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Mesenchymal stem cells and their secretome partially restore nerve and urethral function in a dual muscle and nerve injury stress urinary incontinence model

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Deng K, Lin DL, Hanzlicek B, Balog B, Penn MS, Kiedrowski MJ, Hu Z, Ye Z, Zhu H, Damaser MS. Mesenchymal stem cells and their secretome partially restore nerve and urethral function in a dual muscle and nerve injury stress urinary incontinence model. Am J Physiol Renal Physiol 308: F92–F100, 2015. First published November 5, 2014; doi:10.1152/ajprenal.00510.2014.—Childbirth injuries muscles and nerves responsible for continence. Mesenchymal stem cells (MSCs) or their secretome given systemically could provide therapeutic benefit for this complex multisite injury. We investigated whether MSCs or their secretome, as collected from cell culture, facilitate recovery from simulated childbirth injury. Age-matched female Sprague-Dawley rats received pudendal nerve crush and vaginal distension (PNC+VD) and a single intravenous (iv) injection of 2 million MSCs or saline. Controls received sham injury and iv saline. Additional rats received PNC+VD and a single intraperitoneal (ip) injection of concentrated media conditioned by MSCs (CCM) or concentrated control media (CM). Controls received a sham injury and ip CM. Urethral and nerve function were assessed with leak point pressure (LPP) and pudendal nerve sensory branch potential (PNSBP) recordings 3 wk after injury. Urethral and pudendal nerve anatomy were assessed qualitatively by blinded investigators. Quantitative data were analyzed using one-way ANOVA and Holm-Sidak post hoc tests with P < 0.05 indicating significant differences. Both LPP and PNSBP were significantly decreased 3 wk after PNC+VD with saline or CM compared with sham-injured rats, but not with MSC or CCM. Elastic fiber density in the urethra increased and changed in orientation after PNC+VD, with a greater increase in elastic fibers with MSC or CCM. Pudendal nerve fascicles were less dense and irregularly shaped after PNC+VD and had reduced pathology with MSC or CCM. MSC and CCM provide similar protective effects after PNC+VD, suggesting that MSCs act via their secretions in this dual muscle and nerve injury.

paracrine action; elastin; external urethral sphincter; pudendal nerve; urinary incontinence

VAGINAL DELIVERY CAN INJURE the muscles, nerves, and connective tissues responsible for continence (25). These injuries are correlated with both postpartum stress urinary incontinence (SUI) as well as development of SUI later in life (8). Current treatment options for SUI either have poor long-term efficacy or have a high revision rate (11, 18, 29).

Mesenchymal stem cells (MSCs) can differentiate into mesenchymal cell types and also secrete trophic factors which facilitate regeneration (13). MSCs and their secretions can act systemically as well as locally via mechanisms including immune modulation and metabolic regulation (2), suggesting their potential to facilitate regeneration of complex injuries with multiple mechanisms and sites of injury, such as childbirth.

Intraurethral injection of muscle-derived progenitor cells are currently in clinical trials for SUI (5). However, since the maternal injuries of childbirth are not limited to the urethra, systemic delivery of stem cells has the potential to improve recovery of multiple injury sites and may lead to better long-term outcomes. Systemic delivery of cells has been tested with positive results in a vaginal distension (VD) model of simulated childbirth injury in rats (9, 23); however, this model does not simulate the maternal nerve injuries that occur in childbirth.

We have developed a dual-injury rat model, consisting of VD and pudendal nerve crush (PNC), which better mimics the maternal injuries of childbirth than VD alone since it produces multiple mechanisms and sites of injury, similar to human childbirth (16). This model consistently decreases leak point pressure (LPP) and pudendal nerve function for 4–5 wk, indicative of SUI (16). The aim of this study was to determine whether intraperitoneal (ip) injection of concentrated conditioned media (CCM) cultured from rat bone marrow-derived MSCs or intravenous (iv) injection of rat bone marrow-derived MSCs could prevent SUI and preserve pudendal nerve function in this dual-injury rat model.

MATERIALS AND METHODS

Study Design

This study was approved by the Institutional Animal Care and Use Committee of the Cleveland Veterans Affairs Medical Center. In the first of 2 experiments, 44 adult female Sprague-Dawley rats (250–300 g) underwent sham injury with a 1-ml saline treatment intravenously (iv;
n = 15), PNC+VD with 1 ml saline iv (n = 13), or PNC+VD with 2 million MSCs suspended in 1 ml saline iv (n = 16). All treatments were given 1 h after the end of PNC+VD. Five rats in each group were euthanized 3 wk after injury for anatomic assessment of the urethra and pudendal nerve. The remaining rats underwent LPP testing with simultaneous external urethral sphincter (EUS) electromyography (EMG), and pudendal nerve sensory branch potential (PNSBP) recordings 3 wk after injury to assess restoration of pudendal nerve function.

