Early Intravenous Infusion of Mesenchymal Stromal Cells Exerts a Tissue Source Age-Dependent Beneficial Effect on Neurovascular Integrity and Neurobehavioral Recovery After Traumatic Cervical Spinal Cord Injury

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ABSTRACT

Localized vascular disruption after traumatic spinal cord injury (SCI) triggers a cascade of secondary events, including inflammation, gliosis, and scarring, that can further impact recovery. In addition to immunomodulatory and neurotrophic properties, mesenchymal stromal cells (MSCs) possess pericytic characteristics. These features make MSCs an ideal candidate for acute cell therapy targeting vascular disruption, which could reduce the severity of secondary injury, enhance tissue preservation and repair, and ultimately promote functional recovery. A moderately severe cervical clip compression/contusion injury was induced at C7-T1 in adult female rats, followed by an intravenous tail vein infusion 1 hour post-SCI of (a) term-birth human umbilical cord perivascular cells (HUCPVCs); (b) first-trimester human umbilical cord perivascular cells (FTM HUCPVCs); (c) adult bone marrow mesenchymal stem cells; or (d) vehicle control. Weekly behavioral testing was performed. Rats were sacrificed at 24 hours or 10 weeks post-SCI and immunohistochemistry and ultrasound imaging were performed. Both term and FTM HUCPVC-infused rats displayed improved ($p < .05$) grip strength compared with vehicle controls. However, only FTM HUCPVC-infusion led to significant weight gain. All cell infusion treatments resulted in reduced glial scarring ($p < .05$). Cell infusion also led to increased axonal, myelin, and vascular densities ($p < .05$). Although post-traumatic cavity volume was reduced with cell infusion, this did not reach significance. Taken together, we demonstrate selective long-term functional recovery alongside histological improvements with HUCPVC infusion in a clinically relevant model of cervical SCI. Our findings highlight the potential of these cells for acute therapeutic intervention after SCI. STEM CELLS TRANSLATIONAL MEDICINE 2019;8:639–649

SIGNIFICANCE STATEMENT

Although mesenchymal stromal cell therapy is an attractive therapeutic approach for traumatic spinal cord injury, the importance of tissue source age in therapeutic efficacy remains largely unknown. For this reason, this study aimed to compare human umbilical cord-derived fetal cells of distinct ages. Importantly, this work identified several key similarities and differences on several long-term histological as well as functional parameters. Therefore, these results can help to optimize cell treatment strategies for clinical translation.

INTRODUCTION

More than 1.3 million individuals currently live with spinal cord injury (SCI) in Canada and the U.S. (www.christopherreeve.org), with approximately 13,000 new injuries registered annually and most cases (55%–75%) affecting the cervical region [1, 2]. Traumatic SCI is characterized by an acute mechanical insult followed by a series of secondary lesional and peri-lesional events including acute vascular disruption [3–7], cell death [8, 9], ischemia, ion channel disruption, inflammation [10], demyelination, and fibro-gliotic scarring [11–14]. Of these events, inflammation and scarring have been characterized as dual-edged processes, whereby they limit the extent of damage, but also paradoxically exacerbate the condition [15–19] and inhibit endogenous regeneration [20, 21].

Significant advances in surgical techniques and clinical management [22–29] have been enabled by a better understanding of SCI pathophysiology and improved technology [30, 31], which have translated to reduced mortality and severity of disability [32]. Although surgical intervention is crucial for alleviating any ongoing mechanical trauma to the spinal cord after the initial injury [33], it can lead to reperfusion injury of the lesioned tissue [34–38]. As such, minimally invasive neuroprotective approaches designed to mitigate secondary injury and enhance tissue preservation are needed to complement existing surgical paradigms, and have already been shown to result in substantial improvements in tissue preservation and functional recovery after SCI [39, 40].

Targeting vascular disruption and promoting neovascularization with pharmacological agents such as vascular endothelial growth factor (VEGF), erythropoietin, fibroblast growth factor (FGF1), FGF2, Ang-1, Platelet-derived growth factor (PDGF), and statins has proven promising [18, 41–44], but does not allow for dynamic modulation of the peri-lesional concentrations of pro-angiogenic factors [41, 45, 46]. Mesenchymal stromal cells (MSCs) are multipotent, tissue-specific cells that are currently the leading candidates for tissue engineering and cell therapy due to their ability to rapidly expand, low immunogenicity, and secretory profile—which allows for dynamic modulation of many pathological microenvironments. They are derived from the lateral mesoderm, and can be isolated from the placenta, umbilical cord blood, perivascular tissue, and bone marrow. Currently, it is known that a common pericyte ancestry connects many MSCs. One such MSC, known as the human umbilical cord-derived perivascular cell (HUCPVC), has recently arisen as a promising candidate for cell therapy due to the greater proliferative potential and osteogenic/chondrogenic/adipogenic capacity relative to the well-characterized bone marrow-derived MSCs (BMSCs) [47]. Intravenously administered adult BMSCs have been shown to provide important benefits in models of SCI [48–51]. Despite their low levels of engraftment, they have been shown in both SCI and other Central Nervous System (CNS) conditions [52–55] to promote neovascularization and functional recovery [56]. As such, there are already several clinical trials testing the efficacy of systemic BMSC infusion for the treatment of SCI [57–60].

