Biodistribution of Glial Progenitors in a Three Dimensional-Printed Model of the Piglet Cerebral Ventricular System

Rohit K. Srivastava,^{1,2} Anna Jablonska,^{1,2} Chengyan Chu,^{1,2} Lydia Gregg,³ Jeff W.M. Bulte,^{1,2}
Raymond C. Koehler,⁴ Piotr Walczak,^{1,2,5} and Miroslaw Janowski^{1,2,6}

White matter damage persists in hypoxic-ischemic newborns even when treated with hypothermia. We have previously shown that intraventricular delivery of human glial progenitors (GPs) at the neonatal stage is capable of replacing abnormal host glia and rescuing the lifespan of dysmyelinated mice. However, such transplantation in the human brain poses significant challenges as related to high-volume ventricles and long cell migration distances. These challenges can only be studied in large animal model systems. In this study, we developed a three dimensional (3D)-printed model of the ventricular system sized to a newborn pig to investigate the parameters that can maximize a global biodistribution of injected GPs within the ventricular system, while minimizing outflow to the subarachnoid space. Bioluminescent imaging and magnetic resonance imaging were used to image the biodistribution of luciferase-transduced GPs in simple fluid containers and a custom-designed, 3D-printed model of the piglet ventricular system. Seven independent variables were investigated. The results demonstrated that a low volume (0.1 mL) of cell suspension is essential to keep cells within the ventricular system. If higher volumes (1 mL) are needed, a very slow infusion speed (0.01 mL/min) is necessary. Real-time magnetic resonance imaging demonstrated that superparamagnetic iron oxide (SPIO) labeling significantly alters the rheological properties of the GP suspension, such that, even at high speeds and high volumes, the outflow to the subarachnoid space is reduced. Several other factors, including GP species (human vs. mouse), type of catheter tip (end hole vs. side hole), catheter length (0.3 vs. 7.62 m), and cell concentration, had less effect on the overall distribution of GPs. We conclude that the use of a 3D-printed phantom model represents a robust, reproducible, and cost-saving alternative to in vivo large animal studies for determining optimal injection parameters.

Keywords: ventricle, CSF, brain, glial progenitors, bioluminescence, MRI, iron oxide

Introduction

NEONATAL HYPOXIA/ISCHEMIA (NH-I) leads to significant white matter injury [1]. Several neuroprotective strategies, for example, the use of calcium blockers, nitric oxide, growth factors, neuropeptides, argon gas, hypothermia, inhibitors of excitatory amino acids, and free radicals, have been attempted in experimental and clinical settings [2]. Hypothermia has been shown to be promising in several studies, but only for mild to moderate NH-I [1].

Stem or progenitor cells have been found to be beneficial in a wide array of animal models of neurological disorders [3,4]. Previously, our group has shown that intraventricular delivery of human glial progenitors (GPs) at the neonatal stage is capable of replacing host abnormal glial cells and rescuing the life span of dysmyelinated mice [5]. However, this effect was not observed after stereotactic injection of neural progenitors directly into the brain parenchyma of children suffering from dysmyelination [6]. We have found that one of the factors influencing survival of GPs is the

¹Division of MR Research, Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

²Cellular Imaging Section and Vascular Biology Program, Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

³Visualization Core Laboratory, Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

⁴Department of Anesthesiology and Critical Care Medicine, Translational Tissue Engineering Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

⁵Department of Neurology and Neurosurgery, University of Warmia and Mazury, Olsztyn, Poland.

⁶NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland.

transplantation site, which might also affect the clinical outcome [7]. Thus, a direct rodent to patient translation should be performed with caution [8]. In fact, the use of large animals to test therapeutic approaches before clinical translation is increasingly recommended, especially if questions cannot be adequately addressed in rodent models [9].

GPs exhibit therapeutic effects after intracerebral transplantation in a murine model of NH-I [10]. However, the miniature size of the murine brain does not allow for investigation of the transplantation challenges related to the long migration distances required to reach the entire brain parenchyma in humans [11]. Due to the lack of a significant therapeutic effect after intraparenchymal injection of cells in patients with dysmyelination [6], we have turned to the cerebral ventricles as an attractive gateway to introduce cells across the entire neuroaxis. We selected a unique large animal model of NH-I in piglets [12,13], to maximize the clinical relevance and facilitate a smooth bench-to-bed translation of GP-based therapy of NH-I [14]. However, the intraventricular route of cell delivery has not been studied in piglets so far, and thus, the parameters of cell infusion in this model are unknown. Optimization of the transplantation procedure itself would require a large number of large animals, and therefore, we sought a non-animal alternative according to the “three Rs” tenet: replacement, reduction, and refinement of animal use in scientific research [15]. To accomplish this, we took advantage of recent technological advances in three dimensional (3D) printing, creating a model of piglet cerebral ventricles to optimize experimental conditions before entering the veterinary surgical suite. In the future, a similar model of the human cerebral ventricles may also be established before entering the clinical arena.

In this study, we investigated the main variables affecting the biodistribution of GPs injected into fluid compartments, mimicking the cerebral ventricles to fine-tune seven infusion parameters for a precise and predictable intraventricular delivery of cells in the piglet model. These parameters included the speed of infusion, volume of cell suspension, catheter tip design, cell labeling, cell concentration, cell origin, and catheter length. The overall goal is to achieve a wide distribution of GPs within the ventricular system due to the global character of NH-I as a disease, while avoiding outflow into the intrathecal space, as fibrous arachnoid and *pia mater* covering this area may constitute a stronger barrier for cell migration than the ependyma [16]. We initiated our studies with simple fluid phantoms and then progressed to a custom-designed 3D-printed model of piglet cerebral ventricles. All phantom materials were made transparent in to perform bioluminescent imaging as a convenient, quantitative readout of cell distribution, while magnetic resonance imaging (MRI) was reserved for more advanced settings before injection in animals.

