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Human neural stem cell transplant location–dependent neuroprotection and motor deficit amelioration in rats with penetrating traumatic brain injury

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BACKGROUND: Penetrating traumatic brain injury induces chronic inflammation that drives persistent tissue loss long after injury. Absence of endogenous reparative neurogenesis and effective neuroprotective therapies render injury-induced disability an unmet need. Cell replacement via neural stem cell transplantation could potentially rebuild the tissue and alleviate penetrating traumatic brain injury disability. The optimal transplant location remains to be determined.

METHODS: To test if subacute human neural stem cell (hNSC) transplant location influences engraftment, lesion expansion, and motor deficits, rats (n = 10/group) were randomized to the following four groups (uninjured and three injured): group 1 (Gr1), uninjured with cell transplants (sham+hNSCs), 1-week postunilateral penetrating traumatic brain injury, after establishing motor deficit; group 2 (Gr2), treated with vehicle (media, no cells); group 3 (Gr3), hNSCs transplanted into lesion core (intra); and group 4 (Gr4), hNSCs transplanted into tissue surrounding the lesion (peri). All animals were immunosuppressed for 12 weeks and euthanized following motor assessment.

RESULTS: In Gr2, penetrating traumatic brain injury effect manifests as porencephalic cyst, 22.53 ± 2.87 (% of intact hemisphere), with p value of <0.0001 compared with uninjured Gr1. Group 3 lesion volume at 17.44 ± 2.11 did not differ significantly from Gr2 ($p = 0.36$), while Gr4 value, 9.17 ± 1.53, differed significantly ($p = 0.0001$). Engraftment and neuronal differentiation were significantly lower in the uninjured Gr1 ($p < 0.05$), compared with injured groups. However, there were no differences between Gr3 and Gr4. Significant increase in cortical tissue sparing ($p = 0.03$), including motor cortex ($p = 0.005$) was observed in Gr4 but not Gr3. Presence of transplant within lesion or in penumbra attenuated motor deficit development ($p < 0.05$) compared with Gr2.

CONCLUSION: In aggregate, injury milieu supports transplanted cell proliferation and differentiation independent of location. Unexpectedly, cortical sparing is transplant location dependent. Thus, apart from cell replacement and transplant mediated deficit amelioration, transplant location–dependent neuroprotection may be key to delaying onset or preventing development of injury-induced disability. (*J Trauma Acute Care Surg.* 2020;88: 477–485. Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.)

LEVEL OF EVIDENCE: Preclinical study evaluation of therapeutic intervention, level VI.

KEY WORDS: Traumatic; brain injury; neural stem cells; transplantation; neuroprotection; rat.

Traumatic brain injury (TBI) is a burgeoning public health problem worldwide.[1] Penetrating TBI (pTBI), particularly involving firearms, is an increasingly serious issue in the United States within the military[2] and civilian contexts. Severe pTBI occurs when an object breaches the skull and dura, with direct damage to the brain parenchyma.[3] Neurosurgical interventions (decompression and hematoma removal) manage primary injuries such as hemorrhage; laceration and contusion help reduce intracranial pressure. Timely neurosurgical intervention, improved neuroimaging, and acute trauma management have lowered the firearm fatality rate[4] but left survivors with lifelong debilitating impairments.[5] Secondary mechanisms such as hypoxia, hypotension, ischemia, and systemic inflammatory response also increase the mortality and morbidity.[6,7] The goal in the hospital is to limit the impact of secondary injury, which would otherwise magnify the initial harm. Unfortunately, many drugs showing significant promise in preclinical studies did not translate through clinical trials to become therapeutics.[8,9] Primary axotomy at the time of the injury triggers self-destructive mechanisms (e.g., Wallerian degeneration) in the severed distal portion and proximal soma.[10] Incomplete clearance[9,11,12] of damaged axonal fragments and accumulated β-amyloid precursors[11,13] for several years[14] provoke chronic inflammation,[15–17] which contributes to delayed remote neurodegeneration and secondary axotomy.[18] Thus, host immune response, although important for repair of injury, may cause further damage to healthy tissue.[19] Secondary mechanisms are not readily amenable to medical treatments.[20] In most cases of pTBI, a permanent cavity *gliotic porencephalic cyst* is evident in survivors,[21,22] which expands because of inflammation driven

Submitted: June 4, 2019, Revised: August 30, 2019, Accepted: September 17, 2019, Published online: October 15, 2019.

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This study was presented at the 25th annual meeting of Military Health System Research Symposium, August 27, 2017, in Kissimmee, Florida; National Neurotrauma Symposium, June 2017, in Louisville, KY; and Society for Neuroscience, November 13, 2017, in Washington, DC.