Since fetal and embryonic cells tend to exhibit greater proliferative potential in culture, and have better postimplantation survival, migration [61], and integration with the host CNS, we compared human umbilical cord-derived fetal cells of distinct ages [47, 62–87] to adult BMSCs. Specifically, we compared the therapeutic efficacy of term birth HUCPVC, first-trimester HUCPVC (FTM HUCPVC) and adult BMSCs on functional recovery post-SCI. Furthermore, as we have previously reported the reduction of acute vascular permeability and parenchymal hemorrhage by the early infusion of MSCs, this work aimed to characterize the long-term effects on tissue preservation and repair [88].

In this study, we show that early intravenous HUCPVC infusion in a clinically relevant model of cervical SCI leads to long-term improvements (10 weeks post-SCI) in multiple tissue and functional readouts. To the best of our knowledge, this is the first study to compare MSCs derived from different donor ages and sources, and the first to identify similarities and differences between their efficacies on several chronic histological and functional parameters after traumatic SCI. Additionally, we have applied an innovative ultrasound technique to accurately measure acute lesional volume (24 hours post-SCI) and later cavitation (10 weeks post-SCI) in live animals [89]. Together, this minimally invasive and effective approach to cell therapy has significant translational implications for the acute treatment of traumatic cervical SCI and other CNS injuries.

MATERIALS AND METHODS

Experimental Groups

Passage-matched (passage 6 to 7) HUCPVC, FTM HUCPVC, and adult BMSCs were cultured under identical conditions and compared as detailed below. Experimental animals were randomly assigned to a cell treatment or vehicle control group and given a coded designation until data acquisition and processing was complete.

Cell Isolation

Two to three centimeters of term umbilical cord was purchased from LifeLine Stem Cells (New Haven, IN), and dissected in sterile Hank's Buffered Saline (Gibco, Canada) containing 1% gentamycin using aseptic technique. The vein and arteries were carefully removed. Pieces of Wharton's jelly (umbilical cord matrix) were then transferred to collagenase I and II (1 mg/ml, Gibco), diced into small pieces and incubated for up to 2 hours at 37°C on an orbital shaker. Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS, Gibco) was then added to the viscous cell suspension prior to trituration and centrifugation at 2,000 rpm for 10 minutes at room temperature.

FTM HUCPVCs were isolated as per a published protocol [47] and were obtained from the CReATe Fertility Centre (Sunnybrook Research Institute Research Ethics Board Project Identification Number 454-2011). An in vitro characterization and comparison between term and FTM HUCPVCs had been previously reported [47]. Other cell types used included passage-matched adult human BMSCs which were obtained commercially (PT-2501, Lonza, Switzerland). The expression level of CD44 was revalidated for the three cell lines (Supporting Information Fig. S1).

Cell Culture

Cells were seeded into uncoated tissue culture T175 flasks (Greiner, Canada) in α-MEM (Gibco) containing 10% batch-tested fetal bovine serum (FBS, HyClone), 1% sodium pyruvate, 1% glutamax, and 0.1% gentamicin (Sigma–Aldrich, Canada). This culture was referred to as passage 1. After 2–5 days, the nonadherent HUCPVCs were discarded. Fresh complete medium was then added, and the adherent cells were grown to a maximum of 70%–80% confluence before passaging, harvesting for infusion or cryogenic storage. The medium was completely replaced twice a week.

Subconfluent cultures at passage number 6–7 were passaged at no more than 70%–80% confluence using trypsin-like enzyme (Sigma–Aldrich). The cells were resuspended in 1 ml fresh medium and dissociated by gentle trituration. After passing the cell suspension through a 70 μm filter to remove any clumps, total cell count and viability was determined hemocytometrically using the trypan blue dye (Sigma–Aldrich, U.K.) exclusion assay. Cells were then reseeded into fresh uncoated 140 mm tissue culture dishes (Greiner) at 5×10^5 cells per 20 ml. In order to prevent reaggregation prior to infusion, cells were resuspended in Hank's buffer containing 2 mM EDTA and were kept on ice for no more than 2 hours after dissociation.

SCI Model and Cell Infusion

All experiments involving animal use were approved by the University Health Network Animal Care Committee (Animal Use Protocol no. 979). Careful consideration was given to approved standards of reporting for all procedures and data described (Animals in Research: Reporting in vivo Experiments, ARRIVE and Minimum Information about a Spinal Cord Injury Experiment, MIASCI) [90–94]. Adult female Wistar rats (250–300 g) received a moderate–severe C7-T1 35-g clip-compression SCI for 1 minute under isoflurane anesthesia with a 1:1 mixture of O_2/NO_2 (1 liter/minute). One hour following SCI, 2.5 million cells in 1 ml Hank's buffer/2 mM EDTA were then intravenously infused through the tail vein. The vehicle control consisted of 1 ml Hank's buffer with 2 mM EDTA. Infusion via the tail vein was performed manually over 5 minutes and animals were bulk randomized into treatment groups. As an additional control, lysed cells were also infused via the tail vein at 1 hour following SCI. The cells were lysed via 1 minute of pulse sonication and resuspended in fresh vehicle. The animals were also caged singly postoperatively and kept on a 12-hour light/dark cycle. Manual bladder expression (thrice a day), cyclosporine (Sandimmune, Novartis, Switzerland) injections (10 mg/kg s.c. once daily) and buprenorphine injections (0.05 mg/kg s.c. twice daily) were performed at regular intervals (a.m., noon, or p.m.).