Materials and Methods

Overall experimental design

For an in vitro assessment of GP biodistribution after transcatheter infusion, we first used simple fluid-filled containers to answer basic inquiries, followed by a custom-printed 3D model of piglet cerebral ventricles to mimic more closely in vivo conditions. In each setting, we selected regions of interest (ROIs) or distances to calculate readouts,

which were typically relative values such as ratios of single or grouped ROIs. Luciferase(+) human and mouse GPs were used throughout this study [17]. For testing of infusion settings inside an MRI scanner, we labeled GPs with superparamagnetic iron oxide (SPIO) as previously described [7]. This study was approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

Small fluid-filled container phantoms

NMR tubes (New Era Enterprises, NE-LI0-7, 10 mm in diameter and 178 mm in height) with their long axes positioned vertically were filled with PBS (Gibco; 10010-023) (Supplementary Fig. S1A, B). Evans blue was selected as a prototype small molecule due to its dark blue color, which allowed continuous observation of injection dynamics. A glass pipette (Fisher Brand; 13-678-6B) was inserted into the NMR tube and fixed in the center of the tube by a custom-made 3D-printed ring to precisely direct the injection jet down (Supplementary Fig. S1C, D). The tip of the glass pipette was positioned 12 cm below the top of the NMR tube to approximate the pressure of cerebrospinal fluid (CSF). Polyethylene tubing (BD INTRAMEDIC™, 0.76 mm 1.22 mm inner and outer diameter, respectively) was inserted into the glass pipette so that one end was positioned at the tip of the glass pipette and the opposite end was outside the tube and connected to a syringe loaded with 10% w/v solution of Evans blue. We selected this size of tubing to mimic a catheter suitable for the SmartFrame setup (MRI Interventions), which can be used for stereotactic intraventricular injection. One milliliter of Evans blue solution was injected using a syringe infusion pump over a period of 10 min. Two paradigms of injection were investigated: continuous (0.1 mL/min) and pulsatile (five 5-s-long boluses evenly distributed within a 10-min time period administered at a speed of 1.0 mL/min). The injections were captured using a standard color camera. Then, the width of the Evans blue solution jet stream was measured (Photoshop, Adobe) and calculated (Excel, MS) as a percentage of the width of the NMR tube. A total of 12 injections were performed (6 for each injection paradigm).

The same experimental setup with tubing introduced through a glass pipette fixed inside an NMR tube and connected to a syringe placed in an infusion pump was used for GP cell infusion. NMR tubes were coated with poly-L-lysine (PLL, Sigma; 1002303656) and laminin (Invitrogen; 23017-015) to facilitate cell adhesion to the walls of the NMR tubes. The distribution of GPs within the NMR tube was evaluated using bioluminescence imaging (BLI) as previously described [18]. Initially, we employed the same two paradigms as described for the Evans blue solution infusion, but since we did not observe differences between them, we tested another variant. We hypothesized that since cells are larger and probably heavier, they should be propelled for a longer duration of time to achieve the difference. Thus, we added a third paradigm with 10-s-long boluses, also evenly distributed within a 10-min time period administered at a speed of 1.0 mL/min. Two ROIs were chosen for cell counting, one at the tip of the NMR tube and the second at a distance of one ROI diameter apart from the first ROI. The results are expressed as the ROI-1/ROI-2 ratio. After completing the infusions with a vertical position

of the NMR tube, the same experiments were performed with the NMR tube positioned horizontally. The distance from the catheter tip to the center of the cell collection was then measured. A total of six injections were used per experimental paradigm, for a total of 36.

We then used a glass beaker as a large fluid container mimicking enlarged human cerebral ventricles to check the maximum area covered by cells after infusion. A small thermocol box was used as a lid for the beaker to cover it completely and make a support for the glass pipette, which was used to guide the catheter (Supplementary Fig. S1B). Otherwise, experimental settings for the stem cell infusion were the same as for the NMR tube. The area of the bottom of the beaker covered by stem cell was quantified. Initially, we performed comparison of the pulsatile (5-s and 10-s boluses) and slow, continuous infusion. Then, the correlation between the duration of boluses (10, 20, 30, 40, 50, and 60 s) and area covered by the stem cells was calculated.

Custom-designed, 3D-printed model of the cerebral ventricles: design of the 3D-printed model

A 3D model of the piglet cerebral ventricles was developed by the Visualization Core Laboratory at Johns Hopkins University based on MRI-based semiautomatic segmentations (OsiriX 64-bit, Switzerland) of a 7-day-old piglet weighing 1.5 kg. T2-weighted MRI scans were obtained at 11.7T both in live animals and postmortem ($n=2$). Only the fluid-containing compartment of the cerebral ventricles was segmented. The choroid plexus was treated as the rest of the brain parenchyma and omitted from the segmentation, as it is connected to the ventricular wall. Three-dimensional design software (ZBrush, Pixologic, and Cinema4D, Maxon, Germany) was used to generate an external shell with a 1.1–2.5 mm wall thickness that enclosed the final 3D surface of the ventricular system and to subsequently add ports. The model was 3D printed (Connex3 Objet260 system; Stratasys) in the Carnegie Center for Surgical Innovation at Johns Hopkins University using Veroclear material, a transparent photopolymer (Fig. 1). The volume of the cerebral ventricles in our 3D-printed model is 0.7 mL, with the fol-

lowing dimensions (measured without ports): length=35 mm, height=20 mm, and width=30 mm. The port of entry for the catheter is placed at the border of frontal horn and body of the lateral ventricles, which corresponds to the trajectory typically used for ventricular shunt placement, a frequent emergency pediatric neurosurgery procedure. This location has a high translational value as it is easily accessible in piglets and provides optimal access to the cerebral ventricles in humans. The tip of the catheter protrudes 1 mm into the ventricular system, and two lengths of catheters were used: 0.3 and 7.62 m, respectively. Ports to initiate the physiological flow of CSF and facilitate cleaning the system were, respectively, added to the inferior and anterior horns of the lateral ventricles. A port added to the caudal tip of the fourth ventricle corresponded to the foramen of Magendie, and was connected to horizontal tubing that mimicked the intrathecal/subarachnoid space. The distal end of this tubing was further connected to a 15 cm tall vertical tube, which was used to lock the relative physiological pressure in the system, which was set at 12 cm H₂O.

After proper and tight connection of all ports, the 3D-printed phantom was completely filled with PBS until a height of 12 cm was achieved in the vertical tube to mimic CSF pressure. Fluid circulation was forced through the lateral horns to maximally correspond to the physiological direction of CSF flow (Supplementary Fig. S2). Extra precautions were taken to avoid the introduction of air bubbles, as this could affect the physiology of CSF circulation. Since the rate of CSF flow in neonatal pigs has not been reported in the literature, we calculated it from the equation of CSF flow in newborn and adult humans and adult pigs, and applied it throughout all experiments as 0.001 mL/min.