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DOI: 10.1097/TA.0000000000002510

by progressive tissue loss.[9,23] Such long-term neuronal loss is especially important in humans[2,11] because of limited pool of human neural stem cells (hNSCs) and consequent low neurogenic potential.[24] In contrast to rodents, relative absence of reparative neurogenesis in primates[25] impedes attempts to repair injured central nervous system (CNS) circuitry by boosting endogenous hNSC proliferation.[26] However, because NSCs have the capacity to replace lost brain cells,[27] stimulate recovery by repairing damaged CNS,[28] stabilize TBI lesion,[29] and modulate neuroinflammation,[30,31] cell transplantation is considered as viable option. Numerous studies including those from our group have shown benefits of hNSC transplants in rodent models of severe focal TBI.[32–34] Transplantation of human fetal NSCs (hNSCs) within 24 hours of experimental TBI reduced microglial activation and alleviated cognitive deficits.[30,35] Recently, our laboratory demonstrated robust and durable engraftment of fetal origin hNSC from Neuralstem Inc. (NSI-566) after pTBI in rodents with amelioration of cognitive deficit.[34] The U.S. Food and Drug Administration approved these NSCs for use in phase I/II clinical trials for other CNS disorders but not yet for TBI.[28,36,37] The lack of effective Food and Drug Administration–approved TBI therapies and the limited potential of endogenous repair in human TBI provide a strong rationale to test neural stem cell (NSC) transplantation in severe pTBI.

In transplantation studies, directed at both focal TBI and SCI, authors have speculated that the limiting membrane surrounding the gliotic cavity may constitute a barrier to axonal regeneration and passage of putative trophic substances.[38,39] To determine the best site, we hypothesized that hNSC transplant location (perilesional vs. intralesional), with respect to pTBI lesion, will influence grafted cell survival and endogenous tissue loss. To test this, 1 week after pTBI, hNSCs were transplanted within the lesion (intralesion) or area surrounding it (perilesion). At 12 weeks posttransplantation the following were measured: (1) histological quantification of stained brain sections for tissue loss, (2) transplanted cell engraftment as a primary outcome, and (3) motor deficit development as a secondary outcome.

MATERIALS AND METHODS

Study Design and Animal Procedures

All animal procedures followed guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Animal Research: Reporting of In Vivo Experiments, and were approved by U.S. Army Medical Research and Material Command, Animal Care and Use Review Office, and University of Miami's Institutional Animal Care and Use Committees. Male Sprague-Dawley rats (no less than 280 g) were randomized to the following four groups. (1) pTBI treated with vehicle (media, no cells), (2) uninjured rats with cell transplants (sham+hNSCs), and PTBI groups with cell transplants either (3) into tissue surrounding the lesion (peri) or (4) into lesion core (intra). Unilateral pTBI was produced in rats under anesthesia using aseptic surgical procedures (see Table 1 for animal usage). Briefly, an incision was made along the midline of the skull. The pTBI probe was aligned at 50 degrees from vertical and 25 degrees from midline at a point 2 mm lateral and 4.5 mm rostral from bregma. A burr hole was made at the location, and the pTBI probe was inserted 12 mm into the brain via the burr hole. The probe was inflated to 6.33 mm diameter for 40 milliseconds and then retracted from the brain. The scalp was closed with 12 mm wound clips and cleaned again with chlorohexidine. Buprenorphine was administered subcutaneously (0.01 mg/kg).

A priori, desired effect size was set to 0.7 based on previous publications[34,40] and using G*Power3.1 with power set at 0.80, α at 0.05; the sample size after accounting for 10% attrition was adjusted to n = 10 for the histopathology; and a sample size of n = 15 was needed for the motor outcome. Sample size calculations were authenticated with My Sample Size (https://www.mysamplesize.com/). Investigators blinded to the study design and experimental groups digitized brain sections images, manually counted green fluorescent protein (GFP)–positive cell numbers, and performed quantitation in histological sections using unbiased stereology.

One week following injury, animals were anesthetized again for transplantation. Following a midline scalp incision, the right frontal pole (−3.5 mm AP [anterio-posterior], −2 mm medio-lateral [ML] to bregma) was exposed. Using a gas tight 250-µL Hamilton syringe filled with 1 million GFP expressing NSI566 stem cells in media at 50,000 cells/µL cell deposited within lesion a single bolus. For injection into penumbra, the cell-filled microsyringe was aligned to +2.72 mm AP and +1.5 mm ML (from bregma), advanced ventrally to 6 mm depth for first cell drop, and raised to 4 mm below surface for second drop; then, two drops were deposited at +3.5 mm ML at two depths. Next, drops were at −2.28 mm AP with similar ML; thus, with eight cell drops, the corners of a stereotactically defined "5 mm box" (+2.72 to −2.28 mm bregma) were covered.[34] All animals were immunosuppressed with tacrolimus, mycophenylate, and depomedrol as described earlier for 12 weeks.[34] Perfusion, histology, and chemical stains were done using previously published standard protocols. Approximately 11 mm blocks of cerebrum between +3.72 mm and −6.78 mm bregma were processed into 35-µm-thick serial sections, for a total 14 series. Each series has 22 sections that are 0.5 mm apart and thus span both lesion and transplant. One such series, mounted on glass slides, was stained with hematoxylin and eosin. Glass slides with brain sections were scanned to produce images. Lesion volume analysis was done on these images of using modified version of CalLesion.[41] The pTBI porencephalic cyst intersects with the lateral ventricle across the rostrocaudal axes of the brain, so as to be consistent with previous pTBI studies, lesion area was defined as the area of expanded ventricle plus lesion (porencephalic cyst) minus the area of contralateral ventricle expressed as percent of left hemisphere.[40]

