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ABSTRACT

This research demonstrates the regenerative effects of mesenchymal stem cells (MSCs) on the injured anal sphincter by comparing anal sphincter pressures following intramuscular and serial intravascular MSC infusion in a rat model of anal sphincter injury. Fifty rats were divided into injury (n = 35) and no injury (NI; n = 15) groups. Each group was further divided into i.m., serial i.v., or no-treatment (n = 5) groups and followed for 5 weeks. The injury consisted of an excision of 25% of the anal sphincter complex. Twenty-four hours after injury, 5×10^5 green fluorescent protein-labeled MSCs in 0.2 ml of phosphate-buffered saline (PBS) or PBS alone (sham) were injected into the anal sphincter for i.m. treatment; i.v. and sham i.v. treatments were delivered daily for 6 consecutive days via the tail vein. Anal pressures were recorded before injury and 10 days and 5 weeks after treatment. Ten days after i.m. MSC treatment, resting and peak pressures were significantly increased compared with those in sham i.m. treatment ($p < .001$). When compared with the NI group, the injury groups had anal pressures that were not significantly different 5 weeks after i.m./i.v. treatment. Both resting and peak pressures were also significantly increased after i.m./i.v. MSC treatment compared with treatment with PBS ($p < .001$), suggesting recovery. Statistical analysis was done using paired t test with Bonferroni correction. Marked decrease in fibrosis and scar tissue was seen in both MSC-treated groups. Both i.m. and i.v. MSC treatment after injury caused an increase in anal pressures sustained at 5 weeks, although fewer cells were injected i.m. The MSC-treated groups showed less scarring than the PBS-treated groups, with the i.v. infusion group showing the least scarring. STEM CELLS TRANSLATIONAL MEDICINE 2014;3:760–767

INTRODUCTION

Cell-based therapies have shown encouraging results in improving function in cardiac tissues [1–3] and the urinary sphincter [4–6] after a myocardial infarction and stress urinary incontinence, respectively. Fecal incontinence affects approximately 11% of the population, with equal incidence in elderly men and women [7, 8]. Most therapies to treat fecal incontinence target the anal sphincter [9–12] or bulk the sphincter [13–16]. Others have a mode of action that is currently ill defined such as sacral nerve stimulation [17–20]. In severely symptomatic patients, the options are to replace the external anal sphincter with autologous tissue such as graciloplasty or implantation of an artificial anal sphincter. However, none of these options is efficacious in the long term or without complications related to the surgery or the device [21–25].

The anal sphincter is a complex muscle that comprises both striated and smooth muscle, constituting the external anal sphincter muscle and internal anal sphincter, respectively. Adipose-derived stem cells [26–29], muscle-derived stem cells [30], and mesenchymal stem cells (MSCs) [31] have been used to improve functioning of

the injured myocardium and the urinary sphincter in animal models. In the anal sphincter, the effect of MSCs has been studied, although most of the studies have used ex vivo outcome testing [32–37]. In a prior preliminary study [38], we determined the effect of intramuscular and intravenous injection of bone marrow-derived MSCs on anal sphincter pressures after anal sphincter injury or pudendal nerve crush. Our results showed improvement in resting and peak pressures after both i.m. and i.v. MSC treatment compared with sham treatments.

In this study, we changed our model from anal sphincterotomy to a partial anal sphincter excision (PSE) because the rat anal sphincter recovered spontaneously after an anal sphincterotomy. Our aim in the current experiment was to determine whether serial i.v. infusion or a single i.m. treatment of MSCs improved recovery of the anal sphincter after PSE.

METHODS

Animal Model

The research protocol was approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Fifty age-matched female Sprague-Dawley

rats (240–260 g) were randomly allocated into injury (n = 35) and no-injury (NI; n = 15) groups (Fig. 1), which were subjected to treatment with MSCs or saline or did not receive any treatment.

Anal pressures were recorded before treatment and 10 days and 5 weeks after treatment. The i.m.-treated rats received 5×10^5 green fluorescent protein (GFP)-labeled MSCs in 0.2 ml of phosphate-buffered saline (PBS) or 0.2 ml of saline into the anal sphincter; the i.v. treatment groups received the same dose (5×10^5) daily for 6 consecutive days via a tail vein injection starting 24 hours after injury.

We have demonstrated in our previous studies that chemokine stromal derived factor-1 (SDF-1) and monocyte chemotactic protein-3 (MCP-3) are maximally upregulated at 24 hours after a direct anal sphincter injury, decline soon thereafter, and are not present 3 weeks after injury [39]. We therefore timed the MSC/saline injections to be administered 24 hours after injury except in the group treated 3 weeks after injury.