Stem cell infusion

SPIO-labeled and SPIO-unlabeled human or mouse GPs were infused into the 3D-printed phantom through the catheter port (Supplementary Fig. S2). Since, the size of mouse and human GPs is comparable, the cell volume fraction was constant without the need for adjustments. GPs were prepared for infusions as previously described [7]. For

FIG. 1. Design strategy for printing a 3D model of the piglet brain ventricle. A 3D model of the piglet cerebral ventricular system was created based on semiautomatic MRI segmentation of a T2-weighted MRI series of a live and postmortem piglet brain, together with photographic images of the gross and sliced specimen. ©2019 Johns Hopkins University.

TABLE 1. LIST OF ALL INDEPENDENT VARIABLES USED IN THE STUDY

Independent variables		
1. Infusion speed (mL/min)	0.01, 0.1, or 1.0	
2. Cell suspension volume (mL)	0.1, or 1.0	
3. Cell labeling	SPIO labeled or nonlabeled	
4. Cell species	Human or mouse	
5. Cell dose (cells/mL)	10^5 , 10^6 , or 10^7	
6. Catheter tip	End hole or side hole	
7. Catheter length (m)	Short (0.30) or long (7.62)	

SPIO, cells were incubated overnight with $10 \mu\text{L/mL}$ of Molday ION Rhodamine B (BioPAL). The distal end of the injection tubing was colored black to avoid the possible overlap of bioluminescence signal between the tubing and the 3D model.

Independent variables. Several independent variables were investigated in this study: speed of infusion (0.01 vs. 0.1 vs.

1.0 mL/min), volume of cell suspension (0.1 vs. 1.0 mL), type of catheter tip (end hole vs. side hole), length of catheter (long vs. short), cell labeling (nonlabeled vs. SPIO-labeled cells), cell concentration, and cell origin (human vs. mouse) (Table 1).

Dependent variables. We delineated several ROIs that are critical for the assessment of cell distribution (Fig. 2A). The ratios of specific ROIs were calculated to normalize the biodistribution values across various experimental conditions, allowing a direct comparison. Seven ratios were selected as dependent variables (Fig. 2B)

Imaging

Bioluminescence imaging. Bioluminescence was measured using an IVIS Spectrum/CT instrument (Perkin Elmer). D-luciferin (0.15 mg/mL) was added to the cell suspension before starting the infusion. BLI was quantified within ROIs and expressed as photon flux (p/s).

Real-time MRI. The 3D phantom was placed inside the gantry of the MR scanner. An undersampled radial FLASH

B	Dependent variables	ROIs	C	Dependent variables	Description
	1. Ventricle ratio	ROI 1+ROI 2+ROI 5+ROI 6/Total		1. Ventricle ratio	Sum of BLI signal intensity of anterior and medial part of lateral ventricle; 4 th ventricle; posterior and inferior parts of the lateral ventricle divided by total signal intensity.
	2. Central ratio	ROI 1/Total		2. Central ratio	BLI signal of anterior and medial part of lateral ventricle divided by total signal intensity.
	3. Lateral ratio	ROI 5+ROI 6/Total		3. Lateral ratio	Sum of BLI signal intensity of both posterior and inferior parts of the lateral ventricle divided by total signal intensity.
	4. CP ratio	ROI 1+ROI 2/Total		4. CP ratio	Sum of BLI signal intensity of anterior and medial part of lateral ventricle; 4 th ventricle divided by total signal intensity.
	5. Posterior ratio	ROI 1/Total		5. Posterior ratio	BLI signal intensity of anterior and medial part of lateral ventricle divided by total signal intensity.
	6. Lateral L/R ratio	ROI 5/ROI 6		6. Lateral L/R ratio	Division of BLI signal intensity of both Posterior and inferior parts of the lateral ventricle.
	7. Around catheter	ROI 4/ROI 1		7. Around Catheter	Signal intensity of 4 th ventricle divided by anterior and medial part of lateral ventricle.

FIG. 2. (A) Top view of 3D-printed model of the piglet brain ventricular system with delineated ROIs from which the BLI signal was obtained. (B, C) Calculation methods for seven dependent variables derived from the ROIs. “Total” = photon flux acquired from all ROIs (ROI 1-ROI 6). BLI, bioluminescence imaging; ROI, region of interest. ©2019 Johns Hopkins University.

pulse sequence was used with a 3T Prisma Fit MR scanner (Siemens) to visualize cell infusion into the 3D phantom. Images were reconstructed in real time with temporally regularized nonlinear inversion (NLINV) using sysGen/TYAN Octuple-GPU, which supports an ultra-high speed of 100 frames/s. Acquisitions were based on the following parameters: repetition time = 7.05 ms, echo time = 6 ms, average = 16, matrix size of 192×192 , field of view (FOV) = 210 mm, and 201 slices with 2.5-mm slice thickness.

Statistical analysis

All statistical calculations were performed using SAS 9.4. The comparison between means was performed using the least square means (LSM) embedded within PROC MIXED, while PROC CORR was employed to calculate correlations based on a *r* Person test. Graph pad prism (6.0) was used for image preparation. The 'r' software was used for presentation of correlation between dependent variables. Unless otherwise stated, all *P*-values were calculated using the LSM.

Results

Biodistribution of cells infused into simple fluid-filled containers

We investigated whether modifying the infusion parameters is able to modulate the biodistribution of a solution/suspension introduced through a catheter into a fluid compartment mimicking the cerebral ventricles. We also studied whether changes in that biodistribution could be predicted through prior infusion of a small molecule solution, as we previously demonstrated for the case of intra-arterial stem cell delivery [19]. Initially, we compared two clinically relevant scenarios by infusing 1.0 mL of solution/suspension over a period of 10 min: (1) continuous flow at a 0.1 mL/min speed and (2) pulsatile flow executed through 5-s-long boluses at a speed of 1.0 mL/min. We started with infusion of 10% Evans blue solution, due to its clear visibility and size being similar to clinically used gadolinium chelates. We found that a fast bolus (5s) provided a wider Evans blue distribution than the slow infusion (70.74 vs. 35.85, respectively, $P < 0.001$) (Fig. 3A), demonstrating that tuning of infusion parameters can be used for controlling the biodistribution of the injected solution. The same experiment was repeated using the GP suspension (10^5 cells/mL) but no difference between the two infusion variants was found (12.01 vs. 12.55, respectively, $P = 0.832$) (Fig. 3B). However, lengthening the injection bolus to 10 s resulted in a difference between slow infusion and fast boluses (12.01 vs. 2.94, respectively, $P < 0.003$) (Fig. 3B). Thus, the cell biodistribution can be modified with changes of infusion parameters, but small molecules such as Evans blue and gadolinium are not good predictors of cell biodistribution in fluid compartments.