Very High-Resolution Ultrasound

The injured spinal cord was assessed by presacrificial echography with very high-resolution ultrasound (VHRUS) done on a 44 MHz probe (Vevo 770, Visualsonics, Canada) as described previously [95]. Three-dimensional (3D) VHRUS acquisitions were made in B-mode. The 3D files were analyzed with ImageJ software as previously described [95] with minor modifications. Instead of using a set region of interest, the bright pixel lesions were delineated by three independent blinded observers within a 15-sagittal slice (102 μm) stack to generate a reproducible lesion volume measurement at 24 hours post-SCI. At 10 weeks post-SCI, the TrakEM2 plugin [96] within Fiji [97] was used to measure the cord and cavity volume.

Drabkin's Assay

Parenchymal hemorrhage was assessed using a modified version of the manufacturer's instructions (D5941, Sigma–Aldrich). The sample was sonicated in 100 μl of deionized distilled H_2O (ddH_2O) and subsequently centrifuged at 13,000 rpm for 15 minutes. The supernatant was collected and added to the Drabkin's Reagent (Drabkin's Reagent powder in 1 liter of ddH_2O and 0.5 ml of 30% Brij 35 solution) and allowed to stand for 15 minutes while the hemoglobin-cyanomethaemoglobin reaction took place. Colorimetric measurements were performed using the Wallac 1420 spectrophotometer (Perkin Elmer; Ramsey, MN, http://www.perkinelmer.com/) at 540 nm. The results were normalized to tissue weight (in grams) and calculated based on a bovine blood hemoglobin (H2500, Sigma–Aldrich) standard curve.

Evans Blue Assay

Rats were infused with 2% Evans blue (EB) in PBS 30 minutes prior to sacrificial perfusion with 250 ml PBS. Five millimeters of lesional and peri-lesional spinal cord tissue was homogenized in 500 μl of dimethylformamide (DMF) and incubated at 50°C for 24 hours. The samples were subsequently centrifuged at 13,000 rpm for 15 minutes. The supernatant was collected and 150 μl was aliquoted into a 96-well flat bottom glass plate (Zeisser, Germany) and colorimetric measurements were performed using the Wallac 1420 spectrophotometer (Perkin Elmer) at 620 nm. The results were normalized to tissue weight (in grams) and EB concentration was calculated based on a standard curve in DMF as previously described [98].

Immunohistochemistry

Spinal cords were isolated from rats perfused with 250 ml of chilled PBS followed by 50 ml of chilled 4% paraformaldehyde (PFA). Spinal cords were then postfixed in 4% PFA overnight at 4°C before transfer to 30% sucrose (at 4°C) and storage in octamer-binding transcription factor (OCT) (at 4°C for 1–2 days then −20°C). Embedding was done in fresh OCT and 20 μm thick cross-sections were generated.

Slides were baked for 15 minutes at 55°C so cryosections would adhere permanently to the slides. After blocking in PBS + 2% FBS + 0.1% Triton X-100 for 1 hour at room temperature, primary antibodies diluted in the same diluent solution were applied overnight at 4°C. After three washes in PBS, secondary antibodies (1:200 dilution) were applied as necessary. Hoechst 33242 was used as a nuclear counterstain. Slides were mounted in Mowiol after five PBS washes. Negative staining controls (primary or secondary antibody alone) were used to assess baseline image acquisition parameters. The latter were kept consistent for each fluorescent channel between slides and treatment conditions.

To assess vascularity, endothelial cells were labeled with a DyLight 594-conjugated tomato lectin from *Lycopersicon esculentum agglutinin* (LEA, DL-1177, VectorLabs, Canada, 1:300). Myelination and axonal density were quantified using fluro-myelin (F34651, Molecular Probes; Eugene, OR, http://probes.invitrogen.com, 1:100) and anti-NF200 (N0142, Sigma–Aldrich, 1:200), respectively. Astrogliosis and glial scarring were quantified using anti-Glial fibrillary acidic protein (GFAP) (AB5541, Millipore; Burlington, MA, http://www.millipore.com, 1:200) and anti-Chondroitin sulfate proteoglycan (CSPG) (Clone CS-56, C8035, Sigma, Canada, 1:200) antibodies, respectively. All appropriate goat secondary antibodies (Alexa Fluor) were used at 1:200 dilution. Unbiased estimation of spinal cord diameter, tissue sparing, and gray-white matter ratio was carried out on StereoInvestigator software (MBF Bioscience; Williston, VT, https://www.mbfbioscience.com/) on a Nikon Eclipse E800 microscope for longitudinal cryosection slides.

Image Acquisition and Analyses

Images were acquired at 20× magnification. From three sections per rat, various fields spanning a minimum of 5 mm rostrocaudal to the injury site were stitched automatically postacquisition using StereoInvestigator software on a Nikon Eclipse E800 microscope. Images were then thresholded (based on negative control slides) and binarized, and the area of fluorescent staining was determined as a proportion of the fixed total area of the lesional and peri-lesional spinal cord.

