure 3. (A) Representative images of contractile response to electrical stimulation each group. (B) Mean results of contractility. Each bar shows the mean values (standard deviation). #$P<0.01$ compared with the Lower Urinary Tract Symptoms (LUTS) group. *$P<0.05$ compared with the Lower Urinary Tract Symptoms (LUTS)+imMSC (immortalized MSCs) group.

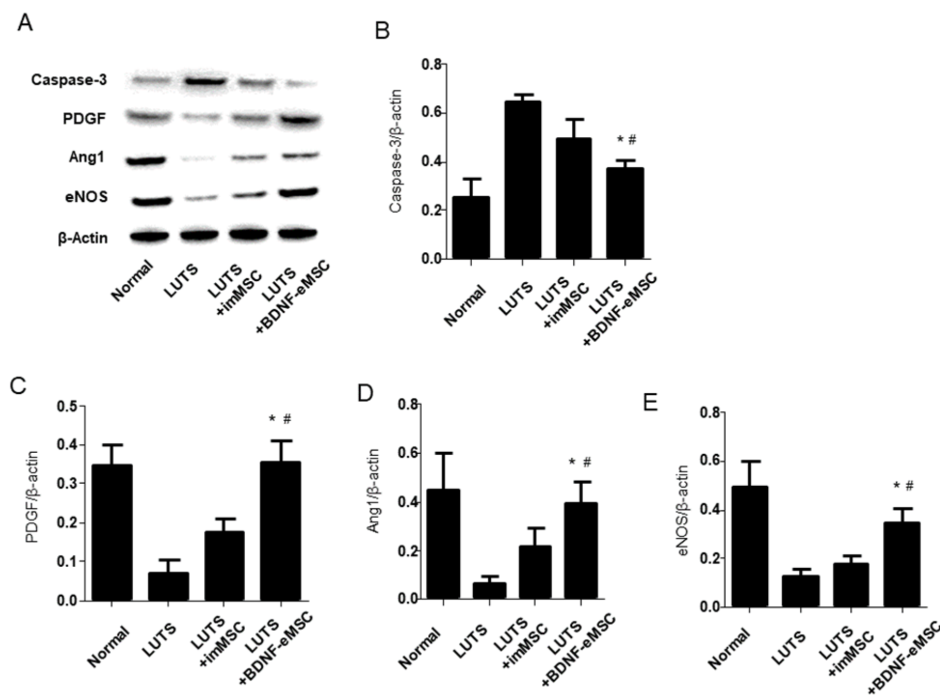

Figure 4. (A) All groups were compared for Caspase-3, PDGF, Ang 1, and eNOS in pelvic nerve by western blot. (B) Quantity analysis of western blot, including Caspase-3/β-actin, PDGF/β-actin, Ang 1/β-actin, and eNOS/β-actin. Each bar shows the mean values (standard deviation). #P<0.01 compared with the Lower Urinary Tract Symptoms (LUTS) group. *P<0.05 compared with the Lower Urinary Tract Symptoms (LUTS)+imMSC (immortalized MSCs) group.

quantitative analysis (Figure 4C, D), we found Ang1 and PDGF expression in the LUTS+BDNF-eMSC group is more than in the LUTS group (P<0.01). And Ang1 and PDGF expression in LUTS+BDNF-eMSC group is higher than in the LUTS+imMSC group (P<0.05). In a high BDNF Surrounding, cells need more growth factor like Ang1 and PDGF to support it.

3. BDNF-eMSCs decreased apoptosis in pelvic nerve

At last, we detected the apoptosis of pelvic nerve tissue by Caspase-3 western blot for each group to find if the MSCs treatment would reduce the apoptosis in the injured tissue. The results of Figure 4B showed that without treatment the apoptosis in the damaged pelvic nerve was still higher than others (P<0.01). But after stem cells treatment the apoptosis was decreased apparently. And more importantly, the apoptosis in the LUTS+BDNF-eMSC group is higher than in the LUTS+imMSC group, which reveals that in a high BDNF Microenvironment apoptosis is improved and damaged tissue becomes easier to recover.