The NI group received MSCs via either i.m. (NI-MSC-IM) or serial i.v. injection (NI-MSC-IV). For the injury groups, the injury was an excision of 25% of the internal and external anal sphincter muscle. The injury was carried out under a dissecting microscope by identifying the anal sphincter after an incision was made in the ventral aspect in all rats. The anal sphincter was dissected from the 10 o'clock to 2 o'clock position and excised. Both muscles were partially excised because in rats the internal sphincter is very small and difficult to distinguish from the external anal sphincter even under the dissecting microscope. Hemostasis was secured by application of pressure. The injury was carried out in a consistent fashion at the same location in all rats by a single operator. The injury groups (PSE) were divided into different treatment groups that received (a) saline (PSE-PBS; n = 10) directly intramuscularly into the anal sphincter (PSE-PBS-IM) or by serial i.v. infusions (PSE-PBS-IV) or (b) MSCs (PSE-MSC; n = 10) directly into the anal sphincter (PSE-MSC-IM) or by serial i.v. infusions (PSE-MSC-IV) or (c) no treatment (PSE-NT, n = 5). The rats were evaluated with anal pressure testing and immunofluorescence 9 days after treatment. A group of rats from the NI-MSC-IM (n = 3) and NI-MSC-IV (n = 3) groups and all rats from the PSE-NT (n = 5), PSE-MSC-IM (n = 5), and PSE-MSC-IV (n = 5) groups were further followed with repeat anal pressure testing, immunofluorescence, and histology at 5 weeks.

The control animals (NI-MSC-IM, NI-MSC-IV, NI-PBS, PSE-NT, PSE-PBS-IM, and PSE-PBS-IV) were evaluated with anal pressure testing 9 days after treatment. The NI-MSC-IM, NI-MSC-IV, and PSE-NT groups were also evaluated with anal pressure testing and histology at 5 weeks. The treatment groups PSE-MSC-IM and PSE-MSC-IV were evaluated with anal pressure testing and immunofluorescence at 9 days and anal pressure testing, immunofluorescence, and histology at 5 weeks after treatment.

To test the hypothesis that administration of MSCs 3 weeks after injury will not produce homing of these cells because of loss of cytokine signaling, a separate injury group (n = 10) underwent administration of i.m./i.v. MSCs (PSE-MSC-IM-3, n = 5; PSE-MSC-IV-3, n = 5) 3 weeks after injury and were followed with anal sphincter testing and immunofluorescence at 10 days after treatment and anal pressure testing and histology at 5 weeks after treatment.

Anal Pressure Testing

Based on our previous animal model [40, 41], anal pressure monitoring was done under anesthesia using a size 4 saline-filled balloon

(Kent Scientific, Torrington, CT, http://www.kentscientific.com) connected to a pressure transducer (Grass Astromed PT300, Grass Technologies, Warwick, RI, http://www.natus.com) through a PE-190 tubing, amplifier (Astromed Inc., Model P122), and digital data recording system (Dash 8×, Astromed Inc.). Data were recorded for a mean of 30 minutes in animals anesthetized with an i.p. injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight).

Anal Pressure Analysis

Anal pressure data were characterized in terms of resting pressure (RP), peak contraction pressure (PP), number of peaks per contractions (NP), time to peak contraction (PT), contraction time (CT), and interval between contractions (CI) [41]. The anal pressures for resting, peak pressures, number of peaks, peak time, and contraction interval were analyzed with two-way analysis of variance with Bonferroni correction. Significant differences were set at $p < .05$. Data are presented as mean $\pm$ SEM.

Cell Culturing

Cell culturing was done as previously described [38]. Virgin female Sprague-Dawley rats were euthanized and bone marrow was harvested from the tibia and femurs by gently flushing the bone with 1 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The cells were centrifuged at 2,500 rpm for 5 minutes with three changes of PBS. The washed cells were placed with 25 ml of DMEM (Gibco, Invitrogen) containing 10% fetal bovine serum and 1% antibiotic and antimycotic solution (Gibco 15240, Invitrogen) and were incubated at 37°C. At this stage, the cells were identified as passage 9 (P0). The medium was changed 3 days later to remove nonadherent cells. Every 3–4 days, the medium was changed according to cellular confluence. When 70%–80% confluence was achieved, the adherent cells were detached after incubation with 0.05% trypsin and 2 mM EDTA for 5–10 minutes.

At P4, cultures were negatively selected for MSCs. Cellular sorting for MSCs was performed with the EasySep phycoerythrin (PE) selection kit according to the manufacturer's instructions (STEMCELL Technologies, Vancouver, BC, Canada, http://www.stemcell.com). CD45$^+$ and CD34$^+$ cellular markers were depleted using 10 μl of each of the primary PE-conjugated antibodies: mouse anti-rat CD45$^+$ (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) and mouse anti-CD34$^+$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com) for every 10^6 cells.

