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Original Article

SWI evaluation of mesenchymal stem cells labelled with SPIO-PLL in the treatment of hypoxic-ischemic brain damage in neonate rats

Aminou Mohamadou[1*], Jie Bian[1], Chuang Sun[1*], Natacha Raissa Doudou[1], Fangyuan Wu[1], Speville Johnson[2], Vuai Silima Miza[3]

[1]Department of Radiology and Nuclear Medicine, Second Affiliated Hospital, Dalian Medical University, Dalian, Liaoning, China; [2]Department of Psychiatry and Mental Health, Dalian Medical University, Dalian, Liaoning, China; [3]Department of Microbiology, Dalian Medical University, Dalian, Liaoning, China. [*]Equal contributors.

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Abstract: The aim of this study was to evaluate the distribution and effectiveness of bone marrow mesenchymal stem cells labelled with SPIO-PLL in the treatment of hypoxic-ischemic brain damage. A total of 50 healthy male SD rats were randomly dived into 5 groups, the control group, HIBD, Lateral Ventricle, Tail Vein, and Magnetized group, with (n = 10) in each group. The control group underwent a sham operation procedure, while the experimental group received the hypoxic-ischemic procedure (permanent ligation of the left carotid artery, followed by 2 hours in the anaerobic box with an 8% oxygen concentration). BMSCs were harvested from rat femurs and tibias by flushing the cavities of the bones with needles filled with DMEM, supplemented with 10% FBS. Approximately 11.2 mg Fe/mL SPIO was diluted with 25 μg/mL cell medium. Afterward, 1.5 μg/ml PLL was added to the final concentration. Confirmation of results was performed using conventional MRI, SWI, and Phase values. It was noted that the speed of regeneration of the brain tissue depended on the route of treatment administration. Distribution is faster through the lateral ventricle than the tail. SWI phase values, along with immunohistochemical and TUNEL assays, confirmed that BMSCs labelled with SPIO-PLL significantly reduced brain ischemia over time. Finally, neurological function analysis revealed that BMSCs significantly improved the neurological behavior of the rats after exposure to hypoxic ischemia. BMSCs labelled with SPIO-PLL play an important role in the treatment of HIBD. Therefore, intracerebroventricular injections and the use of magnets are effective measures in the treatment of early stage hypoxic ischemic brain damage.

Keywords: Hypoxic-ischemic brain damage, SPIO-PLL, susceptibility weighed imaging, bone marrow mesenchymal stem cells

Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) or hypoxic ischemic brain injury (HIBI) is a neurological condition that causes neonatal death, occurring in three to five out of every 1,000 live births [1]. About 10 to 60% of people that experience HIBD die as infants, while 25% of those that survive this injury exhibit a variety of serious neurological disorders, lowering life quality. Consequently, these factors increase the economic and social burden to their families [2].

Despite improvements in both maternal and neonatal care, HIBD is considered a main clinical issue causing acute mortality and severe neurologic impairments in neonates [3, 4]. This disorder is attributed to asphyxia *in utero* or at birth that results in diminished cerebral blood flow, cell damage, impaired vascular regulation, and metabolic disorders [5]. Effects of HIBD manifest as complications in the central nervous system (CNS), such as epilepsy, cerebral palsy, mental retardation, and learning disabilities, followed by a series of physiological and biochemical events [6]. Hypoxemia, over a prolonged period, can lead to cardiac hypoxia, which has been associated with reduced cardiac output leading to diminished blood flow to the brain [7, 8]. Notably, diagnosis of HIBD in both preterm and full-term neonates is often

difficult because it can occur in the perinatal, antenatal, or early postnatal period [9]. Due to the detrimental effects of HIBD, it is important to identify the best strategies for identification and treatment, as well monitoring of prognosis.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells (SCs) found in almost all tissues where they occur, either as mural cells or pericytes on the outer side of blood cells [10]. Bone marrow mesenchymal stem cells (BMSCs) have an effective critical component of stem cell therapy in HIBD and other conditions. Therefore, they are broadly used in processes that involve tissue regeneration in the connective tissues, such as bone, cartilage, or adipocytes. A recent breakthrough in stem cell therapy involves the use of MSCs to treat brain diseases that have a limited ability to regenerate, such as HIBD [11].