We then used the same experimental setup with the NMR tubes placed horizontally to mimic various possible head positions during the cell transplantation procedure, and again, we found no difference in biodistribution between slow infusion and 5s fast boluses (3.54 vs. 3.72, respectively, $P = 0.241$). Lengthening of the bolus injection to 10 s resulted in a difference between slow infusion and fast boluses (3.54 vs. 3.99, respectively, $P < 0.009$) (Fig. 3C). In the experiment with horizontal orientation of the catheter and NMR tube, a positive correlation was observed between the

time of infusion and the distance traveled by GPs ($r = 0.684$, $P < 0.05$) (Fig. 3D). The cell distribution therefore only depends on the infusion parameters and not on the spatial orientation of the NMR tube. The correlation between the range of cell biodistribution and bolus duration indicates that the latter could potentially be used for better control of the biodistribution of intraventricularly transplanted cells.

Subsequently, we inquired whether the size of the fluid compartment affects cell biodistribution, which addresses various sizes of ventricular systems in patients with brain pathologies, where ventricular enlargement is observed such as after hypoxic/ischemic brain injury [20]. We found a difference between slow continuous and 5-s and 10-s fast bolus infusion into the large fluid compartment (3.15 vs. 4.56 vs. 7.14, $P < 0.05$) (Fig. 3F). The area covered by GPs correlated highly with the bolus duration ($r = 0.953$, $P < 0.0001$) (Fig. 3F). Thus, while we saw some similarities between small and large fluid compartments, such as correlation of bolus duration and the distance that cells traveled, the distribution of differences between various injection parameters was distinct. Moreover, cell infusion into large fluid compartments is more reproducible as substantiated by a lower variability in the cell biodistribution pattern and a higher correlation between the speed of infusion and biodistribution characteristics. The lower reproducibility observed in smaller fluid compartments may depend on less predictable and more turbulent flow. The observed differences in cell biodistribution between small and large fluid compartments with turbulent flow as a potential factor warranted a more detailed assessment focused on cell biodistribution in a smaller and more complex fluid compartment, with better relevance to in vivo scenario, that is, a 3D-printed model of a piglet cerebral ventricular system.

Custom-designed 3D-printed model of piglet cerebral ventricles

We investigated how the infusion parameters can affect the biodistribution of cells in a model of the piglet cerebral ventricular system. We found no difference between infusions of 1.0 mL of cell suspension (10^5 cells/mL) at a constant speed of 0.1 mL/min and pulses of 10-s-long boluses. This is in contrast to the simple larger fluid containers described above, indicating that the specific size and shape of a fluid compartment play an important role in determining cell biodistribution (Fig. 4A, B, G). Moreover, we found that the majority of cells went outside the ventricular system. Only ~30% of cells stayed within the ventricles, with the remaining 70% flowing into the subarachnoid-mimicking space, which is highly undesirable. We then decreased the volume of the injected cell suspension by an order of magnitude to 0.1 mL, while increasing the cell concentration to keep the same total number of injected cells. This reduced the time of infusion to 1 min, which significantly improved the lack of outflow from the ventricular system, with ~75% of cells remaining within the ventricles. (Fig. 4C, G). The speed of infusion was then decreased to 0.01 mL/min, with the duration of injection increasing to 10 min, while the other parameters remained the same. This injection paradigm allowed nearly all injected cells to stay inside the ventricles, with many cells in close proximity to catheter tip (Fig. 4D, G). Thus, a slow speed might be particularly suitable for effective delivery of cells to certain areas of the

FIG. 3. (A) Time course of Evans blue distribution range after injection at different speeds (slow continuous: 0.1 mL/min and pulsatile (5-s bolus): 1 mL/min) and time intervals (at start and after 5-s). Box plot shows the least square means for the two speeds and time intervals ($P < 0.001$). (B) BLI of Luc-GPs injected in NMR tubes positioned vertically at different speeds (slow continuous speed: 0.1 mL/min; pulsatile (5-s bolus): 1 mL/min; and pulsatile (10-s bolus): 1 mL/min). Box plot shows distribution of GPs and least square means for the three speeds of cell infusion (slow continuous vs. 5-s bolus, $P = 0.832$; 5 s vs. 10 s, $P < 0.003$). (C) BLI of Luc-GPs in NMR tubes positioned horizontally with travel distance for the three different speed least square means box plot (slow continuous vs. 5-s bolus, $P = 0.241$; 5 s vs. 10 s, $P < 0.009$). (D) Correlation between time of infusion and distance traveled by GPs ($r = 0.684$, $P < 0.05$). (E) BLI of luc-GPs injected in a large beaker glass at different speeds (slow continuous speed: 0.1 mL/min; pulsatile (5-s bolus): 1 mL/min; and pulsatile (10-s bolus): 1 mL/min) showing range of cell distribution. The red circles indicate the regions of interest chosen to assess the cell distribution within the large glass beaker. (F) Area of cell distribution and least square means for the three speeds of infusion in a large beaker glass and correlation between time of infusion and area of cell distribution ($r = 0.953$, $P < 0.0001$).

ventricular system, which is desirable for focal brain diseases. Two more extreme injection conditions were subsequently tested: a very fast injection of 1.0 mL/min with a smaller volume of 0.1 mL completed in 6 s and a slow injection of 0.01 mL/min with a higher volume of 1.0 mL performed over 100 min. Interestingly, both experimental conditions were effective in keeping cells within the ventricular system (Fig. 4E–G), while the slow infusion had a tendency to keep cells around the catheter tip. In summary, if we can decrease the total volume of injection to 0.1 mL; then, we can use a wide array of infusion speeds ranging from 0.01 to 1.0 mL/min, with the caveat that at the lowest speed, more cells are located next to the catheter tip. If high delivery volumes such as 1.0 mL are needed, for the piglet

ventricular system, a low speed of 0.01 mL/min is then recommended. Cells tend to be retained within the lateral ventricles at low speeds, while at higher speeds, they are routed to the fourth ventricle.