Long-Term Neurobehavioral Assessment

All neurobehavioral assessments were performed weekly for 10 weeks after SCI by examiners blinded to the experimental group. Whole-body limb function and trunk stability was evaluated with
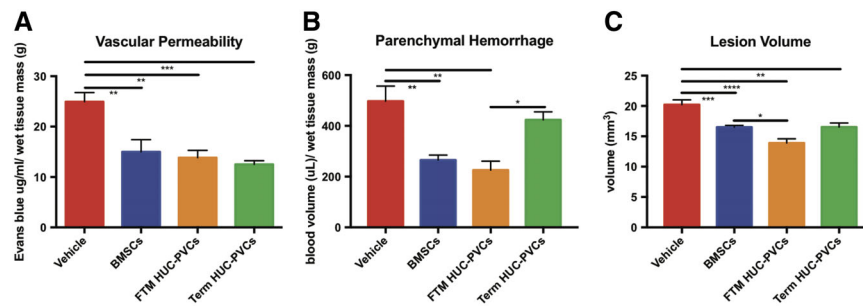
Figure 1. Early intravenous cell infusion reduced acute (1 day post-spinal cord injury) vascular pathology. **(A):** Cell infusion reduced vascular permeability as assessed by Evan's blue dye extravasation (n = 4–5 per group). **(B):** Bone marrow-derived mesenchymal stromal cells and first-trimester human umbilical cord perivascular cells reduced parenchymal hemorrhage as assessed by the Drabkin's assay (n = 4–5 per group). **(C):** Very high resolution ultrasound quantified acute lesion volume was also reduced by all cell types (n = 5 per group). Data are expressed as mean ± SEM. One-way analysis of variance (Tukey's multiple comparison). *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$; ****, $p \leq .0001$. Abbreviations: BMSCs, bone marrow-derived mesenchymal stromal cells; FTM, first trimester; HUC-PVCs, human umbilical cord perivascular cells.

the inclined plane test, where animals were placed on a horizontal plane and the incline angle was incrementally raised until they were no longer able to maintain their position [99]. Hind limb locomotion was assessed using the 22-point (0–21) Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale, as previously described [99]. Fore limb function was assessed with a grip strength meter (SDI Grip Strength System, model DFM-10; San Diego Instruments, San Diego, CA, http://www.sandiegoinstruments.com), as previously described [100].

Statistical Analyses

Statistical analyses were performed with GraphPad Prism software (La Jolla, CA). Each test is described in the corresponding figure legend. Unless otherwise stated, one-way analysis of variance and Bonferroni's multiple comparisons tests were performed, with the alpha significance threshold set to 0.05.

RESULTS

Early Intravenous MSC Infusion Reduced Acute (24 Hours Post-SCI) Vascular Pathology

Vascular permeability (Fig. 1A), parenchymal hemorrhage (Fig. 1B), and acute lesion volume (Fig. 1C) were reduced following infusions of all cell types compared with the vehicle control. Interestingly, although there was no significant difference in effect between the cell sources on vascular permeability, there were differences in hemorrhage and VHRUS-quantified lesion volume. Specifically, FTM HUCPVC had significantly reduced parenchymal hemorrhage compared with term cells (Fig. 1B) and reduced lesion volume compared with BMSCs (Fig. 1C).

To confirm that these vascular effects were indeed due to live cell infusion, and not simply a passive mechanical effect on the spinal cord vasculature and given the lack of significant difference between MSC sources on vascular permeability, we infused lysed cells in fresh vehicle. However, this resulted in immediate mortality upon infusion (data not shown).

Early Intravenous MSC Infusion Improved Long-Term (10 Weeks Post-SCI) Fore Limb Functional Recovery

A battery of standard weekly functional tests were conducted for 10 weeks following SCI and cell infusion (Fig. 2). Although all animals started off within the same weight range (250–270 g), the

rats treated with FTM HUCPVC had more weight gain relative to the rats receiving other cells types (Fig. 2A). This resulted in a significantly higher presacrificial weight than rats receiving other treatments. The effect of FTM HUCPVC-infused rats over vehicle controls was not reflected in other functional readouts at the same time points, except for grip strength (Fig. 2B). Furthermore, the grip strength of HUCPVC versus FTM HUCPVC-infused rats was significantly different from 5 to 10 weeks post-SCI. The two green asterisks at 2 and 4 weeks post-SCI represent the greater grip strength of term HUCPVC compared with the vehicle control, with a p = .0504 and .0514, respectively. As aforementioned, no significant differences were observed in hind limb motor function (Fig. 2C) and BBB scores between groups.

Although there were no significant differences in inclined plane between conditions at 1 and 8 weeks post-SCI (Fig. 2D), HUCPVC-infused rats performed better than vehicle-infused controls at 9 and 10 weeks post-SCI (p = .0394 and .0042, respectively) and all cell-infused rats were better than vehicle controls at 10 weeks post-SCI (with BMSC versus vehicle at p = .009, and FTM HUCPVC versus vehicle at p = .0012).

Early Intravenous MSC Infusion Improved Long-Term (10 Weeks Post-SCI) Neurovascular and Neuroanatomical Integrity

Neurovascular and neuroanatomical integrity (Fig. 3) was assessed by quantification of vascular density (LEA), myelination (fluoromyelin), and axonal density (NF200), respectively. All parameters were expressed as the proportion of the area within a 5 mm length of lesioned spinal cord with positive staining. FTM HUCPVC and BMSC infusion resulted in significantly higher (p < .05) vascularity, myelination, and axonal density compared with vehicle-infused control rats at 10 weeks post-SCI; Fig. 3). There was no significant difference between cell sources for any of these three parameters, although HUCPVC infusion consistently resulted in higher values for these parameters than vehicle controls without reaching statistical significance.