Green Fluorescent Protein Labeling

Green fluorescent protein labeling was done using a standard procedure in the laboratory. After sorting and MSC selection, when the cells reached 80%–90% confluence, MSCs were transfected with a lentivirus vector pCCLsin.ppt.hPGK.GFP.pre (a generous gift from the Cossu Laboratory), which uses a human phosphoglycerate kinase promoter to constitutively express GFP, and were processed overnight by incubation in a mixture of normal medium (6 ml), polybrene (6 μl), and 10× multiplicity of infection (10 million viruses for each million cells). Transduction proceeded overnight and the medium was changed after incubating for 6–8 hours. MSCs were checked for GFP-labeled cells under immunofluoroscopy and expanded until P12–P20 when they were used for the study. Cultures were then trypsinized and spun at 2,500
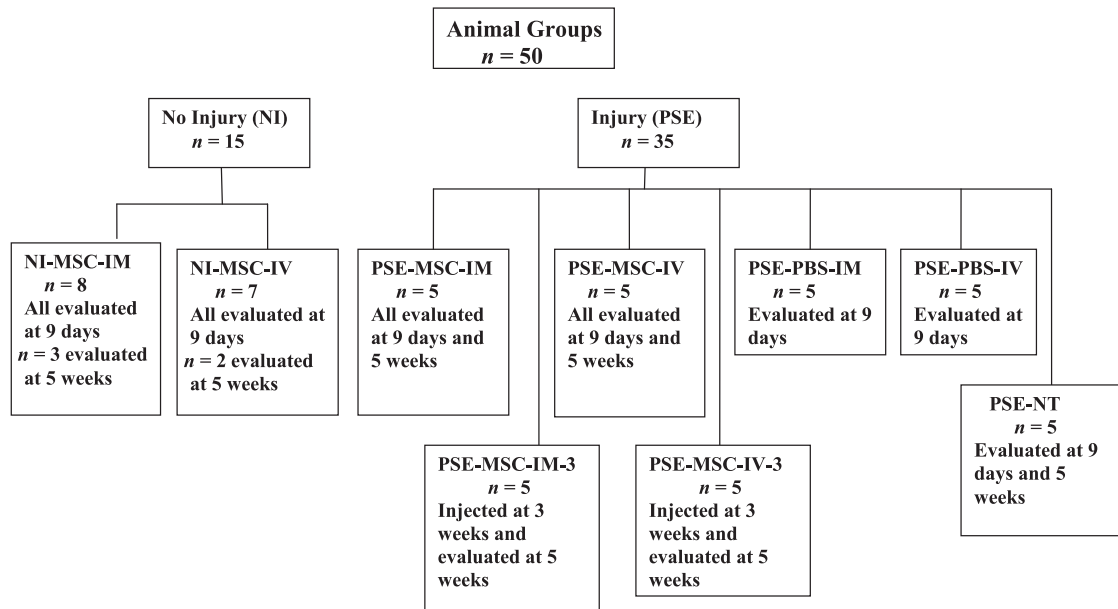
Figure 1. The animal groups and animal numbers for the study. Abbreviations: IM, intramuscular; IV, intravascular; MSC, mesenchymal stem cell; NI-MSC-IM, no injury, receiving intramuscular MSCs; NI-MSC-IV, no injury, receiving intravascular MSCs; NT, no treatment; PSE, partial anal sphincter excision; PSE-MSC-IM, injury, receiving intramuscular MSCs; PSE-MSC-IV, injury, receiving intravascular MSCs; PSE-MSC-IV-3, injury, receiving intravascular MSC 3 weeks after injury; PSE-NT, injury, receiving no treatment; PSE-PBS-IM, injury, receiving saline intramuscular MSCs; PSE-PBS-IV, injury, receiving intravascular saline.

rpm for 5 minutes. Cells were resuspended in PBS (0.2 ml for 2 million cells) for the treatments.

Histology

After rats were euthanized, the anal sphincters were excised. A part of each specimen was immersion fixed in 10% formalin for light microscopic analysis using H&E and Masson's staining.

Immunofluorescence

The anal sphincter was dissected, immersion fixed in formalin, paraffin embedded, sectioned (5 μm), and prepared for immunofluorescence studies to localize MSCs via GFP labeling. Antigen retrieval was performed by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 1 hour and then cooled for 20 minutes. The slides were then washed in PBS for 5 minutes three times at room temperature. Slides were then incubated with 1% universal blocking buffer for 3 hours at 37°C to reduce any nonspecific binding of IgG. Slides were then incubated overnight with rabbit anti-GFP (SC 8334, Santa Cruz Biotechnology; 1:200) and mouse anti-smooth muscle α-Actin (SC 1306, Santa Cruz Biotechnology; 1:30) antibodies overnight at 4°C. Slides were then washed with PBS and incubated for 2 hours with Alexa Fluor 488 (A21206, Invitrogen; 1:800). The slides were again washed with PBS and then incubated for 2 hours with goat anti-mouse IgG Texas red (SC 2781, Santa Cruz Biotechnology; 1:100). After extensive washing with PBS, the cover slips were mounted with aqueous mounting medium (Vectashield Mounting Medium) with DAPI (4[prime],6-diamidino-2-phenylindole) as a nuclear counterstain (H-1200; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com).