Imaging of GP biodistribution using MRI

Since the biodistribution of GPs is highly dependent on the infusion parameters, we hypothesized that performing the procedures under real-time MRI guidance could be beneficial and potentially allow fine-tuning of infusion parameters, as we have previously shown for intra-arterial cell delivery [19]. Cells were labeled with SPIO to make them visible on MRI. We have shown previously that EPI-based

FIG. 4. (A–G) Box plots of the distribution of GPs in the 3D-printed model of the piglet cerebral ventricular system injected with an end-hole catheter. (H) Correlation diagram of all independent variables. (I) Summary of all different experimental conditions for injection of GPs. (J) Representative BLI images for all experimental conditions. Data are presented as medians, third quartiles, and extreme values from six independent experiments for each paradigm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on multivariate analysis. HVBO, high-volume bolus; HVNS, high-volume normal speed; LVNS, low-volume normal speed; LVSS, low-volume slow speed; CP ratio, central posterior ratio; Lateral L/R ratio, lateral left/right ratio; GP, glial progenitor. ©2019 Johns Hopkins University.

real-time MRI suffers from artifacts, which complicates interpretation of imaging data [21]. However, recent advances in the field of MRI with ultrafast, undersampled FLASH sequences with real-time reconstruction capable of imaging at a rate of 100 f/s [22] allowed us to visualize the administration of SPIO-labeled GPs in real time. We injected 1.0 mL of cell suspension at a speed 0.1 mL/min, which should have resulted in the majority of cells leaving the ventricular system, but the opposite was observed (Fig. 5). Besides SPIO labeling, the main difference between the studies inside and outside the MR scanner was the need of using a long catheter (7.62 m or 25 feet) to achieve a safe distance between the infusion pump and the gantry of the MR scanner. To further investigate this discrepancy and to elucidate the role of SPIO labeling and length of the catheter, we performed the same experiments outside the MR scanner with BLI serving as a readout.

Effect of SPIO labeling on the biodistribution of GPs

Since certain higher cell numbers than 10^5 cells/mL are applicable in therapeutic settings, we tested amounts one

and two orders of magnitude higher (10^6 and 10^7 cells/mL). We confirmed that SPIO labeling prevents outflow of cells into the intrathecal space for 10^5 and 10^6 cells/mL, but for 10^7 cells/mL, the effect was opposite (Fig. 6). SPIO-labeled cells remained inside the ventricular system at the same ratio across all three cell concentrations, but for nonlabeled cells there was an increase of cells staying within the ventricular system for the highest concentration (10^7 cells/mL). Hence, we cannot make conclusions regarding the destination of nonlabeled GPs based on the biodistribution seen during MRI-guided procedures of labeled GPs.

Effect of catheter length on the distribution of GPs

Since there are currently no MRI-compatible precise infusion pumps available that can operate at low infusion speeds, the pump has to be located 7.62 m from the middle of the MR scanner gantry. Thus, we compared long (7.62 m) tubing with more typical, short (30 cm) tubing. Use of the longer tubing increased the amount of cells within the ventricular system, although the effect was small (Fig. 7). Nevertheless, the length

FIG. 5. Real-time MRI of SPIO-labeled and BLI of nonlabeled GPs in the 3D-printed model of the piglet ventricular system. An abundant presence of GPs was observed in the ventricular area (A) with few cells in the intrathecal space (B). BLI showing the distribution of nonlabeled GPs in the ventricular area and in the intrathecal space (C). MRI, magnetic resonance imaging; SPIO, superparamagnetic iron oxide. The dotted lines in image (C) indicate the vertical cross sections of ventricle area (A) and intrathecal space (B) on the 3D printed model when seen under real time MRI (shown in corresponding red circles). ©2019 Johns Hopkins University.

of the tubing needs to be taken into account as another variable when planning intraventricular cell delivery.

Effect of catheter tip design on the distribution of GPs

Intra-arterial catheters are typically designed with end holes, while intraventricular catheters have side holes, which are designed for CSF drainage rather than for injections. We found that the type of tip does not affect the outflow of GPs outside the ventricular system. To some

extent, it only affects the cell concentration in proximity to the catheter tip rather than the entire ventricular system (Fig. 8). Thus, if a more localized cell distribution inside the ventricles is required, such as is the case for stroke, an end-hole catheter is more desirable, while for disseminated diseases, a side-hole catheter might be more beneficial, although the differences are relatively small (Supplementary Table S1). As observed before, the dominant factors affecting cell distribution are the injection speed and volume, and not the catheter tip design (Supplementary Tables S2 and S3).

FIG. 6. (A–G) Differences in distribution between SPIO-labeled and nonlabeled GPs in the 3D-printed model of the piglet ventricular system for three different concentration of cells. (A–G). A slow continuous speed (0.1 mL/min) and 1 mL of total cell suspension volume were used. Data are presented as medians, third quartiles, and extreme values from six independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on multivariate analysis. NS, non significant.

FIG. 7. Difference in GP distribution between short and long tubing and between SPIO-labeled and nonlabeled cells. (A–G) Labeled cells and long tubing prevent cellular outflow from the ventricular system. One milliliter of GPs with a concentration of 10^5 cells/mL and a slow continuous speed (0.1 mL/min) was used with both short and long tubing. (H) Image of the short (0.3 m) and long (7.62 m) tubing used in this experiment. Data are presented as medians, third quartiles, and extreme values from six independent experiments for each paradigm (short and long tubing, SPIO-labeled and non-labeled cells) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on multivariate analysis. LTNL, long tubing nonlabeled; LTL, long tubing labeled; STNL, short tubing nonlabeled; STL, short tubing labeled.