Immunohistochemistry, Imaging, and Analysis

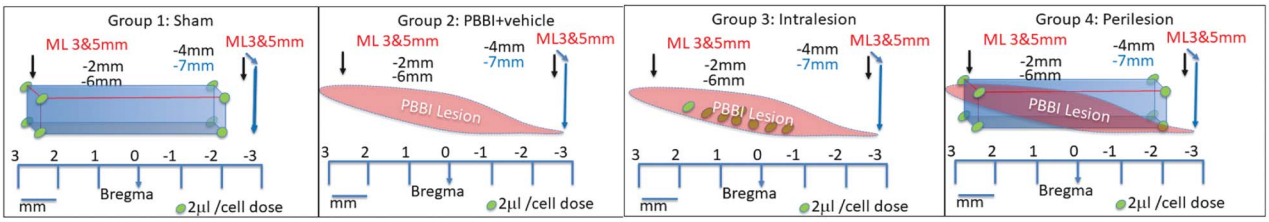
Immunohistochemistry was performed on the brain sections using a standard protocol described previously.[15,34] Volumetric GFP cell counts were generated using the physical fractionator method in StereoInvestigator (version 10.6, Stereo Investigator; MBF Bioscience, Williston, VT) on evenly spaced (0.2 mm apart) brain sections and then used to estimate the total cell survival as described earlier.[34] Cortical thickness measurements and three-dimensional (3D) renderings were produced using Neurolucida as described earlier.[34]

Footfault Test, a Motor Assessment

The animals' ability to integrate motor responses was assessed using the footfault test validated previously.[42] The rats

TABLE 1. Experimental Design. Animal Numbers, Experimental Group Allocation, and Outcome Assessments

Sources of Variation		Experimental Groups			
		Sham	Vehicle	Intralesion	Perilesion
Experiment Conditions	Injury	No	Yes	Yes	Yes
	Transplantation at 1wk post PTBI	Yes	No	Yes	Yes
	Survival post injury (Weeks)	13	13	13	13
	Immunosuppression	Yes	Yes	Yes	Yes
	Sample size (n/group)	10	10	10	10



Effect of variations on outcomes measured		Sham	Vehicle	Intralesion	Perilesion
Experiment Outcomes Median (Interquartile range)	Lesion volume (% left hemisphere.	0.46 (1.58)	24.94 (12.98)	15.1 (9.56)	7.54 (6.92)
	Spared tissue (% left hemisphere)	102.0 (4.0)	63.5 (30.68)	58.0(29.18)	84.5(24.44)
	Spared motor cortex (% left hemisphere)	99 (7.56)	23.5(19.37)	8.0(16.06)	29.0(16.38)
	Engraftment % input hNSC counts, % animals	24.48 (33.26)	N/A	53.09 (89.85)	79.58(120.61)
	%hNSC neuronal differentiation (GFP+NeuN+)	6.28(7.51)	N/A	15.66(8.43)	13.38(7.01)
	Left front foot fault %	6.49(8.65)	23.0(10.8)	17.33(7.19)	17.0(6.99)

In the top half of the table, the first column lists sources of variation, second to fifth columns list the four experimental groups and rows contain their values, illustration shows the transplant location relative to lesion, lower portion of the table lists outcomes, and rows present values obtained for each outcome in corresponding experimental condition.

were placed onto a suspended wire mesh (40 × 150 cm; height, 50 cm; mesh size, 5 cm), and the correct and incorrect placements (footfaults) of the affected forelimb were recorded for 60 seconds per trial from a recorded video (four trials per session).

Statistical Analysis

Presence of graft was used as an inclusion criterion for behavior analysis, as the hypothesis depends on successful engraftment of transplant. One animal from each transplant group had

to be excluded because of poor engraftment, defined as a presence of less than 1% or less of input cells.[39] End-points were compared by analysis of variance (ANOVA) followed by post hoc and paired *t* test analyses when appropriate (Sigma Stat, San Jose, CA). All data are presented as the mean ± SEM, and *p* values of <0.05 were considered significant.[40,43]

RESULTS

The experimental design and details shown in Table 1 state the sources of variation in the study. Unilateral pTBI as described elsewhere[44] produces progressive tissue loss.[40] Representative sections from each experimental group (Fig. 1A) show that, when compared with sham, the vehicle-treated animals had an enlarged ventricle with a large porencephalic cyst, a thinner cerebral cortex, and substantial subcortical tissue loss. In both pTBI+hNSCs groups, 90% of the intralesion and perilesion animals had an engraftment of greater than 1% of the input cells. In the sham group, only 60% of the animals had successful hNSC engraftment, and transplanted cells appeared to persist as a thin sliver of tissue without visible damage to the normal brain parenchyma or migration of cells from the transplantation site. Engraftment of hNSCs in the sham group was thus significantly less than that in the pTBI +hNSCs groups. Cortical thinning as seen in vehicle-treated rats