DISCUSSION

With the development of surgical techniques, postoperative survival rate of patients with pelvic tumors has improved [16]. The number of pelvic surgeries, including radical prostatectomy, rectal surgery, and hysterectomy, has been increasing annually. Many of these patients could be injured in MPG, resulting in NB, and hence a compromised quality of life [17], with functional, psychological, and socioeconomic disorders, such as urinary tract infections (UTI) and pressure ulcers [18]. Although there are many ways to alleviate the complications caused by NB, the latter needs to be treated directly to repair the bladder tissue and recover its functionality.

In this study, rat models of NB were established first, followed by injection of engineered stem cells into the rats for treatment. MSCs are known to repair tissue function by clustering around the damaged tissues. We used a gene transfer technique that allowed the engineered imMSCs to express more BDNF than in normal imMSCs. We found that after injection of imMSCs, the average bladder pressure of the NB rats

was significant. In addition, both bladder muscle tissue and apoptosis reduction were significant.

Stem cells are known to improve proliferation and angiogenesis by expressing Ang1 in vivo [19]. Moreover, PDGF is a biological factor that stimulates the recovery of injured tissue [15]. In this study, we demonstrated that engrafted stem cells improved the bladder wall and restored bladder function in the NB rat. Furthermore, we detected the expression of Ang1 and PDGF in the bladder wall.

Western blot results showed expression of both Ang1 and PDGF to be increased upon stem cell injection. Expression of eNOS was also found to be upregulated, further validating improvement of the injured bladder [20].

Taken together, our results showed engrafted stem cells to accelerate the rebuilding of bladder tissue by upregulating Ang1 and PDGF expression. High eNOS expression due to the stem cells also improved the restoration of damaged bladder, thereby contributing to functional improvement of the bladder [20-22].

We had previously observed the functional efficacy of SDF-1-overexpressing MSCs in an NB rat model [10]; there was a significant improvement in contraction interval, voiding contractions, voided volume, and voiding frequency. Our current findings regarding the effect of BDNF-expressing MSC therapy on LUTS in an NB rat model mainly indicated an increase in contraction pressure, besides the significant improvement in both contraction interval and bladder contractile function. These changes might be attributed to reduced fibrosis and apoptosis. Overall, our results suggested that impaired bladder contractility could be related to nerve injury, as was seen in a previous study [10] that evaluated denervated bladder contractility after MPG electrocauterization.

In this study, we investigated the changes in bladder function with acetylcholine as an autonomic transmitter. Although cholinergic neurotransmitters play a major role in controlling voiding, several reports have addressed the potential action of non-adrenergic non-cholinergic neurotransmitters as well. We evaluated the efficacy and safety of BDNF-expressing MSC therapy for LUTS in an NB rat model, and found imMSCs to have definite effects on nerve tissue repair; they caused functional recovery and tissue repair in the rat model. The findings suggested that treatment with BDNF-expressing MSCs may be a therapeutic strategy to improve bladder function in patients with LUTS.

요 약

이 연구는 신경인성 방광 쥐 모델에서 줄기세포에 의해 발현된 뇌유래신경영양인자가 하부요로 증상에 미치는 영향을 조사하였다. 48마리의 Sprague-Dawley 쥐를 정상군, 하부요로증상군, 하부요로증상+imMSC군 및 하부요로증상+BDNF-eMSC군으로 무작위 선정하였다. 하부요로증상모델은 골반신경절 손상에 의해 유도되었으며 방광 기능평가는 마취 하에 실시하였고, 수축성 검사 및 웨스턴 블롯 분석을 위해 방광 조직을 절제하였다. 뇌유래신경영양인자 발현 중간엽줄기세포 치료가 하부요로증상에 미치는 영향도 평가되었으며 뇌유래신경영양인자 발현 중간엽줄기세포는 방광 조직의 섬유화를 억제하였고 Caspase-3 발현을 감소시켰다. 결론적으로, 뇌유래신경영양인자 발현 중간엽줄기세포는 하부요로증상 쥐 모델에서 세포 사멸의 억제와 함께 방광의 기능 및 수축성의 회복을 가져왔다.

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Author's information (Position): Jeon SH[1], M.T.; Park MY[2], Professor.

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