Tissues were analyzed by upright spectral laser scanning confocal microscope (Model TCS-SP, Leica Microsystems, Heerbrugg,

Switzerland, http://www.leica.com) equipped for blue argon (for DAPI), green argon (for Alexa Fluor 488), and red krypton (for Alexa Fluor 594) laser. GFP-positive cells were also counted per $\times$20 field objective and scanned for 10 fields.

RESULTS

Baseline Pressures

Baseline resting pressures (10.4 $\pm$ 0.2 vs. 9.1 $\pm$ 0.2 cm H_2O) and peak pressures (14.3 $\pm$ 0.5 vs. 13.1 $\pm$ 0.4 cm H_2O) were not significantly different for both control and test (PSE-MSC-IM, PSE-MSC-IV) rats, respectively. Except for NP, which was significantly higher in control rats (9.96 $\pm$ 0.64 vs. 7.22 $\pm$ 0.45 cm H_2O, $p < .001$), no significant differences were seen on the pressure parameters PT (12.24 $\pm$ 0 0.78 vs. 12.46 $\pm$ 2.41 cm H_2O), CT (35.10 $\pm$ 2.55 vs. 26.23 $\pm$ 1.68 cm H_2O), and CI (24.91 $\pm$ 2.78 vs. 27.53 $\pm$ 2.37 cm H_2O) for both control and test rats, respectively.

Anal Pressures After Injury and No Treatment

For the PSE-NT group, the baseline resting pressure was 6.76 $\pm$ 0.25 cm H_2O; the 5-week resting pressure was 7.18 $\pm$ 0.35 cm H_2O. The baseline peak pressure was 8.03 $\pm$ 0.05 cm H_2O; the 5-week peak pressure was 7.8 $\pm$ 0.47 cm H_2O.

Anal Pressures After No Injury and Treatment

Analysis of the NI-MSC-IM and NI-MSC-IV groups at 10 days and 5 weeks showed no significant difference from the pretreatment group in resting (NI-MSC-IM: 9.27 $\pm$ 0.27 cm H_2O; NI-MSC-IV: 8.93 $\pm$ 0.21 cm H_2O) or peak pressures (NI-MSC-IM: 12.85 $\pm$ 0.67 cm H_2O; NI-MSC-IV: 11.38 $\pm$ 0.27 cm H_2O).
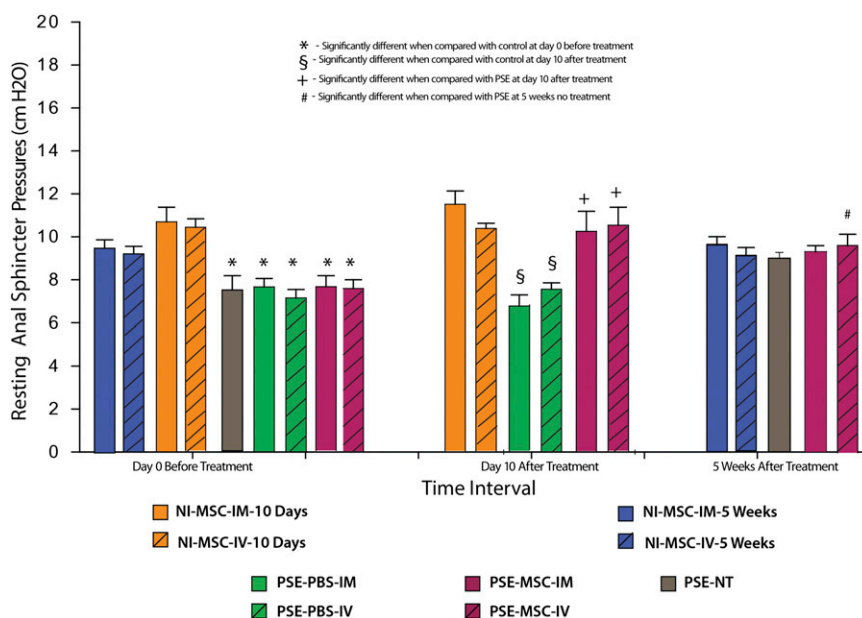
Figure 2. Comparison of resting pressures after partial anal sphincter excision or no injury, before treatment and 9 days (day 10) after treatment with MSCs or saline given i.m. or via serial i.v. infusions or no treatment. Five-weeks data reported for after injury (PSE) and no treatment or treatment with i.m. or i.v. MSCs. Each bar represents mean ± SEM. Shaded bars represent i.v. treatment of the same group. Abbreviations: IM, intramuscular; IV, intravascular; MSC, mesenchymal stem cell; NI-MSC-IM, no injury, receiving intramuscular MSCs; NI-MSC-IV, no injury, receiving intravascular MSCs; NT, no treatment; PSE, partial anal sphincter excision; PSE-MSC-IM, injury, receiving intramuscular MSCs; PSE-MSC-IV, injury, receiving intravascular MSCs; PSE-MSC-IV-3, injury, receiving intravascular MSC 3 weeks after injury; PSE-NT, injury, receiving no treatment; PSE-PBS-IM, injury, receiving saline intramuscular MSCs; PSE-PBS-IV, injury, receiving intravascular saline.