Effect of cell species on the distribution of GPs

In all previous experiments, we used immortalized human GPs as they are easy to grow in large quantities [7,17]. We compared immortalized human GPs to primary mouse GPs, for the various experimental conditions. For therapeutic transplantation purposes, primary GPs will be used at high concentrations, preferably with SPIO labeling. We did not

find any significant difference in distribution between mouse and human GPs (Fig. 9).

Discussion

While there is rapid progress in many areas of stem cell research, studies on the conditions and potential variables of cell injection that may affect cell delivery lag behind [23].

FIG. 8. (A–G) Distribution of variability among independent variables (catheter, speed and volume) with annotated number (H) values contributing to each dependent variable.

To the best of our knowledge, this is the first study systematically investigating relevant parameters determining the biodistribution of cells delivered into the cerebral ventricles. We found that the infusion speed and the volume of cell suspension are major determinants of cell distribution in a 3D-printed model of the piglet cerebral ventricular system. Labeling of GPs with SPIO affected their biodistribution in this model system, and thus, predictions regarding the destination of nonlabeled cells based on their labeled counterparts should be made with caution. Use of a long catheter also facilitated the persistence of cells within the ventricular system. The combination of SPIO labeling and long travel

distance may cause cell clumping, which may increase cell retention within the ventricular system and prevent outflow to the intrathecal space. Surprisingly, the cell concentration does not have a clear influence on cell distribution. There is also no major difference between the biodistribution of human and mouse GPs. The single end-hole catheter design deposits more cells in the vicinity of the catheter tip, while the side-hole design prevents cells from collecting at the catheter tip, but the difference has a negligible effect on the overall distribution of cells in the ventricular system.

Therefore, the reported sedimentation of cells in the occipital horn of the cerebral ventricle in a pediatric patient

with cerebral hypoxia/ischemia [18] might have been modifiable through adjustment of infusion parameters. While real-time MRI-guided imaging of cell delivery to the ventricular system would be preferable, a change in cell properties caused by the labeling process can represent a confounding factor. Positron emission tomography might be an interesting alternative, despite its limited spatial and temporal resolution, as due to its very high sensitivity, it may require negligible doses of radiotracer, therefore exerting minimal influence on cell properties [24]. There is also a rapid progress in magnetic particle imaging (MPI) cell tracking [25–27] and hybrid MPI-MRI tomography [28] with potential higher sensitivity, reducing the dose of SPIO needed for cell labeling [29].

It has been previously found that cell infusion parameters can affect cell viability [30]. Delivering a small volume of cells over an extended period of time reduces mechanical forces acting on cells and the creation of damaging pressure gradients [31]. However, there is still a high variability in the velocity of infusion used for cell transplantation, even in patients when using the same route of injection. Intracerebral transplantation of neural stem cells has been performed at a speed of 5 $\mu\text{L}/\text{min}$ [32], while the infusion velocity of intraspinally injected bone marrow stem cells was as high as 300 $\mu\text{L}/\text{min}$ [33]. The speed of infusion has also been recognized as an important factor of intra-arterial cell delivery to the brain [34]. Along with our finding of the potential value of real-time MRI to follow cell biodistribution within the ventricular system, it has been recently shown that this noninvasive method of cell monitoring is also important for following cell biodistribution after intraparenchymal delivery [35].

We found, surprisingly, that SPIO labeling affects the biodistribution of GPs infused in our 3D-printed model of the piglet ventricular system. Because it was previously reported that SPIO cell labeling does not affect cell sedimentation [18], we conclude that pushing the cell suspension through a long catheter with buildup of pressure inside the system may explain this paradox. Within this context, it has been shown that the secretome of mesenchymal stem cells can be modulated by passage through a catheter [36]. This phenomenon is probably independent from the previous findings of lowered neural stem cell motility following SPIO labeling [37].

The relatively broad cell biodistribution within the cerebral ventricles observed in our model system makes this route an attractive approach to deliver cells to vast areas of the brain in disseminated diseases such as NH-I. The cell suspension is directly injected to the fluid compartment, mitigating a potential stress of injection in the relatively rigid brain parenchyma. It has been reported that intracerebral injection may lead to tears of the brain parenchyma, inducing an inflammatory response [7]. There is currently much interest in optimization of intraparenchymal cell transplantation parameters [38,39]. However, direct placement of cells in the brain parenchyma is still the preferred option for localized diseases such as Parkinson disease [40] and small to middle-size strokes [41]. Finally, the field of tissue engineering has shown that cells may benefit from scaffolding with certain biomaterials [42]. Tissue engineering has been attempted to modulate the stroke cavity [43] and the feasibility of MRI-guided intrathecal trans-

plantation of hydrogel-embedded GPs has been shown in large animals [44]. Biomaterials may also be further used to modulate intraventricular cell delivery.

Limitations

While we attempted to closely reproduce the *in vivo* conditions, including the shape, size, and flow through the ventricular system, we were not capable of emulating cardiac pulsations that are contributing to the CSF pressure, which may affect convective mixing in the ventricles. Thus, it is possible that the amount of cell dispersion might have been underestimated in our model. We have also injected cells to the ventricular system at just one site, while other access points, although less clinically applicable, could have led to a different biodistribution. Our model allows for monitoring the immediate cell biodistribution only, and does not allow long-term observation of cell migration within the ventricular system and brain parenchyma. We have also not studied potential gradients of chemokines and cytokines present in pathological conditions such as ischemia, which could also affect the biodistribution of floating cells. While our experiments indicate that the buildup of pressure, which cells experience inside longer catheters and that can affect their clumping and biodistribution, we did not measure the ejection pressure, which was previously shown to be an important factor in needle-based cell injections [45].

Conclusions

A low volume of suspension such as 0.1 mL is beneficial in preventing outflow of cells from the piglet ventricular system into the subarachnoid/intrathecal space. A lower speed of infusion and use of an end-hole catheter facilitate focal delivery of cells near the catheter tip. If higher volumes of suspension such as 1.0 mL are necessary, then a much slower speed such as 0.01 mL/min is needed. Ultra-fast real-time MRI of SPIO-labeled cells is feasible, but the labeling itself can affect the biodistribution of GPs within the ventricular system. Our 3D-printed model of the piglet ventricular system allowed us to compare many injection paradigms with many repetitions, greatly reducing the number and cost of large animal experiments.