Early Intravenous MSC Infusion Reduced Long-Term (10 Weeks Post-SCI) Glial Scarring

Astrogliosis (GFAP) and glial scarring (CSPG) were also examined (Fig. 4). Both parameters were expressed as the proportion of the area within a 5 mm length of lesioned spinal cord with positive staining. Infusion of all three cells resulted in significantly
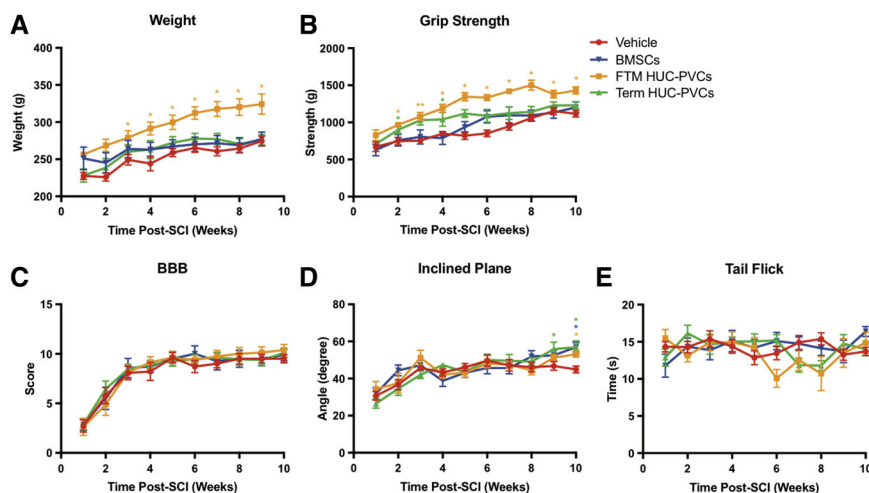
Figure 2. Early intravenous first-trimester human umbilical cord perivascular cell (FTM HUCPVC) infusion enhanced long-term (10 weeks postspinal cord injury [SCI]) functional recovery. Weekly standardized behavioral testing was applied for 10 weeks after SCI and cell infusion (vehicle, $n = 15$; bone marrow-derived mesenchymal stromal cells, $n = 8$; FTM HUCPVCs, $n = 13$; term HUCPVCs, $n = 15$) by examiners blinded to the experimental groups (two-way analysis of variance, Bonferroni's multiple comparison post hoc test). Animals were weighed weekly **(A)** alongside fore limb motor function assessed with a grip strength meter **(B)**; hind limb locomotion assessed by using the 22-point (0–21) Basso, Beattie, and Bresnahan Locomotor Rating Scale **(C)**; whole-body limb function as well as trunk stability was evaluated with the inclined plane test **(D)**; and thermal allodynia via the tail flick test **(E)**. Data are expressed as mean $\pm$ SEM. *, $p \leq .05$; **, $p \leq .01$. Abbreviations: BBB, Basso, Beattie, and Bresnahan Locomotor Rating Scale; BMSCs, bone marrow-derived mesenchymal stromal cells; FTM, first trimester.

reduced glial scarring compared with vehicle-infused control rats. There was no significant difference between any of the cell sources.

No Significant Differences in Other Long-Term (10 Weeks Post-SCI) Neuroanatomical Assessments

Cavitation, tissue sparing, gray:white matter ratio, and spinal cord diameter were measured at 10 weeks following SCI and cell infusion (Fig. 5). Cavitation was assessed by VHRUS immediately prior to sacrifice, and while trends toward reduced cavitation with cell infusion were clear (Fig. 5A); no statistically significant effects relative to the vehicle control were observed for any cell type. The other neuroanatomical parameters assessed via StereoInvestigator software after histological processing (tissue sparing, gray:white matter ratio, and spinal cord diameter) were similar among the experimental groups (Fig. 5C, 5E).

DISCUSSION

In this study, we compared the efficacy of acute intravenous infusion of term HUCPVCs, FTM HUCPVCs, and BMSCs on acute neurovascular readouts and functional recovery after traumatic cervical SCI. In general, we found that acute systemic infusion of term HUCPVCs, FTM HUCPVCs, or BMSCs was able to reduce vascular permeability and lesional volume, as assessed by EB and VHRUS, respectively. Similarly, all three cell types conferred improvements in astrogliosis and scarring. However, while both BMSCs and FTM HUCPVCs were able to confer improvements in parenchymal hemorrhage, we did not observe any significant improvements with term HUCPVCs. Furthermore, the efficacy of BMSCs and FTM HUCPVCs continued to be reflected at the tissue level with increased vascular density, myelination, and axonal preservation relative to the vehicle control. Surprisingly, no gross anatomical/pathological

differences in cavitation, tissue sparing, or gray:white matter ratio were observed with cell infusion. Finally, in terms of functional improvement, FTM-HUCPVC was the only cell type that resulted in lasting chronic effects in fore limb function and weight gain.

Our findings here mirror our recent study with human brain-derived vascular pericytes, whereby an acute infusion was able to mitigate acute neurovascular readouts and result in chronic functional improvement after traumatic cervical SCI [88]. In this study, we find that this effect is not exclusive to brain-derived pericytes, but also extends to umbilical cord-derived MSCs. Furthermore, this study demonstrates a clear age-related difference in cell efficacy after SCI, with FTM cells being more efficacious than term cells. Albeit, further work, including more detailed immunohistochemical analysis, is needed to validate these findings.