MRIs are the most reliable and commonly used neuroimaging technique. This technique can show the extent or severity of brain damage, predicting prognosis [12]. In this case, this technique can reveal the location of hypoxic-ischemic lesions in the brain [13]. In prognosis, MRI monitors para-magnetically label cells, such as MSCs [14, 15]. MRI is the modality of choice for HIBD diagnosis and tracking MSCs *in vivo* due to the non-invasive nature. MRIs provide high spatial resolution and high-contrast images.

Susceptibility weighted imaging (SWI) is a new imaging technique of the magnetic resonance sequence used to obtain a high contrast image of iron, blood, and other tissues [16]. SWI uses phase or magnitude images, or a combination of both, obtained with an echo sequence of high-resolution 3D gradient, compensated full flow in the directions. It also combines phase image filtering, thin slices, high-lighting susceptibility, and better data interpretation for the images [17]. Therefore, SWI is a technique based on differences in magnetic susceptibility or properties of tissues that are attributed to paramagnetic substances, such as de-oxyhemoglobin, iron, and ferritin [18]. SWI has been widely used as an additional tool in clinical imaging to visualize tissue structural changes caused by various pathological neurologic conditions, such as traumatic brain injury and cerebral infarction [19].

Materials and methods

Production of the SD rat HIBD model

Seven-day old male SD 7 rats, weighing 10.5~14.8 g, were purchased from Dalian Medical University Laboratory Animal Centre. The animals were then placed into sevoflurane anaesthesia induction boxes, set to 2% and 300 mL/min gas flow. Pulps were placed in the supine position, fixed on the single tube pavement covering the anaesthesia table. Limbs were fixed and the necks were disinfected using routine iodine-disinfectant. An approximately 10 mm longitudinal incision was made to separate subcutaneous tissue and superficial fascia, exposing the left carotid artery triangle of the medial margin of the sternocleidomastoid muscle. The left common carotid artery was ligated permanently with 7.0 surgical lines. Wounds were sutured and the animals were put into their original rearing environment to recover from surgery for 2 hours. They were then transferred to a 37°C oxygen deficiently chamber, with a control concentration of 8 (7%-9%) for hypoxia, with the rats fully awake. Oxygen concentrations were monitored by an oxygen analyzer. Humidity was set at 70%±5% and hypoxia induction lasted for 2 hours. In the sham operation group, the left common carotid artery was isolated after anaesthesia, but not ligated. Wounds were sutured without anoxic treatment. No rats died during the procedure.

Animal grouping

The animals were randomly divided into 5 groups, each with 10 rats: control group, HIBD group, lateral ventricle, tail vein group, and magnetized group. MRI examinations were performed at 0 hours, 24 hours, 1 week, and 2 weeks.

Isolation and purification of bone marrow mesenchymal stem cells

Bone marrow stem cells were harvested from the 10 SD rats. Primary MSCs were isolated from the bone marrow of the tibias and femurs of the rats by their adherence to plastic *in vitro* culturing. The rats were sacrificed by cervical dislocation, following anesthetization by intraperitoneal injections with 10% chloral hydrate (0.35 mL/100 g). Under sterile conditions, tibias and femurs were harvested, adherent soft tissue was removed, and the ends of the bones

were excised toward the start of the marrow cavity. Fresh bone marrow was harvested, aseptically, by flushing the cavities of the bones with needles filled with Dulbecco's modified Eagle's mediumlow glucose (DMEMLG; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone). A single cell suspension was prepared by gentle pipetting, several times, and passing the cell suspension through a 200-mesh metal strainer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were seeded into each tissue culture flask at a density of 90 cells/mL and cultured in an incubator (Forma Scientific; Thermo Fisher Scientific, Inc.) containing 5% CO_2 at 37°C. After 72 hours, nonadherent cells were removed. The medium was renewed and changed every 3 days. Once cells reached 80% confluency, as determined by phase contrast microscopy (Eclipse 80i; Nikon Corporation, Tokyo, Japan), they were placed into culture flasks at a 1:2 ratio.