Author Disclosure Statement

All authors declare that no competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

References

1. Wang S, EX Wu, CN Tam, HF Lau, PT Cheung and PL Khong. (2008). Characterization of white matter injury in a hypoxic-ischemic neonatal rat model by diffusion tensor MRI. *Stroke* 39:2348–2353.

2. de Paula S, S Greggio and JC DaCosta. (2010). Use of stem cells in perinatal asphyxia: from bench to bedside. *J Pediatr* 86:451–464.
3. Davidson JO, G Wassink, LG van den Heuvel, L Bennet and AJ Gunn. (2015). Therapeutic Hypothermia for Neonatal Hypoxic-Ischemic Encephalopathy - Where to from Here? *Front Neurol* 6:198.
4. Lee JA, BI Kim, CH Jo, CW Choi, EK Kim, HS Kim, KS Yoon and JH Choi. (2010). Mesenchymal stem-cell transplantation for hypoxic-ischemic brain injury in neonatal rat model. *Pediatr Res* 67:42–46.
5. Lyczek A, A Arnold, JY Zhang, JT Campanelli, M Janowski, JWM Bulte and P Walczak. (2017). Transplanted human glial-restricted progenitors can rescue the survival of dysmyelinated mice independent of the production of mature, compact myelin. *Exp Neurol* 291:74–86.
6. Gupta N, RG Henry, J Strober, SM Kang, DA Lim, M Bucci, E Caverzasi, L Gaetano, ML Mandelli, et al. (2012). Neural stem cell engraftment and myelination in the human brain. *Sci Transl Med* 4:155ra137.
7. Janowski M, C Engels, M Gorelik, A Lyczek, S Bernard, JW Bulte and P Walczak. (2014). Survival of neural progenitors allografted into the CNS of immunocompetent recipients is highly dependent on transplantation site. *Cell Transplant* 23:253–262.
8. Haley MJ and CB Lawrence. (2016). Obesity and stroke: can we translate from rodents to patients? *J Cereb Blood Flow Metab* 36:2007–2021.
9. Savitz SI, SC Cramer, L Wechsler and S Consortium. (2014). Stem cells as an emerging paradigm in stroke 3: enhancing the development of clinical trials. *Stroke* 45: 634–639.
10. Porambo M, AW Phillips, J Marx, K Ternes, E Arauz, M Pletnikov, MA Wilson, JD Rothstein, MV Johnston and A Fatemi. (2015). Transplanted glial restricted precursor cells improve neurobehavioral and neuropathological outcomes in a mouse model of neonatal white matter injury despite limited cell survival. *Glia* 63:452–465.
11. Srivastava RK, JWM Bulte, P Walczak and M Janowski. (2018). Migratory potential of transplanted glial progenitors as critical factor for successful translation of glia replacement therapy: the gap between mice and men. *Glia* 66: 907–919.
12. Cui D, D Sun, X Wang, L Yi, E Kulikowicz, M Reyes, J Zhu, ZJ Yang, W Jiang and RC Koehler. (2017). Impaired autophagosome clearance contributes to neuronal death in a piglet model of neonatal hypoxic-ischemic encephalopathy. *Cell Death Dis* 8:e2919.
13. Lee JK, B Wang, M Reyes, JS Armstrong, E Kulikowicz, PT Santos, JH Lee, RC Koehler and LJ Martin. (2016). Hypothermia and Rewarming Activate a Macrogial Unfolded Protein Response Independent of Hypoxic-Ischemic Brain Injury in Neonatal Piglets. *Dev Neurosci* 38:277–294.
14. Huang L, F Zhao, Y Qu, L Zhang, Y Wang and D Mu. (2017). Animal models of hypoxic-ischemic encephalopathy: optimal choices for the best outcomes. *Rev Neurosci* 28:31–43.
15. Fenwick N, G Griffin and C Gauthier. (2009). The welfare of animals used in science: how the “Three Rs” ethic guides improvements. *Can Vet J* 50:523–530.
16. Oliveira JM, L Carvalho, J Silva-Correia, S Vieira, M Majchrzak, B Lukomska, L Stanaszek, P Strymecka, I Malysz-Cymborska, et al. (2018). Hydrogel-based scaffolds to support intrathecal stem cell transplantation as a gateway to the spinal cord: clinical needs, biomaterials, and imaging technologies. *NPJ Regen Med* 3:8.
17. Gorelik M, M Janowski, C Galporthawela, R Rifkin, M Levy, B Lukomska, DA Kerr, JW Bulte and P Walczak. (2012). Noninvasive monitoring of immunosuppressive drug efficacy to prevent rejection of intracerebral glial precursor allografts. *Cell Transplant* 21:2149–2157.
18. Janowski M, P Walczak, T Kropiwnicki, E Jurkiewicz, K Domanska-Janik, JW Bulte, B Lukomska and M Roszkowski. (2014). Long-term MRI cell tracking after intraventricular delivery in a patient with global cerebral ischemia and prospects for magnetic navigation of stem cells within the CSF. *PLoS One* 9:e97631.
19. Walczak P, J Wojtkiewicz, A Nowakowski, A Habich, P Holak, J Xu, Z Adamiak, M Chehade, MS Pearl, et al. (2016). Real-time MRI for precise and predictable intra-arterial stem cell delivery to the central nervous system. *J Cereb Blood Flow Metab* 37:2346–2358.
20. Jozwiak S, A Habich, K Kotulska, A Sarnowska, T Kropiwnicki, M Janowski, E Jurkiewicz, B Lukomska, T Kmiec, et al. (2010). Intracerebroventricular Transplantation of Cord Blood-Derived Neural Progenitors in a Child With Severe Global Brain Ischemic Injury. *Cell Med* 1:71–80.
21. Janowski M, P Walczak and MS Pearl. (2016). Predicting and optimizing the territory of blood-brain barrier opening by superselective intra-arterial cerebral infusion under dynamic susceptibility contrast MRI guidance. *J Cereb Blood Flow Metab* 36:569–575.
22. Olthoff A, AA Joseph, M Weidenmuller, B Riley and J Frahm. (2016). Real-time MRI of swallowing: intraoral pressure reduction supports larynx elevation. *NMR Biomed* 29:1618–1623.
23. Amer MH, F Rose, KM Shakesheff, M Modo and LJ White. (2017). Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges. *NPJ Regen Med* 2:23.
24. Weist MR, R Starr, B Aguilar, J Chea, J Miles, E Poku, E Gerdts, X Yang, S Priceman, et al. (2018). Positron emission tomography of adoptively transferred chimeric antigen receptor T cells with Zirconium-89 oxine. *J Nucl Med* 59: 1531–1537.
25. Bulte JW, P Walczak, M Janowski, KM Krishnan, H Arami, A Halkola, B Gleich and J Rahmer. (2015). Quantitative “Hot Spot” Imaging of Transplanted Stem Cells using Superparamagnetic Tracers and Magnetic Particle Imaging (MPI). *Tomography* 1:91–97.
26. Zhou XY, ZW Tay, P Chandrasekharan, EY Yu, DW Hensley, R Orendorff, KE Jeffris, D Mai, B Zheng, PW Goodwill and SM Conolly. (2018). Magnetic particle imaging for radiation-free, sensitive and high-contrast vascular imaging and cell tracking. *Curr Opin Chem Biol* 45:131–138.
27. Wang P, PW Goodwill, P Pandit, J Gaudet, A Ross, J Wang, E Yu, DW Hensley, TC Doyle, et al. (2018). Magnetic particle imaging of islet transplantation in the liver and under the kidney capsule in mouse models. *Quant Imaging Med Surg* 8:114–122.
28. Vogel P, S Lothar, MA Ruckert, WH Kullmann, PM Jakob, F Fidler and VC Behr. (2014). MRI Meets MPI: a bimodal MPI-MRI tomograph. *IEEE Trans Med Imaging* 33:1954–1959.
29. Bulte JWM. (2018). Superparamagnetic iron oxides as MPI tracers: a primer and review of early applications. *Adv Drug Deliv Rev* [Epub ahead of print]; DOI: 10.1016/j.addr.2018.12.007.