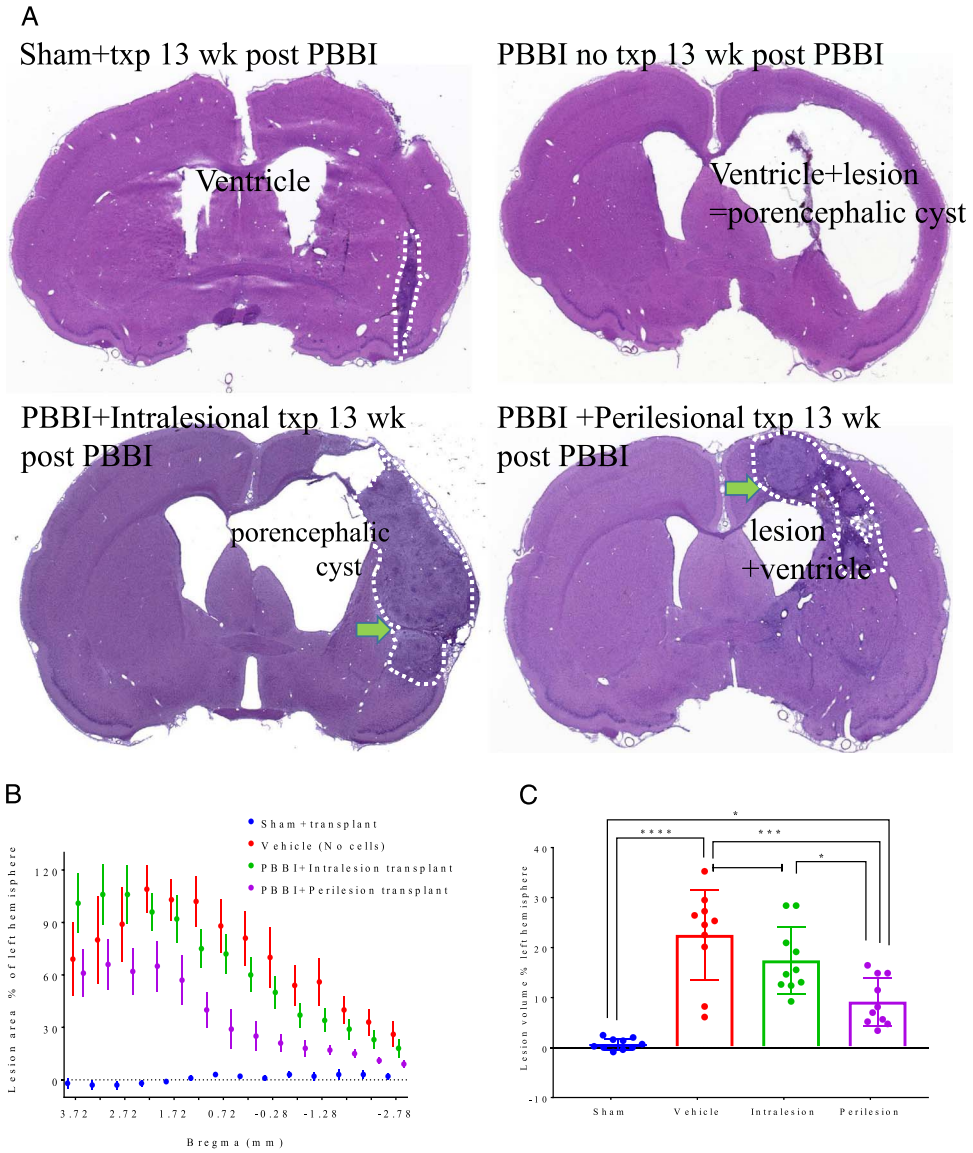


Figure 1. Human neural stem cell transplants mitigate PTBI lesion. Representative sections at minus 0.78 mm from bregma show the injury effect (unilateral porencephalic cyst in vehicle compared with sham in top panel). In both the intralesion and perilesion sections (middle panel), transplanted hNSCs (darker purple with white dotted outline) are indicated by green arrow and persistence of porencephalic cyst only in intralesion (*A*). Changes in lesion area across the brain are shown in (*B*), while those in group lesion volume are shown as a scatter plot (one dot per rat) (*C*). One-way ANOVA followed by Tukey analysis showed statistically significant injury effect (sham vs. vehicle) and statistically significant therapeutic effect (perilesion vs. vehicle) on the lesion size ($F_{3,33} = 25.41$, $p < 0.0001$). No differences were detected when intralesion was compared with vehicle.