Super-paramagnetic Iron oxide-poly-l-lysine (SPIO-PLL) labelled with BMSCs

Approximately 11.2 mg Fe/mL SPIO was diluted with 25 μg/mL cell medium. Next, 1.5 μg/ml PLL was added to the final concentration. The mixture was then incubated for 2 hours to obtain a SPIO-PLL complex. Cells with active proliferation were extracted and centrifuged, while the supernatant was discarded. The suspension was brown after sedimentation of the cells.

BMSCs labeling with SPIO-PLL

BMSCs were inoculated in the DMEM medium. The SPIO-PLL mixture was added to the culture medium to obtain the final concentrations of SPIO 25 μg/mL and 0.75 μg/mL PLL, then incubated for 24 hours at 37°C and 5% CO_2.

Prussian blue staining detecting the effectiveness of SPIO-PLL labelled with BMSCs

Prussian blue staining was used for detection. Cells with different concentrations of SPIO markers were plated in six-well plates for cell slides. First, the SPIO-containing medium was removed and rinsed 3 times with DMEM. Perl's solution A and B were then mixed in a 1:1 volume ratio and incubated for 30 minutes after the BMSCs had been fixed. An inverted phase contrast microscope was used for observation and photographing. Unmarked cells were used as controls. Positive blue particles in the cytoplasm were positive for Prussian blue staining. Positive cells were counted and cell marker rates of BMSCs in each group were determined.

MRI examinations

MRI scans were performed after ischemia–hypoxia exposure at intervals (0 hours, 24 hours, 1 week, and 2 weeks) under general anesthesia. Anatomic images were acquired using a 3D T1-weighted magnetization-prepared rapid gradient-echo sequence with the following parameters: repetition time, 500 ms; echo time, 11 ms; flip angle, 15; matrix, 320 x 320; and slice thickness, 2.0 mm. The final voxel size was 0.35 mm 3 0.35 mm 3 1.0 mm across the entire head from the tip of the snout to the cervical/thoracic spinal cord junction. Imaging comprised of conventional T2-weighted sequences and echo-planar susceptibility-weighted sequences. Total scanning time was controlled to within 6 minutes to shorten the exposure time of the neonate rats at a low temperature (15°C). Locations, extent, and image timing of ischemic damage on conventional and susceptibility-weighted sequences and phase images were compared. Images were exported into the Digital Imaging and Communications in Medicine format and analyzed using 3D visualization software (AMIRA; Visage Imaging, San Diego, CA).

Evaluation of neurological behavior

Neurological behavior of neonatal SD rats in the HIBD model was evaluated at 0 hours, 24 hours, 1 week, and 2 weeks, respectively, after ischemia and hypoxia. Scores were based on the improved of neurological impairment score (modified Neurological Severity Score, MNSS).

Immunohistochemical analysis

Brain tissues from experimental and control rats were fixed with 4% neutral paraformaldehyde and paraffin wax-embedded. Subsequently, 3-mm thick serial sections of the tissues were cut. The sections were washed carefully with 0.01 M phosphate buffered saline (PBS), three times (10 minutes each), then blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 (PBS-X) for 1 hour at room temperature. After staining, the tissue sections were evaluated by two blinded and experienced investigators that provided a consensus opin-