In the second experiment, 54 adult female Sprague-Dawley rats underwent sham injury with concentrated control media (CM) given intraperitoneally (ip; n = 18), PNC+VD with CM ip (n = 19), or PNC+VD with CCM ip (n = 17). As above, all treatments were given 1 h after the end of PNC+VD, and five rats in each group were euthanized 3 wk after injury for anatomic assessment. The remaining rats in each group underwent LPP measurement with simultaneous EUS EMG and PNSBP recordings 3 wk after injury.

**Stem Cell Harvest and Culture**

Bone marrow from donor female Sprague-Dawley rats was used to obtain MSCs (9). Cells were aspirated from the femur and tibia by flushing the bone with saline and were sorted using intracellular adhesion molecule 1 (ICAM-1) to select for MSCs. The MSCs were transfected with lentivirus to express green fluorescent protein (GFP), and GFP-positive (GFP+) MSCs were cultured to passage 16 (P16) before being injected or used to prepare CCM.

**MSC Characterization**

As described previously (9), P16 MSCs were sorted using flow cytometry for CD29, CD90, CD54, and CD45 to confirm expression of stem cell surface makers; P16 MSCs were cultured in chondrogenic media, osteogenic media, or adipogenic media for 20–35 days to confirm the ability of these MSCs to differentiate into other mesenchymal cell types.

**CCM Preparation**

To create CCM, P16 MSCs were incubated in 15 ml serum-free DMEM for 24 h, and conditioned media was extracted and centrifuged at 4,000 rpm for 75 min and filtered with a sterile 0.22- 

**PNC+VD**

Rats were anesthetized with 2% isoflurane and underwent PNC followed by VD as described previously (16). The pudendal nerve was isolated bilaterally and crushed with a Castroviejo needle holder twice for 30 s. The vagina was then accommodated with increasing sizes of bouge a boule urethral dilators (24 – 32 Fr). A modified 10F Foley catheter was inserted into the vagina, and the balloon was inflated with 3 ml water for 4 h. Sham injury was created by making an incision in the vagina and inserting a Foley catheter for 4 h without inflation. One hour after the end of VD or sham VD, rats received either saline or MSCs iv via the tail vein, or CM or CCM ip.

**In Vivo Urethral Functional Testing**

Functional recordings, including LPP, EUS EMG, and PNSBP, were performed as described previously (16). Under isoflurane inhalant anesthesia, a transvesical catheter was inserted into the bladder dome and fixed with a suture. The abdominal wall was closed, and the catheter was connected to both a pressure transducer (model PT300; Astro-Med, Providence, RI) and syringe pump (5 ml/h, model 200; KD Scientific, New Hope, PA.). Bipolar parallel platinum electrodes (30-gauge needles 2 mm apart) were placed on the surface of the midurethra and were connected to an amplifier (band-pass frequencies: 3 Hz–3 kHz, model P511; AC Amplifier, Astro-Med) and electrophysiologic recording system (10-kHz sampling rate, PowerLab 8/35, ADInstruments, Colorado Springs, CO). The pudendal nerve sensory branch was identified on the ventral side of the pudendal canal and was suspended over recording electrodes connected to an amplifier and the electrophysiologic recording system.

Before the recordings, the animals were anesthetized with urethane (1.2 g/kg ip), and the isoflurane anesthesia was removed since urethane better preserves reflexes of continence and voiding (3). Intravesical pressure was increased for LPP testing with simultaneous EUS EMG recording with the bladder approximately half full by gradually pressing on the bladder with the rat in a supine position (3). When leakage was visualized, the external pressure was rapidly removed. LPP testing was repeated four to six times for each rat. If an active bladder contraction occurred, the results were not analyzed and the test was repeated. After LPP testing, the clitoris was brushed gently with a cotton swab or gauze bilaterally with moderate speed while PNSBP was recorded. The test was repeated six to nine times in each animal.

**Anatomy**

The urethra and pudendal nerve were dissected, stored at −80°C, and sectioned transversely (14 μm). Transverse pudendal nerve sections underwent immunofluorescence to identify axons with primary antibodies, anti-neurofilament 200 and anti-neurofilament 68 (1:100 dilution, Sigma-Aldrich, St. Louis, MO), and the secondary antibody, Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:400 dilution, Invitrogen, Carlsbad, CA).