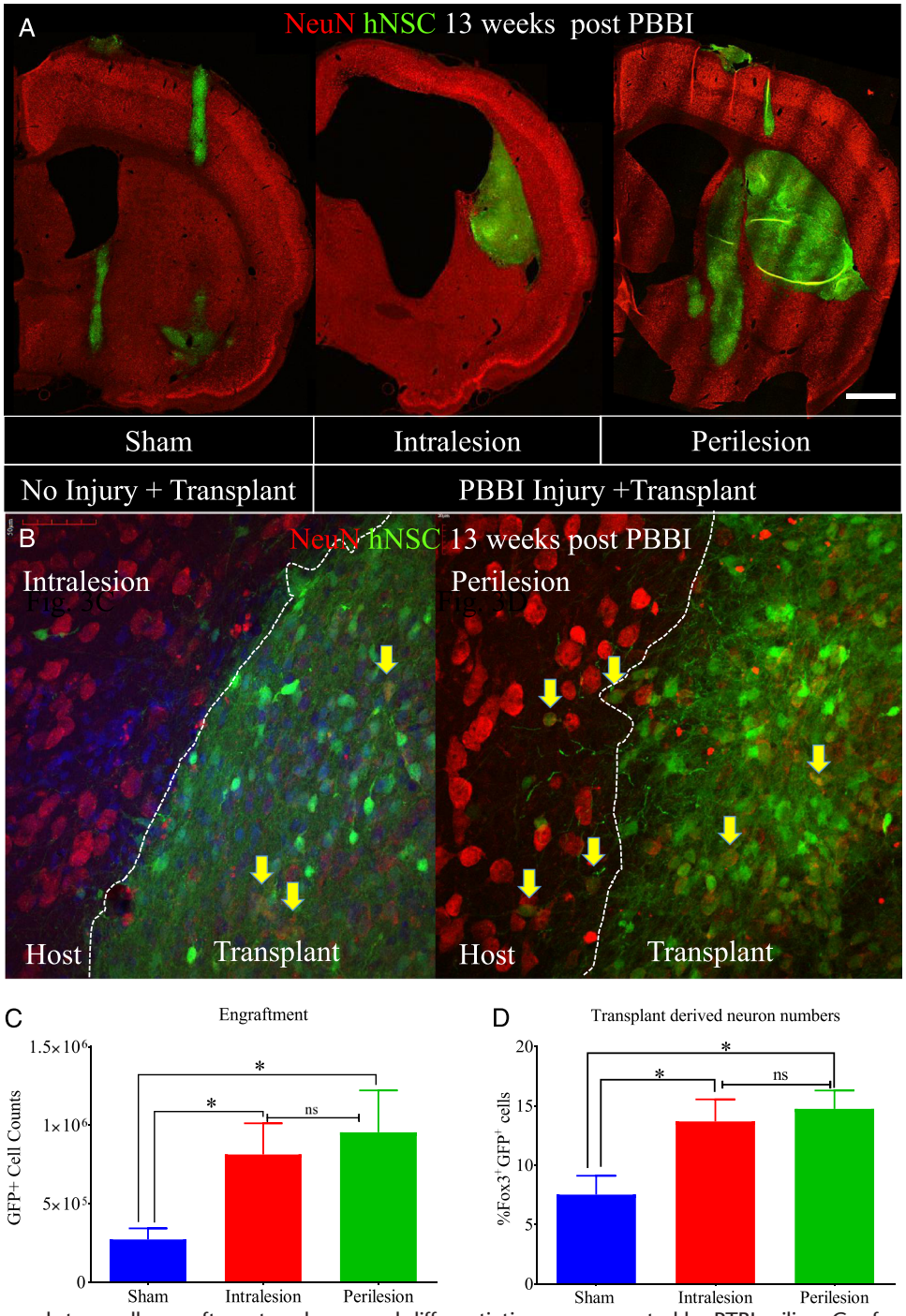


Figure 2. Human neural stem cell engraftment and neuronal differentiation are promoted by PTBI milieu. Confocal images of brain sections immunostained for mature neuronal marker, Fox3 (red fluorescence) with engrafted hNSCs (green fluorescence), show engraftment in sham+hNSCs (left) is less than that in PTBI+hNSC group (middle and right). Note the persistence of secondary injury in intralesion as indicated by tissue loss in cortex and absence of corpus callosum (middle); in contrast, cortical thickness in perilesional and sham groups is comparable. The scale bar is 1 mm (*A*). A magnified view of the transplant border shows examples of host neurons (red fluorescence) and transplant-derived neurons (yellow arrow) in intralesion (left) and perilesional (right) (*B*). Quantitation of GFP (left) and $GFP^{+}Fox3^{+}$ (right) is shown in bar graphs (*C* and *D*). One-way ANOVA did not detect significant differences between groups, albeit both GFP counts and transplant-derived neurons were greater in transplant groups compared with sham and higher in perilesion compared with intralesion. Scale bar size is at bottom right.

was prominent in the intralesional group but less so in the perilesional group (Fig. 1A). Lesion size reduction in both pTBI +hNSCs groups compared with vehicle can be seen at multiple levels across the brain (Fig. 1B). A two-way ANOVA of lesion size across bregma levels showed significant interaction ($F_{39,448} = 2.81$, $p < 0.0001$) between location and treatment and statistically significant differences at multiple bregma levels ($F_{13,448} = 14.19$, $p < 0.0001$) and between vehicle and transplant groups ($F_{3,448} = 145.3$, $p < 0.0001$). These results indicate a strong injury effect of pTBI and robust therapeutic effect of NSC treatment, respectively. A scatter plot showed the presence of a larger lesion in the vehicle-treated group, consistent with the progressive tissue loss seen in this model (significant injury effect). Critically, there was a significant reduction in lesion volume only in the perilesion transplantation group (vehicle vs. perilesion, $p = 0.0024$; intralesion vs. perilesion, $p = 0.0069$) (Fig. 1C).

In the current study, the presence of transplanted NSCs was associated with reduced lesion size in injured animals.