FTM HUCPVCs Exert Their Therapeutic Effects Through Remote Mechanisms

To ascertain the mechanism behind the robust neurovascular and behavioral effects of the FTM HUCPVCs, we looked for the presence of infused cells at or near the lesion site (data not shown). However, we were unable to find any transplanted cells at the lesional and peri-lesional area. Strikingly, our lab and others [88, 101, 102] have recently reported that, while systemically infused MSCs distribute mainly to the lung and spleen, they continue to exert therapeutic effects on the local spinal cord milieu. In these studies, the authors propose two possible mechanisms through which the remotely distributed MSCs exert their therapeutic effect: (a) MSCs serve as cellular decoys, where the immune system targets these exogenous human cells instead of endogenous cells, thus minimizing damage; and (b) MSCs release anti-inflammatory cytokines in the spleen, such as IL10, and impact immune cell recruitment and phenotype.
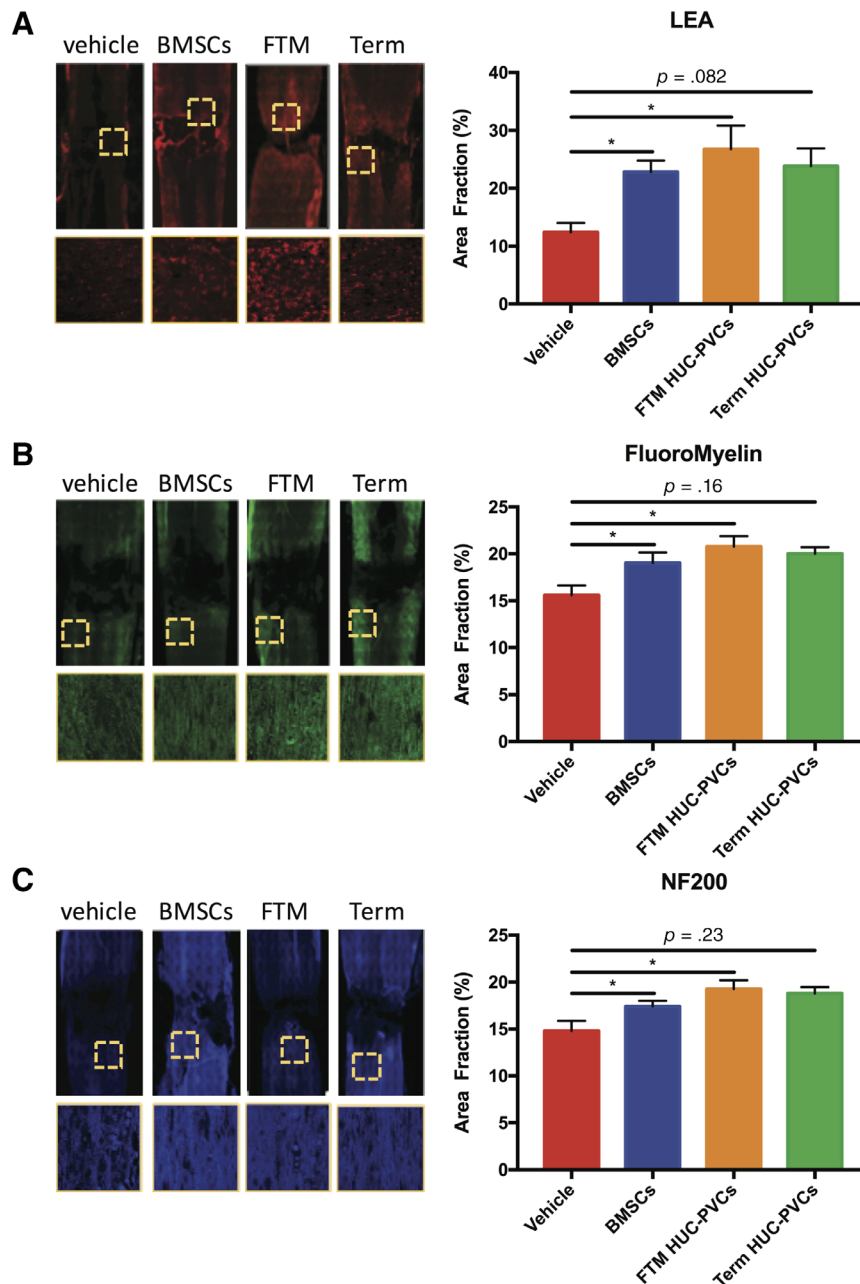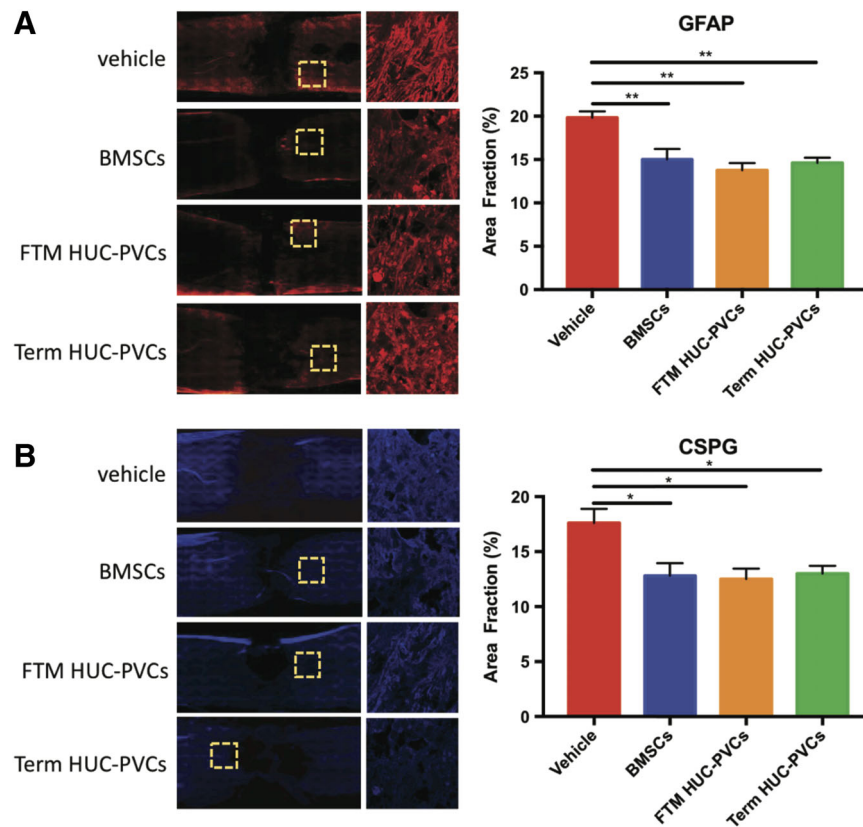
Figure 3. Early intravenous cell infusion improved long-term (10 weeks postspinal cord injury) neurovascular and neuroanatomical integrity. Cell infusion of bone marrow-derived mesenchymal stromal cells and first-trimester human umbilical cord perivascular cells increased vascularity **(A)**, myelination **(B)**, and axonal preservation **(C)** as measured by *Lycopersicon esculentum agglutinin*, fluoromyelin, and NF200, respectively (n = 4–5 per group). All parameters were expressed as the proportion of the positively stained area within a 5 mm length of spinal cord (2.5 mm rostral and 2.5 mm caudal to the lesion epicenter). Data are expressed as mean ± SEM. One-way analysis of variance (Bonferroni's multiple comparison). *, $p \leq$ .05. Abbreviations: BMSCs, bone marrow-derived mesenchymal stromal cells; FTM, first trimester; HUC-PVCs, human umbilical cord perivascular cells.