Anal Pressures After PSE and Treatment

Intramuscular MSC Administration

Nine days after i.m. treatment (day 0), a significant increase in RP ($p = .02$) was seen in the PSE-MSC-IM group when compared with pretreatment (day 10) (13.1 ± 1.2 vs. 9.5 ± 0.5 cm H_2O, respectively) (Fig. 2). A significant pressure increase in RP ($p = .003$) was also seen when the PSE-MSC-IM group was compared with the PSE-PBS-IM group (9.8 ± 0.8 vs. 6.8 ± 0.7 cm H_2O, respectively). Similarly, a significant increase in PP ($p < .001$) was seen when the PSE-MSC-IM group was compared with the PSE-PBS-IM group (13.1 ± 1.2 vs. 8.3 ± 0.6 cm H_2O, respectively).

There were no significant differences found in NP, PT, CT, and CI between the groups (PSE-MSC vs. PSE-PBS; PSE-MSC vs. PSE-NT).

Five weeks after treatment, comparison of RP and PP in the PSE-MSC-IM and PSE-NT groups (11.7 ± 0.7 and 16.6 ± 1.9 cm H_2O, respectively) showed a significant difference ($p = .04$ and .02, respectively), indicating significant pressure improvements.

Resting pressures in the PSE groups were not significantly different compared with those in the NI group at baseline (9.28 ± 0.27 vs. 8.38 ± 0.18 cm H_2O, $p = .18$, respectively) and 5 weeks post-treatment (9.17 ± 0.29 vs. 8.38 ± 0.18 cm H_2O, $p = .09$, respectively) (Fig. 2). Similarly, peak pressures were not significantly different compared with those in the NI group at baseline (12.67 ± 1.13 vs. 12.85 ± 0.67 cm H_2O, $p = .2$, respectively) and 5 weeks post-testing (13.71 ± 1.34 vs. 12.85 ± 0.67 cm H_2O, $p = .98$, respectively) (Fig. 3).

No significant differences were found in NP, PT, CT, and CI when the PSE-MSC-IM group was compared with the PSE-NT group.

Five weeks after treatment in the group that received PSE-MSC injected i.m. at 3 weeks after injury (PSE-MSC-IM-3), significant RP improvement was also seen ($p = .01$) when compared with RP before treatment (day 0). However, when comparisons of RP and PP with PSE-NT groups were made, there was no significant difference.

Intravascular Tail Vein Administration

Nine days after treatment, a significant increase was seen in RP ($p < .001$) in the PSE-MSC-IV-treated group when compared with pretreatment values (day 0) (11.03 ± 0.71 vs. 6.86 ± 0.64 cm H_2O, respectively) (Fig. 2).

A significant pressure increase in RP ($p < .001$) was also seen in the PSE-MSC-IV group compared with the PSE-PBS-IV group (11.03 ± 0.71 vs. 6.94 ± 0.98 cm H_2O, respectively). Similarly, a significant increase in PP ($p < .001$) was also seen when compared with the PSE-PBS-IV group (16.68 ± 1.33 vs. 9.64 ± 0.64 cm H_2O, respectively). In addition, at 5 weeks post-treatment, RP and PP in the PSE-MSC-IV group were significantly increased when compared with those of the PSE-PBS-IV group ($p = .01$ and $p < .001$, respectively) (Fig. 2).

Resting pressures were not significantly different compared with those of the NI group at baseline (8.93 ± 0.21 vs. 9.08 ± 0.5 cm H_2O, $p = .17$, respectively) and at 5 weeks post-testing (8.67 ± 0.47 vs. 9.08 ± 0.5 cm H_2O, $p = .24$, respectively), indicating that the pressure increase in the PSE-MSC group was comparable or similar to the control values (Fig. 2). Similarly, 5 weeks after treatment, PP was not significantly different from that of the NI group at baseline (11.38 ± 0.27 vs. 13.54 ± 0.73 cm H_2O, $p = .32$, respectively) and 5 weeks post-testing (13.74 ± 2.86 vs. 13.54 ± 0.73 cm H_2O, $p = .61$, respectively) (Fig. 3).