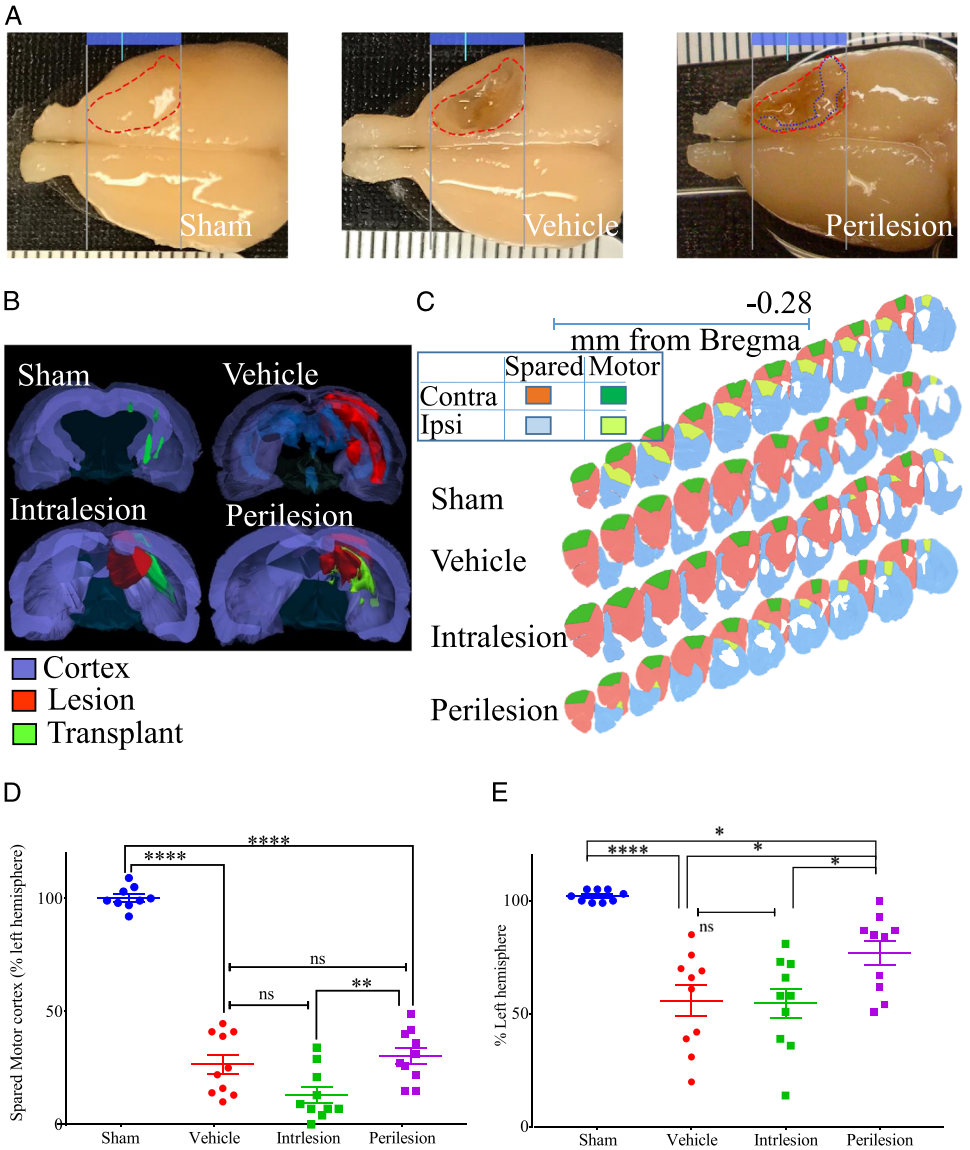


Figure 3. (*A*) Motor cortical tissue (area within red dashed outline) on brain surface in sham group (left) is lost at 13 weeks post-PTBI +vehicle treatment (middle), while some of it is protected in perilesion group (right). Evenly spaced (0.5 mm) serial brain sections spanning the motor cortex (+3.72 mm to –0.28 mm bregma = nine sections) were traced using Neurolucida to render lesion (red), transplant (green), and spared brain tissue (blue) in 3D space. Evidently, lesion volume in vehicle and intralesional groups is larger than that in perilesional group (red area in *B*). Traces of contralateral and ipsilateral hemispheres and motor cortices on HE sections made using modified CalLesion shows extent of ipsilateral brain tissue and proportion of motor cortex in spared cortical tissue (*C*). Quantitation of spared tissue (*D*) and motor cortex (*E*) shows statistically significant difference between pTBI+vehicle versus pTBI+hNSC groups. Sparing of motor cortical tissue in the perilesional group was significantly better than that in intralesional group (*D*). The one-way ANOVA of mean ± SEM spared tissue was significant with $F_{3,32} = 15.63$ and $p < 0.0001$; mean ± SEM spared motor cortical tissue was significant with $F_{3,32} = 125$ and $p < 0.0001$.

Based on this observation, a more detailed quantitative examination of the transplant attributes including the number of GFP fluorescent cells as a measure of total hNSC engraftment and GFP colocalized with anti-neuronal nuclei antibody (NeuN)-positive cell counts as a measure of hNSC neuronal differentiation (NeuN positivity) was performed using unbiased stereology. The extent of hNSC engraftment volume and neuronal differentiation was greater in the injured groups compared with the uninjured transplanted group (Fig. 2A–D), but there were no significant differences between the perilesional and intralesional groups with respect to GFP-positive cell engraftment or graft derived NeuN-positive neuronal numbers (Fig. 2D). Therefore, we sought to determine quantity of host tissue sparing evident from a whole brain image to test for putative *neuroprotective effect* (Fig. 3A). Quantitation of brain sections with Neurolucida revealed increased cortical tissue sparing (Fig. 3B). To test if apparent cortical sparing tissue included motor cortex (identified using a standard rat brain atlas) and to quantitate the effect, modified version of CalLesion (Fig. 3C) was used. A two-way ANOVA of the spared motor cortical tissue revealed that between +3.72 mm and −0.28 mm bregma (region spanning rat motor cortex) there was significant reduction in lesion size (red shape in Fig. 3B) with significant tissue sparing (Fig. 3C, cyan region), specifically, the ipsilateral motor cortex (Fig. 3C, light green). One-way ANOVA revealed statistically significant differences in spared tissue between the two transplant groups; the sparing of the motor cortex was significantly better with perilesional transplantation. Concomitant reduction in lesion area and significant increase in cortical tissue sparing ($F_{3,32}$ = 15.63) (Fig. 3D) specifically in motor cortex ($F_{3,32}$ = 125) (Fig. 3E) suggest that transplantation of hNSCs reduced the progression of pTBI induced cortical tissue loss, which may be associated with the functional improvement (Figs. 2 and 3E). The highest sparing of cortical tissue and motor cortex is in the perilesional group, while the least is in the intralesional with the vehicle group in between.