Immunofluorescence was also used to assess innervation of neuromuscular junctions (NMJ) of the EUS using primary and secondary antibodies as above to identify axons and 4 μg/ml of tetramethylrhodamine-conjugated α-bungarotoxin (Rh-α-BTX; Invitrogen) to identify NMJs. Alexa Fluor 350-conjugated phalloidin (5 μl in 100 μl PBS, Invitrogen) was used to identify striated muscle of the EUS. Additional near sections of the urethra were stained with elastin von Giessen (EVG) stain. Additional urethral sections underwent immunofluorescence to localize GFP+MSC with primary antibody, rabbit anti-GFP (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit (1:500, Invitrogen).

**Data Analysis**

LPP was defined as baseline pressure just before testing subtracted from peak pressure at leakage. Quantitative assessment of EUS EMG and PNSBP signals was performed by selecting four to six 1-s segments for each rat at baseline and peak activity (Figs. 1A and 2A) and determining the mean amplitude and frequency of muscle and nerve activity, as done previously (9). An increase in amplitude and frequency at peak activity was calculated by subtracting baseline from peak activity values. Mean values were calculated for each animal and were used to calculate a mean and SE for each experimental group. One-way ANOVA followed by the Holm-Sidak post hoc test was used. P < 0.05 was used to indicate a statistically significant difference between groups in all cases.

Histology and immunofluorescence were evaluated qualitatively by a blinded observer according to the following criteria. EUS striated muscle morphology and elastin fibers as well as their location, orientation, and quantity of elastin fibers were assessed on urethral EVG-stained slides. Thickness of innervating axons, concentration of motor endplates, and their innervation status were used to qualitatively assess innervation of the EUS. Density of nerve fascicles and axon morphology were used to qualitatively assess pudendal nerves. GFP+ cells were counted at a ×20 field and scanned for 10 fields.
RESULTS

Characterization and Differentiation of MSC

MSCs expressed CD29, CD54, and CD90, but not CD45. Differentiation assays confirmed that MSCs can differentiate into adipogenic, chondrogenic, and osteogenic cells as we have shown previously (9).

MSC or CCM Treatment Improves Urethral Function After Simulated Childbirth Injury

LPP was significantly decreased 3 wk after PNC+VD with saline or CM treatment compared with sham-injured rats (Fig. 1), indicative of urethral injury and decreased function, as previously demonstrated (16). LPP in rats that underwent PNC+VD and were treated with MSCs or CCM was not significantly different from sham-injured animals, demonstrating that both MSCs and CCM facilitate functional recovery of the urethra. The increase in amplitude of EUS EMG with LPP was significantly decreased 3 wk after PNC+VD in rats treated with saline or MSCs (Fig. 1).

In contrast, there were no significant differences between the experimental groups in the increase in EUS EMG firing rate with LPP testing, suggesting that MSC treatment did not affect EUS EMG recovery after PNC+VD. Similarly, the increase in both EUS EMG amplitude and firing rate were not significantly different among the experimental groups treated with CCM or CM (Fig. 1). The increase in amplitude and firing rate of PNSBP during clitoral brushing were both significantly decreased 3 wk after PNC+VD animals treated with either saline or CM compared with sham-injured animals; however, they were not significantly different when injured animals were treated with either MSCs or CCM (Fig. 2), indicating that both MSCs and CCM preserved nerve function after PNC+VD.

Anatomy

MSC or CCM treatment promotes urethral elastogenesis. Under microscopic examination of the urethra, striated muscle fibers of the EUS had fewer striations and were atrophied 3 wk after PNC+VD compared with sham-injured animals. After treatment with MSCs or CCM, the EUS had increased striated muscle layers than those treated with saline or CM. Elastin fibers were observed as consecutive long fibers in sham-injured animals, mainly outside the EUS, and most were parallel to the striated muscle fibers of the EUS (Fig. 3). Elastin fibers external to the EUS were disrupted and twisted after PNC+VD. The density of elastin fibers interior to the EUS increased, and their orientation became radial in all injured animals, regardless of treatment. Density of elastin fibers increased further with either MSC or CCM treatment, indicating that both MSCs and CCM stimulate elastogenesis (Fig. 3).