Age-Related Differences in MSC Efficacy

In our study, we observed profound differences in the efficacy of term HUCPVCs and FTM HUCPVCs at the tissue and behavioral level. Several studies have characterized the impact of aging on various MSC features, including apoptosis [103], proliferation [104, 105], and engraftment capacity [106]. Indeed, age-dependent differences in one or more of these parameters could directly attenuate the efficacy of MSCs through either one of the potential therapeutic mechanisms. Here, we find in accordance with the literature, that FTM HUCPVCs slightly outperformed term HUCPVCs in the reduction of neurovascular disruption, astrogliosis, scarring, and ultimately resulted in lasting functional recovery following cervical SCI. Previous characterization [47] of these two cell types demonstrated that FTM HUCPVCs have expression of the Oct4A and Sox17 proteins while term HUCPVCs did not—which may contribute to the greater proliferative potential of the younger cells. In addition, FTM HUCPVCs showed superior angiogenic potency compared with BMSCs [107], as well as term HUCPVCs (unpublished).

Figure 4. Early intravenous cell infusion reduced long-term (10 weeks postspinal cord injury) glial scarring. Cell infusion reduced astrogliosis **(A)** and glial scarring **(B)** as measured by GFAP and CSPG, respectively (n = 4–5 per group). All parameters were expressed as the proportion of the positively stained area within a 5 mm length of spinal cord (2.5 mm rostral and 2.5 mm caudal to the lesion epicenter). Data are expressed as mean ± SEM. One-way analysis of variance (Bonferroni's multiple comparison). *, $p \leq .05$, **, $p \leq .01$. Abbreviations: BMSCs, bone marrow-derived mesenchymal stromal cells; CSPG, chondroitin sulfate proteoglycan; FTM, first trimester; GFAP, glial fibrillary acidic protein; HUC-PVCs, human umbilical cord perivascular cells.

### FTM HUCPVCs Result in Long-Term Functional Recovery

Our long-term behavioral improvements with FTM HUCPVCs are consistent with previous MSC studies [49–51]. Matsushita et al. [49] used a T9 SCI model of similar functional severity to this study (with BBB plateauing at 10). Similarly, Quertainmont et al. [51] used a slightly more severe T8 SCI model (with BBB plateauing at 8). Both groups intravenously infused 1-million adult rat BMSCs 1 week post-SCI but were also unable to detect the infused cells within the spinal cord, despite reporting significant cell-induced reductions in vascular permeability and long-term functional benefits at 9 weeks postinfusion.

Quertainmont et al. [51] also report enhanced tissue sparing, neovascularization, improved BBB, and grid navigation. We have noticed, however, that the Matsushita et al. [49] study found BBB to stabilize at 10 weeks in untreated rats at a score of approximately 10.2 ± 2.0 (with larger error bars). However, vehicle-infused rats (used as a negative control) stabilized at 10 weeks at an average score of only 8.25 ± 0.81 (with considerably smaller error bars). Furthermore, the reported BBB score at 1 week post-SCI in untreated rats was 2.9 ± 1.3 but approximately five in the cohort of rats which were infused at this time point. The authors do not report comparing untreated and DMEM-treated rats, and no other functional tests are mentioned. As such, a comparison between functional results obtained by Matsushita et al. [49] and this study

is difficult. Other differences are apparent—Quertainmont et al. [51] deprived MSCs of serum for 72 hours prior to infusion via the tail vein. No details regarding culture conditions are provided by Matsushita et al. [49], who infused cells through the femoral vein. Nevertheless, the greater functional improvements reported in these studies are possibly a consequence of better infused allogeneic cell survival. Even with immunosuppression until sacrifice, cell survival within our study was likely much lower.