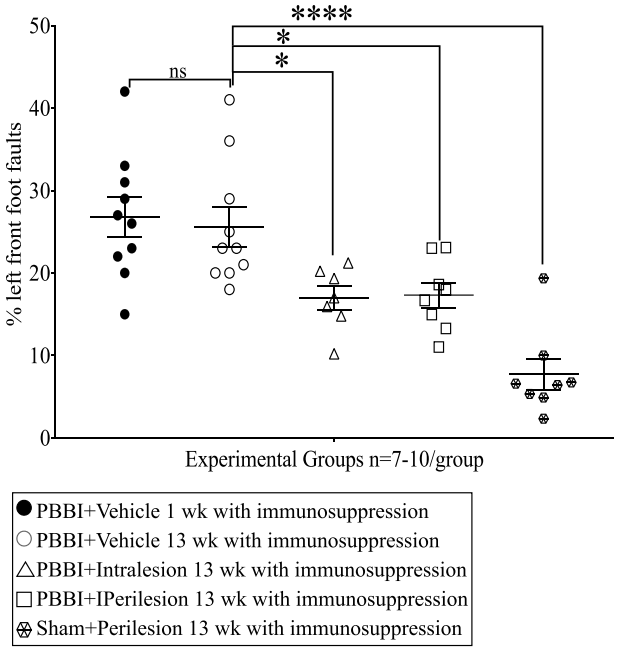


Figure 4. Scatter plot of foot faults as percentage of total steps per group is shown. Comparison between sham (hexagons) and PTBI vehicle-treated groups at 1 week postinjury (closed circles) or at 13 weeks postinjury (open circles) shows significant differences because of stable persistent injury effect. Therapeutic effect is evident upon comparison of vehicle with transplant groups. The one-way ANOVA of mean foot faults ±SEM between groups at 13 weeks was significant with $F_{4,38}$ = 13.95 and $p < 0.0001$.

Using the greater than 1% presence of grafted hNSCs as inclusion criteria, behavioral assessments made at 12 weeks posttransplantation were analyzed to correlate the histological effects of injury and contribution of the hNSCs to spared motor cortical tissue to motor function. One animal from each transplant group was excluded because of poor engraftment (<1% of the input number of cells); footfault videos of three more animals were not available because of equipment failures. The analysis thus included sham+hNSCs (n = 8), PTBI+vehicle (n = 10), PTBI+hNSCs intralesion (n = 7), and PTBI+hNSCs perilesion (n = 8). At 13 weeks postinjury, mean ± SEM front footfault values for the aforementioned four groups were 7.704 ± 1.828, 25.6 ± 2.386, 16.95 ± 1.429, and 17.33 ± 1.522. Footfaults were lower in the sham group and significantly higher in the vehicle-treated group as expected (Fig. 4). The injury effect size (mean difference/pooled standard deviation (sham vs. vehicle)) at 1 week (−4.1) was stable and persisted at 13 weeks postinjury (−3.9) with a p value of <0.0001. The footfault performance of the transplant groups was between these two extremes. One-way ANOVA showed statistically significant difference between groups in mean ± SEM footfaults with $F_{4,38}$ = 13.95 and $p < 0.0001$. The mean difference from vehicle was 8.646 (intralesion) and 8.275 (perilesion), and p value was 0.0470 (intralesion) and 0.0490 (perilesion), respectively.

DISCUSSION

In this study, we tested the hypothesis whether transplant location–dependent effect would be observed in focal brain injury. The results suggest that the location of transplanted cells, with respect to lesion, played a critical role sparing motor cortex and improving longitudinal motor performance. It has been previously reported that the pTBI lesion grows by two-fold between weeks 1 and 10 postinjury in the brain regions, 3.72 mm to −0.28 mm from bregma.[40] Such preclinical data are consistent with clinical observations of progressive tissue loss following TBI in humans[10,23] and, in particular, following cranial gunshot wound.[21,22] In the current study, the vehicle-treated pTBI group showed tissue damage similar to what has previously been reported[40] (Fig. 1A–D). The NSC-mediated reductions in lesion size were greater in animals that received perilesional than intralesional NSC transplants (Figs. 1 and 2A). Despite differences in treatment initiation and survival durations, lesion reduction was comparable with that of pharmacological interventions, human amniotic cell transplants, or amniotic cell culture supernatants.[45] Unlike pharmacological agents, which required repeated administration, transplantation of NSC at a single time-point appears to confer enduring benefits, ameliorating delayed secondary brain tissue loss. The hNSC transplant 1 week after injury primarily *neuroprotective*, that is, stabilized the lesion across the brain, prevented