MSC or CCM treatment promotes NMJ and pudendal nerve regeneration. Axons located in the EUS in sham-injured animals were consistently straight, thick, and innervated discrete organized NMJs (Fig. 4). Three weeks after PNC+VD treated with saline or CM, innervating axons were thinner than in sham-injured animals, and NMJs were less organized and diffusely stained along the edge of striated muscle fibers, indicative of denervation. Some NMJs were visibly not well innervated (Fig. 4). In contrast, after treatment with MSC or CCM, axons innervating the EUS took a more torturous course and had multiple collaterals, indicating ongoing neuroregeneration. The degree of NMJ diffusion along striated muscle fibers visibly decreased (Fig. 4), indicating that MSC and CCM accelerated neuromuscular recovery after simulated childbirth injury.

Pudendal nerve sensory branch fascicles in sham-injured animals were circular and had small compact axons (Fig. 5). Three weeks after PNC+VD and treatment with saline or CM, nerve fascicles were less dense and axons had irregular shapes, indicating injury and the beginning of neuroregeneration. In contrast, after treatment with MSC or CCM, the pudendal nerve demonstrated both normal and abnormal nerve fascicles with more normal fascicles than when treated with saline or CM (Fig. 5), indicating facilitated neuroregeneration or neuropreservation. No GFP+ stem cells were observed in the urethra or vagina 3 wk after injury, suggesting the cells did not permanently engraft and differentiate in this location.

DISCUSSION

Cell-based therapies have shown promising results in both preclinical and clinical studies for bone marrow transplantation (31), wound healing (4), inflammation control (1), and neovascularization (10), as well as for SUI (21), through their direct and indirect effects on tissue regeneration. The mechanism by which stem cells repair and regenerate tissues is not entirely known but includes secretion of paracrine factors in addition to differentiation and proliferation of cells (24). Consistent with our study, the number of cells that engraft and differentiate locally is often too few to explain significantly improved functional and anatomic results (22, 23), suggesting that the effects of their secretions on innate tissues dominates the mechanism (4). Bi et al. (2) reported that MSCs and their conditioned media have similar protective effects on acute kidney injury when given ip and concluded that secretome from stem cells protects against acute tubular injury even at a distance from the target organ, inspiring our administration of CCM ip.

Both preclinical studies and clinical trials of cell therapy for SUI primarily investigate local injection of stem cells into the urethra, with the expectation that these cells will engraft and differentiate at this site of injury to restore urethral function (5,
However, systemic delivery has the potential to better promote healing at multiple sites. Therefore, in this study we investigated the therapeutic effects of iv injection of rat MSCs or ip injection of CCM. We demonstrated that both MSCs and CCM lead to similar levels of recovery and regeneration after PNC/H11001/V.

We have previously demonstrated that MSCs or their secretions improve urethral function after VD (9). In that study, MSCs were given iv and CCM was delivered periurethrally, leaving open the question of whether CCM could have a beneficial effect if given systemically. In the current study, we expanded the investigation of MSCs and MSC secretome treatment to a more complex dual-injury model which better represents the multiple sites and type of maternal injuries during childbirth than either injury alone, since muscles, organs, and connective tissues of the pelvic floor and their innervating nerves are damaged in delivery, leading to both postpartum SUI and redevelopment of SUI later in life (8, 15). Consistent with a multiple injury mechanism, PNC/H11001/V produces a more severe injury that takes longer to recover from.

![Fig. 2. Pudendal nerve sensory branch potential (PNSBP) results. Examples demonstrate the two 1-s segments selected from baseline (●) and brush activity (▲) that were used to calculate PNSBP amplitude difference (μV) and firing rate difference (Hz) during brush stimuli to the rat clitoris (A). Shown are PNSBP amplitude increase after pudendal nerve crush and vaginal distension (injury) or sham injury with MSC or saline treatment (B) as well as with CCM or CM treatment (D) and firing rate increase after injury or sham injury with MSC or saline treatment (C) as well as with CCM or CM treatment (E). Values are means ± SE of data from 8–14 animals. ●, Baseline PNSBP; ▲, brush PNSBP. *Significant difference compared with corresponding sham-injured animals, P < 0.05.](http://ajprenal.physiology.org/short/10.1152/ajprenal.00510.2014)
than either single injury alone (16). In the current study, we delivered CCM ip to determine whether it could have a beneficial effect even if not delivered locally to the site of injury.

LPP and PNSBP demonstrated accelerated recovery with both iv MSC and ip CCM treatment, indicating an effect of the cells and their secretions on the urethra and its innervation by the pudendal nerve. In contrast, neither MSC nor CCM treatment significantly improved EUS EMG despite improved preservation of the innervating axons and motor endplates with either MSC or CCM treatment. Since the EUS demonstrated greater atrophy after PNC/VH compared with sham-injured rats, we conclude that muscle and NMJ regeneration were still ongoing, as we have shown previously (7, 30).