Technical Considerations

The most effective cell administration route for the integration of donor cells within and near the lesion site [108–114] is still debated, even though the invasiveness of intraparenchymal cell injection is well recognized, in addition to the challenge this poses to the application of cell therapy, especially during the acute phase of injury. However, the benefits of intravenous cell administration are clear from this study as well as others [49–51, 88]. Although not yet a fully validated therapy for SCI, the safety of this approach has already been established through preclinical SCI studies [115], its clinical application to other conditions affecting the CNS, including multiple sclerosis [64] and bone marrow transplantations. Nevertheless, this approach is not without its limitations, osteoblastic differentiation of infused MSCs has been
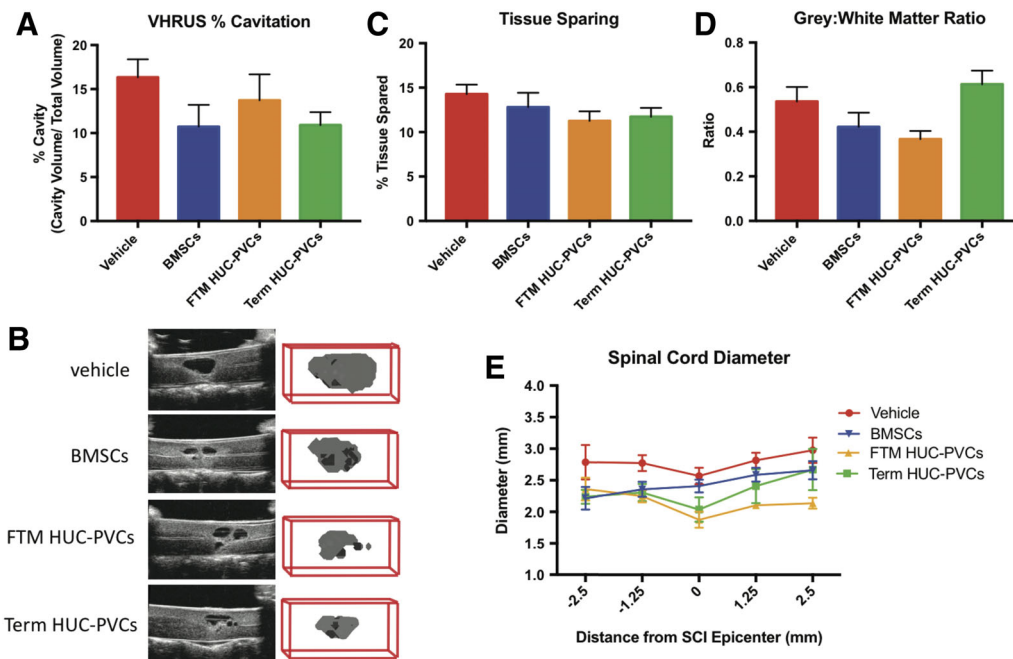
Figure 5. Early intravenous cell infusion did not affect long-term (10 weeks postspinal cord injury) cavitation and tissue sparing. Cell infusion had no effect on cavitation (*n* = 5 per group) measured by very high resolution ultrasound (VHRUS) immediately prior to sacrifice **(A)**. Representative images of the VHRUS scans and three-dimensional cavity reconstructions are shown for all the experimental groups **(B)**. Tissue sparing **(C)**, gray-white matter ratio **(D)**, and spinal cord diameter **(E)** were assessed via StereoInvestigator software after histological processing (*n* = 4–5 per group). Data are expressed as mean ± SEM. One-way analysis of variance (Bonferroni's multiple comparison). Abbreviations: BMSCs, bone marrow-derived mesenchymal stromal cells; FTM, first trimester; HUC-PVCs, human umbilical cord perivascular cells; SCI, spinal cord injury.

reported within lung tumors [116] and although this has not been observed in injury and degeneration models, this possibility should not be dismissed. Furthermore investigation is necessary prior to clinical translation.

Another important point of discussion is the significant difference in weight gain among the experimental groups. Although all animals started off within the same weight range (250–270 g), we acknowledge that the rise in weight with FTM HUCPVCs may also account for some of the improvements in fore limb grip strength. Nevertheless, as weight loss and the need for nutritional support remains a major concern following SCI [117], this finding still supports general improvements in animal wellbeing following FTM HUCPVCs treatment.

Future Work

The mechanisms by which these effects are produced remain to be explored. The lack of detection of infused cells within the peri-lesional area of the spinal cord imposes limits on the identification and characterization of the population of cells responsible for the local benefits observed. On the other hand, it also suggests that acute benefits are preserved chronically even in the absence of infused cells at or near the lesion site. As such, an examination of the secretory profiles of the cells used in this study is warranted to determine possible molecular mediators. Furthermore, to enhance the secretory capacity of these cells, an investigation into cell priming prior to infusion is necessary. It is also important to note that the use of FBS for cell culture has limited translational relevance and is not suitable for good manufacturing practice compliant cell therapy products. For this reason, future work should

substitute FBS for platelet lysate or chemically defined media in cell culture [118].

CONCLUSION

We have shown that early MSC infusion in a clinically relevant model of moderate–severe cervical SCI leads to reduced acute neurovascular pathology, enhanced neuroanatomical readouts, and improvements in fore limb function, and that these effects are donor age-dependent and remotely mediated. All in all, FTM HUCPVC-infusion outperformed commercially available BMSCs and conferred long-term functional recovery, making it a prime candidate for further studies.

AUTHOR CONTRIBUTIONS

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author or CReATe Program Inc. upon reasonable request.

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