Except for NP, which was significantly increased when the PSE-MSC-IV group was compared with the NI-MSC-IV group (9.56 ± 1.11 and 6.52 ± 0.64 cm H_2O, respectively), parameters
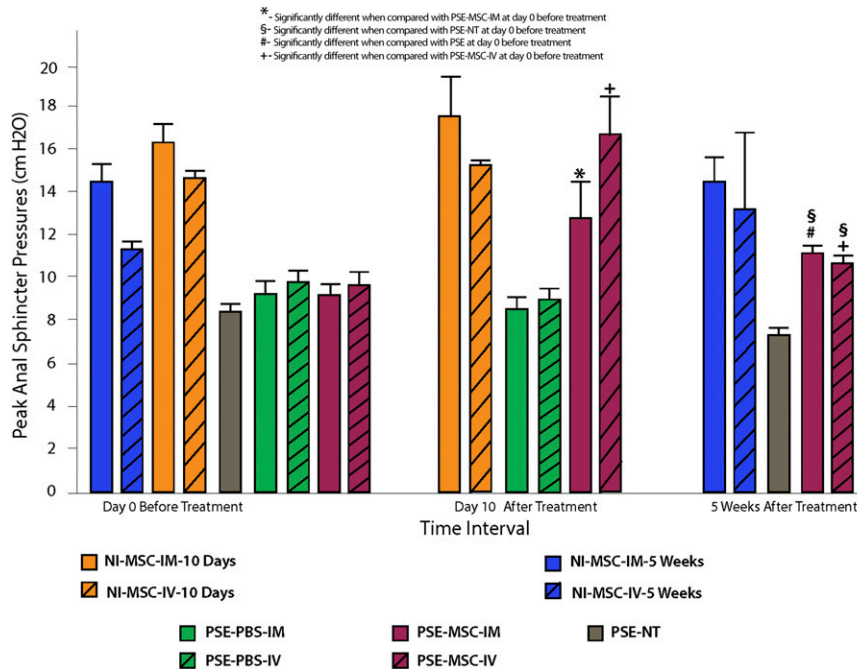
Figure 3. Comparison of peak pressures after partial anal sphincter excision or no injury, before treatment and 9 days (day 10) after treatment with MSCs or saline given i.m. or via serial i.v. infusions or no treatment. Five-weeks data reported for after injury (PSE) and no treatment or treatment with i.m. or i.v. MSCs. Each bar represents mean ± SEM. Shaded bars represent i.v. treatment of the same group. Abbreviations: IM, intramuscular; IV, intravascular; MSC, mesenchymal stem cell; NI-MSC-IM, no injury, receiving intramuscular MSCs; NI-MSC-IV, no injury, receiving intravascular MSCs; NT, no treatment; PSE, partial anal sphincter excision; PSE-MSC-IM, injury, receiving intramuscular MSCs; PSE-MSC-IV, injury, receiving intravascular MSCs; PSE-MSC-IV-3, injury, receiving intravascular MSC 3 weeks after injury; PSE-NT, injury, receiving no treatment; PSE-PBS-IM, injury, receiving saline intramuscular MSCs; PSE-PBS-IV, injury, receiving intravascular saline.

of PT, CI, and CT were not significantly different between the groups (PSE-MSC-IV vs. PSE-PBS; PSE-MSC-IV vs. NI-MSC-IV).

In groups that were i.v. injected with MSCs at the 3-week time point (PSE-MSC-IV-3), at the 5-week time point, a significant increase in RP was seen ($p < .001$) only when compared with the RP before treatment (day 0).

Comparison of Intramuscular and Intravascular Administration After PSE

Ten days after treatment, there were no significant differences in RP when the PSE-MSC-IM and PSE-MSC-IV groups were compared ($p = .37$). In contrast, PP was significantly increased in the PSE-MSC-IV group ($p = .02$). No significant differences were seen for PT, NP, CI, and CT.

Five weeks after treatment, there were no significant differences in RP between the two groups ($p = .4$). PP was also not significantly elevated ($p = .3$). No significant differences were seen for PT, NP, CI, and CT.

Histology

Figure 4A shows the normal anal sphincter with Masson's trichrome staining in the control group. Nine days after treatment, significant sphincter defect (×20) was seen in the injury groups, which had treatment with saline (PSE-PBS-IM, PSE-PBS-IV) or no treatment at all (PSE-NT) (Fig. 4B). An increase in collagen deposition, which produced an intense blue stain, was also appreciated, indicating scar tissue in the area of the defect. This is in contrast to the MSC-treated group, which showed a minimal

sphincter defect (Fig. 4C, 4D). Collagen staining was decreased (less blue staining) compared with that in the PSE-NT or PSE-PBS group, indicating less collagen deposition.

Five weeks after treatment, the no-treatment and saline groups continued to progress with fibrosis on the sphincter defect with collagen deposition, which was more intense 5 weeks after treatment. The PSE-MSC-treated group in either route (i.m. vs. i.v.) showed less fibrosis and more collagen deposition; the sphincter defect was also observed to be more coapted with new tissue formation. The immunofluorescence study showed no GFP-positive cells in any of the groups at 9 days and 5 weeks or in those injected at 3 weeks and evaluated at 5 weeks.

DISCUSSION

Regenerative medicine is a viable option for a symptomatic benign disease process, such as fecal incontinence, which occurs many years after the initial injury as it uses local tissues to regenerate the weakened or disrupted tissues in the anal canal and improve symptoms. The process of chemokine signaling has been studied by us [39]; pro- and anti-inflammatory cytokine upregulation has also been studied [35].