30. Kondziolka D, GT Gobbel, W Fellows-Mayle, YF Chang and M Uram. (2011). Injection parameters affect cell viability and implant volumes in automated cell delivery for the brain. *Cell Transplant* 20:1901–1906.
31. Guest J, F Benavides, K Padgett, E Mendez and D Tovar. (2011). Technical aspects of spinal cord injections for cell transplantation. Clinical and translational considerations. *Brain Res Bull* 84:267–279.
32. Borlongan CV, M Chopp, GK Steinberg, TM Bliss, Y Li, M Lu, DC Hess and D Kondziolka. (2008). Potential of stem/progenitor cells in treating stroke: the missing steps in translating cell therapy from laboratory to clinic. *Regen Med* 3:249–250.
33. Yoon SH, YS Shim, YH Park, JK Chung, JH Nam, MO Kim, HC Park, SR Park, BH Min, et al. (2007). Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: phase I/II clinical trial. *Stem Cells* 25:2066–2073.
34. Guzman R, M Janowski and P Walczak. (2018). Intra-arterial delivery of cell therapies for stroke. *Stroke* 49:1075–1082.
35. Malloy KE, JQ Li, GR Choudhury, A Torres, S Gupta, C Kantorak, T Goble, PT Fox, GD Clarke and MM Daadi. (2017). Magnetic resonance imaging-guided delivery of neural stem cells into the basal ganglia of nonhuman primates reveals a pulsatile mode of cell dispersion. *Stem Cells Transl Med* 6:877–885.
36. Parsha K, O Mir, N Satani, B Yang, W Guerrero, Z Mei, C Cai, PR Chen, A Gee, et al. (2017). Mesenchymal stromal cell secretomes are modulated by suspension time, delivery vehicle, passage through catheter, and exposure to adjuvants. *Cytotherapy* 19:36–46.
37. Berman SMC, Kshitiz, CJ Wang, I Orukari, A Levchenko, JWM Bulte and P Walczak. (2013). Cell motility of neural stem cells is reduced after SPIO-labeling, which is mitigated after exocytosis. *Magn Reson Med* 69:255–262.
38. Rossetti T, F Nicholls and M Modo. (2016). Intracerebral cell implantation: preparation and characterization of cell suspensions. *Cell Transplant* 25:645–664.
39. Bible E, DY Chau, MR Alexander, J Price, KM Shakesheff and M Modo. (2009). Attachment of stem cells to scaffold particles for intra-cerebral transplantation. *Nat Protoc* 4:1440–1453.
40. Rath A, A Klein, A Papazoglou, J Pruszek, J Garcia, M Krause, J Maciaczyk, SB Dunnett and G Nikkha. (2013). Survival and functional restoration of human fetal ventral mesencephalon following transplantation in a rat model of Parkinson's disease. *Cell Transplant* 22:1281–1293.
41. Smith EJ, RP Stroemer, N Gorenkova, M Nakajima, WR Crum, E Tang, L Stevanato, JD Sinden and M Modo. (2012). Implantation site and lesion topology determine efficacy of a human neural stem cell line in a rat model of chronic stroke. *Stem Cells* 30:785–796.
42. Modo M, F Ambrosio, RM Friedlander, SF Badylak and LR Wechsler. (2013). Bioengineering solutions for neural repair and recovery in stroke. *Curr Opin Neurol* 26:626–631.
43. Nicholls FJ, W Ling, G Ferrauto, S Aime and M Modo. (2015). Simultaneous MR imaging for tissue engineering in a rat model of stroke. *Sci Rep* 5:14597.
44. Malysz-Cymborska I, D Golubczyk, L Kalkowski, A Burczyk, M Janowski, P Holak, K Olbrych, J Sanford, K Stachowiak, et al. (2018). MRI-guided intrathecal transplantation of hydrogel-embedded glial progenitors in large animals. *Sci Rep* 8:16490.
45. Wahlberg B, H Ghuman, JR Liu and M Modo. (2018). Ex vivo biomechanical characterization of syringe-needle ejections for intracerebral cell delivery. *Sci Rep* 8:9194.

Address correspondence to:

Miroslaw Janowski, MD, PhD

Division of MR Research

Russell H. Morgan Department of Radiology

and Radiological Science

The Johns Hopkins University School of Medicine

Miller Research Building Room 649

733 North Broadway

Baltimore, MD 21205

E-mail: mjanows1@jhmi.edu

Received for publication August 20, 2018

Accepted after revision February 12, 2019

Prepublished on Liebert Instant Online February 14, 2019