Abnormal elastin fibers have been observed in periurethral tissues of parous women with SUI (14). In addition, lysyl oxidase like-1 knockout mice, which are unable to assemble elastin fibers properly, develop SUI (20), suggesting a role for disruption of elastin in the pathogenesis of SUI. In this study, we observed disruption of urethral elastin fibers 3 wk after PNC+VD. The elastin fibers were reoriented and increased in density near the EUS with either MSC or CCM treatment. Since the EUS demonstrated greater atrophy after PNC+VD compared with sham-injured rats, we conclude that muscle and NMJ regeneration were still ongoing, as we have shown previously (7, 30).

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Stem cells home to injury sites following gradients of chemokines (28), and we have previously demonstrated homing of MSCs to the pelvic organs of rats after VD (6). In contrast, in the current study we were unable to locate MSCs in the urethra and vagina 3 wk after PNC+VD. It is possible that few MSCs injected intravenously migrate to the injury site (26), and most of them might have reached the end of their lifespan 3 wk later (22). Nonetheless, they facilitated regeneration and functional recovery from the injury via either local or systemic effects.

We have previously demonstrated that local neurotrophin therapy facilitates continence recovery after PNC+VD in rats (12). The results of the current study suggest that local delivery of trophic factors is not necessary, as also observed by Voulgari-Kokota et al. (32) in a mouse model of glutamate excitotoxicity. Similarly, Lee et al. (19) demonstrated that secretions released by MSCs distant from the injury site suffice to modulate inflammation, improve tissue repair, and protect against neural dysfunction. In addition, it was recently demonstrated that cell-free derivatives from stem cells have the potential to reduce pulmonary embolism and infarcts from iv injection of stem cells, suggesting utility in treatment with both cells and their derivatives (17). However, since most factors present in CCM have short half-lives (2), multiple injections...
instead of a single treatment could further improve tissue repair and continence recovery.

Our findings suggest that MSCs or CCM could be beneficial for treating postpartum SUI, which occurs after vaginal childbirth in premenopausal women. This treatment also has the potential to be of benefit for premenopausal women with a history of vaginal childbirth. However, to demonstrate the idea that MSCs or CCM could be used to treat SUI months or years

Fig. 4. Examples of immunofluorescence of the EUS showing neuromuscular junctions (red), innervating nerves (green), and striated muscle (blue). Sham-injured animals demonstrated discrete organized motor endplates innervated by straight thick innervating axons (A and D). Three weeks after pudendal nerve crush and vaginal distension (Injury) with saline or CM treatment, innervating axons were thinner and motor endplates were less organized and diffuse (B and E). With MSC or CCM treatment, innervating axons took a more torturous course and had multiple collaterals (C and F).

Fig. 5. Examples of immunofluorescence of transections of the sensory branch of the pudendal nerve distal to the injury. Sham-injured animals demonstrated mostly circular nerve fascicles with small dense compact axons (A and D). Three weeks after pudendal nerve crush and vaginal distension (Injury) and treatment with saline or CM, nerve fascicles were less dense and axons had irregular shapes (B and E). With MSC or CCM treatment, the pudendal nerve demonstrated both normal and abnormal nerve fascicles, with a greater number of normal fascicles than when treated with saline or CM (C and F).
after delivery, one would need to conduct a new experiment using delayed injection of MSCs or CCM to determine whether delayed treatment could also facilitate recovery, as has previously been observed after caesarean neural injury (27).

Despite our findings of equal effectiveness of CCM and MSCs on recovery after PNC+VD, we did not identify the active cytokines in CCM. Further investigations are needed to delineate the content of CCM, the roles of secreted factors, and their interactions. Our study is also limited by the use of a quadrupled animal model of simulated childbirth trauma to investigate SUI. However, no animal model simulates human vaginal delivery and animal models of chronic SUI are not physiologically representative (15), suggesting the need for confirmation in multiple animal models.

Conclusions

Systemically delivered MSCs and CCM prevented SUI after a multifactorial simulated childbirth injury, likely mediated by cell secretions acting via preservation and regeneration of the pudendal nerve, elastogenesis, and enhanced neuromuscular recovery. Both MSC and their secretions independently have the potential to prevent or reduce SUI systemically and may have the potential to treat and/or prevent SUI.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