The models used in studies evaluating the effect of stem cells have been varied. Kang et al. [33] used a cryogenic injury, Aghaee-Afshar et al. [32] and Kajbafzadeh et al. [37] used sphincterotomy alone, and White et al. [36] and Lorenzi et al. [34] used a model of sphincterotomy and repair. Our previous injury model involved a sphincterotomy; however, this recovered over time in the animal model. This does not occur in clinical practice; hence, we
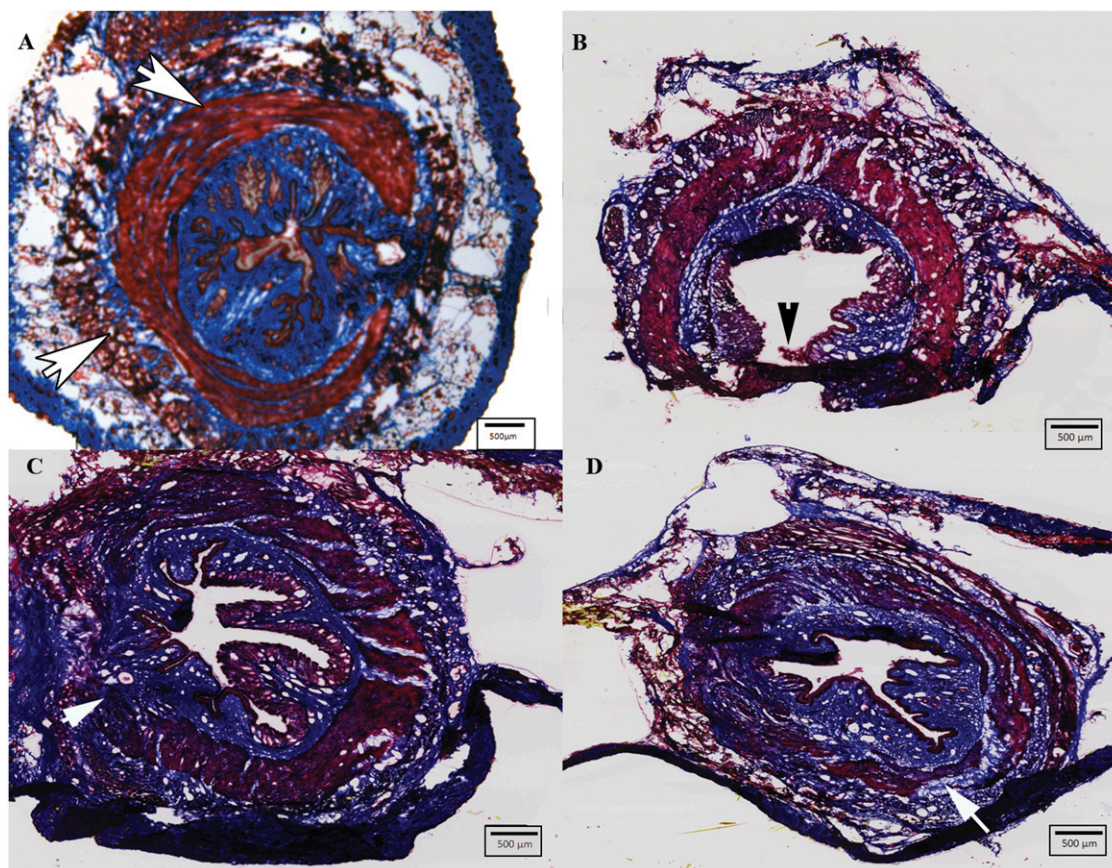
Figure 4.   Histopathology using Masson's trichrome comparing normal anal sphincter **(A)** (arrows indicate internal and external anal sphincter) with injury (partial anal sphincter excision [PSE]) and no treatment **(B)**, showing scar in the lower part; injury (PSE) followed by i.v. infusion of mesenchymal stem cell (MSC) animals euthanized at 5 weeks **(C)** and injury (PSE) followed by i.m. MSC injection **(D)**. Bridging of the gap with cells is seen in the MSC group (**[C, D]**, arrows).

moved to a model in which we excised about 25% of the anal sphincter complex to achieve a comparable result. This model did not recover, and we had a larger defect that did not recover over time and we could report histological findings.

In our previous studies, we demonstrated the effect of MSCs in response to acute injury. We also showed that the improvement in anal sphincter pressures was higher when the MSCs were delivered i.v. [38]. We studied the process of homing because in clinical practice fecal incontinence manifests many years after the acute injury and may be affected by other factors such as surgery, radiation, change in bowel habits, and aging muscles, which compound the symptoms. In this situation, by re-establishing the homing process, cells can be guided to the anal sphincter to have a therapeutic effect, as has been done in cardiac models [42, 43]. This is our aim for future studies.