expansion into regions remote from the focal injury core consistent with the role of endogenous NSCs in TBI.[29] This is in contrast to pharmacological agents, which thus far failed to confer such neuroprotection.[19] Cell death in this pTBI model peaks in injured cortex and striatum between 24 hours and 72 hours, respectively, with delayed cell death in locations remote from injury such as the thalamus occurring around day 7. Additional tissue destruction (both gray and central white matter of the cerebral hemispheres, with ventriculomegaly) continues even up to 10 weeks postinjury.[40,44] The pTBI model in this study recapitulated focal pTBI pathology as seen in humans.[21,22] In this study, transplantation of hNSCs in the perilesional zone/penumbra mitigated secondary injuries up to 3 months postinjury (the longest time point tested in this model for any intervention). Nevertheless, the benefits seen here may not primarily be because of new axons, dendrites, or synapses between transplanted cells and host because such effects would need longer survival times to be established.[28] Longer duration studies, for example, in gyrencephalic brains, may be needed to clearly establish cell replacement effects. Addition of proneurogenic compounds could accelerate and direct development of such connectivity.[46]

Could immunosuppressants have contributed to these observed beneficial effects? The immunosuppression regimen used here has been optimized for NSI-566RSC survival in rats, primates, and humans.[28] There is no information on effect of mycophenylate or tacrolimus on TBI[10]; however, in vitro studies suggest that they could modulate neuroinflammation.[10,47] Short duration combined use of mycophenylate and tacrolimus may mitigate some TBI secondary damage.[47,48] Acute treatment with tacrolimus reduced incidence of nonconvulsive seizures but not cortical atrophy.[49] The robust *cortical neuroprotection* in the perilesional group, but not in identically immunosuppressed intralesional group (both compared with vehicle), is consistent with a lack of beneficial effect of the immunosuppressants alone. Statistically significant differences in motor cortical neuroprotection observed (Fig. 3D) also argue against beneficial effects to immunosuppressant regimen alone.

Engraftment of hNSCs was three times greater in pTBI injured than in sham rats with putatively normal brains (Fig. 3); this effect was much greater for perilesional transplants than intralesional transplanted animals. Thus, the mean survival of transplanted cells when placed in an "8-point box" configuration was near 95% of the million cells transplanted (Fig. 2C), suggesting that the post-pTBI tissue *neurotrophic milieu* is similar for both transplant locations with both mechanistic and therapeutic implications. Perilesional multisite transplantation had a better effect on host tissue.

Limitations of the study are as follows: (1) absence of image-guided verification of (*a*) injury location and (*b*) presence of successful transplantation; better control of both could have reduced the variations in data. (2) No insights into the mechanistic aspects of host-hNSC interactions were explored, so it is not clear if decreased proteinopathy or neuroinflammation contributes to the tissue sparing. (3) Contribution of transplant-derived human neurons, axons, and synapses to motor deficit amelioration remains to be established. Longer study duration, sufficient to demonstrate maximal neuronal differentiation and axonal/synapse formations, could help establish cause and effect relationship between transplant-derived neuronal differentiation and deficit amelioration. (4) Inadvertent reduction in animal numbers weakens confidence in the behavior amelioration.

CONCLUSIONS

Collectively, our data support the use of these NSI-566 human fetal neural origin cells in focal severe TBI, and the study shows that a perilesional transplantation strategy is preferable to intralesional cell deposition. Despite robust engraftment in both sites, the *neuroprotection extent* was better when the cells were placed around lesion. Although further mechanistic and safety studies are warranted, these studies support the rationale for a clinical trial in this indication and support a perilesional stereotactic implantation strategy in humans with TBI.

AUTHORSHIP

R.M.B., D.A.S., and S.G. formulated the hypothesis, designed experiments, and obtained funding and regulatory approvals for the study. M.S.S. and Z. H. performed surgeries. A.M. performed behavior analysis, optimized methods for histology quantitation, and edited the article. L.S.Q., L.D., S.W.L., J.J., and G.R.G. performed histological quantitation and generated illustrations. L.S.Q., L.D., K.N.R., and C.B.A. performed immunohistochemistry. J.J. and M.S.S. used stereology to quantitate cells and render 3D representations. S.G. contributed with microscopy. M.S., Z.H., A.M., S.W.L. R.M.B., D.A.S., and S.G. contributed to the article writing.

ACKNOWLEDGMENTS

We thank Dr. Thomas G Hazel, and Dr. Karl K Johe, Neuralstem, Inc. for the hNSCs (GFP labeled NSI-566 RSC), technical help from Dr Hyun-Joo Park for CalLesion, Student volunteer Isa Ciocca and Ms. Jare of The University of Miami Writing Center for checking the manuscript for grammar. This research is funded by the U.S. Department of Defense W81XWH-16-2-0008, BA150111 CDMRP JPC-6, and approved by United State Army Medical Research and Material Command, Combat Casualty Care Research Program, Fort Detrick, MD.

DISCLOSURE

For all authors, no conflicts are declared. K.N.R. presented at New England Science Symposium, Harvard, in 2018 and is recipient of Asclepius Laboratories Stem Cell and Regenerative Medicine Award.
This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting views of the Department of Defense. Research was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility, under an Institutional Animal Care & Use Committee-approved protocol, in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

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