Infusions of MSCs given serially i.v. or over time have shown improved outcomes in the heart [44] and in diabetes [45] in animal models. In our preliminary study [38], we demonstrated that i.v. infusions had a better effect than i.m. injections. We anticipate that i.v. infusions result in a small number of MSCs reaching the target organ; hence, in this study, we incorporated a serial i.v. infusion model. In our previous study, we demonstrated that cytokine expression declines 9 days after injury [39]; hence, we studied the effect of serial infusions that were injected over 6 days while the cytokines were still upregulated. We also delivered

a greater total number of cells by the i.v. route (3×10^6) than via the i.m. route (5×10^5) given that the majority of cells injected i.v. are localized in the lung or cleared from the system. In this study, we found that both i.m. and serial i.v. injections achieved increased pressures, suggesting that there is little advantage to injecting more cells i.v. rather than fewer cells i.m. This is contrary to the findings of Pathi et al. [35], who found i.m. injections to be more effective. They, however, studied neurophysiological effects ex vivo, and their model was of sphincterotomy and repair and they used a single i.v. injection.

The timing of the injection of stem cells has also been varied. Most studies used injections at the time of injury or injury and repair [33–36], and one study injected 2 weeks after injury, citing the need for a repair process to be in place for a decrease in scar tissue formation as a prerequisite for stem cell-induced tissue repair [32]. We timed the injection 24 hours after injury as our previous studies have indicated maximal cytokine upregulation at this time point [39] and our hypothesis is that MSCs home in response to cell signaling.

Previous studies have used various outcome measures to establish whether MSCs improve outcome. Most of the studies have used ex vivo testing that involved muscle contractility [33, 36] and histopathology alone [34]. Kang et al. [33] used electromyography and Kajbafzadeh et al. [37] used anal manometry and electromyography recording results before and after electrical stimulation.

We used in vivo methods to report anal sphincter pressures. To demonstrate new tissue formation, we used histopathology; immunofluorescence demonstrated the presence of labeled cells at the site of injury in this study.

Our aim in this study was to determine whether serial i.v. infusions offer improved outcomes over a single i.m. dose after anal sphincter injury. Our results show that a single i.m. treatment after injury results in increased pressures similar to serial i.v. injections, and these pressures were sustained 5 weeks after injection and were comparable to the anal pressures of control rats. The advantage of serial i.v. injections is that although a large percentage of the cells is lost in the lungs and spleen, the small number that are engrafted in the anal sphincter complex do so because they are chemoattracted to the site of injury and the results are a consequence of their effects on the local tissues. Intramuscular injections can cause bulking but may not be suitable for larger muscles because of the larger area to be addressed by these injections [46]. MSCs are currently being investigated in clinical trials for stress urinary incontinence as an i.m. injection probably because the urinary sphincter is much smaller in volume than the anal sphincter complex [5, 26, 28, 47]. Our results therefore have significance for directing future clinical trials of stem cell therapy for fecal incontinence by suggesting that i.m. injection of fewer cells will suffice even in this larger muscle. The anal sphincter is easily accessible by i.m. injections, making this a viable route for clinical therapy.

One limitation of this study is that we did not study pudendal nerve damage alone or as a double injury with PSE. In our preliminary study, we found that chemokine expression in response to a pudendal nerve crush occurs at different times as compared with physical injury of the sphincter complex [39]. Therefore, studying the nerve injury would involve different timings of the MSC treatment with a compromise in optimizing the right timing for either the muscle or nerve recovery in a double injury.

The second limitation is that we did not study a group with multiple i.m. injections. This is because we did not want to overload the small anal sphincter complex with a large volume of cells and have the confounding factor of bulking causing an increase in anal pressure. Other limitations are the relatively short duration of the experiment and whether this impacted the outcome in the long term. The mechanism of stem cell-mediated repair was not assessed in this study.

CONCLUSION

Both serial i.v. infusion and i.m. injections of MSCs after PSE result in increased anal pressures. This increase was sustained over the time of the experiment and was comparable to anal pressures in the control animals that did not receive an injury. The process of increased pressures after i.v. infusions can be explained by MSCs homing to the site of injury in response to the cytokine upregulation causing a positive effect. There is a possibility of a bulking effect of an i.m. injection that needs to be explored. Healing of the injury in the control rats was seen to occur by fibrosis, and more new tissue formation was seen in the MSC-treated group. Fibrosis was less with both i.v. and i.m. MSC treatments; however, the i.v.-treated group showed less scar tissue than the i.m.-treated group. Given that MSCs delivered i.m. or i.v. resulted in functional recovery, the i.m. route may be preferable as fewer cells seem to be needed.

AUTHOR CONTRIBUTIONS

L.S.: data acquisition, data interpretation, manuscript writing; M.P. and M.D.: design, data interpretation, manuscript editing; B.B.: data acquisition, data interpretation; M.Z.: conception, design, data interpretation, manuscript writing and editing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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