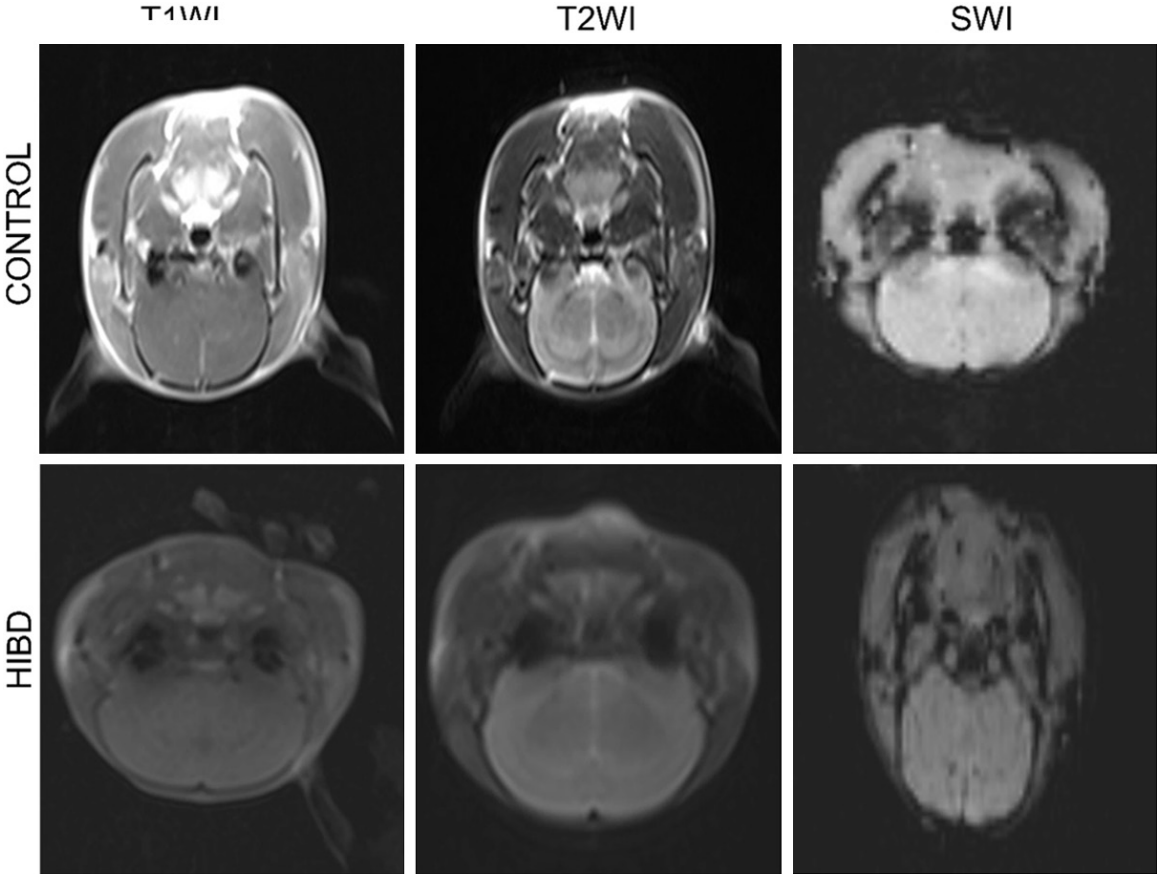

Figure 1. MRI results of the control and HIBD group. MRI results show no obvious abnormal changes at T1, T2, and SWI in the control group and some inhomogeneity, especially in the SWI image in the HIBD group.

ion of staining patterns observed under light microscopy.

Statistical analysis

All data are presented as mean ± SD. One-way ANOVA was used to analyze differences in phase values around the posterior horn of the lateral ventricle. Analyses of variance were used at a 95% significance level. Analysis was performed using SPSS 16.0. *P* values <0.05 indicate statistical significance.

Results

Establishment of the hypoxic ischemic brain damage model

Modified Rice et al., [20] methods were used to establish the brain hypoxic ischemic model. Routine MRI images of the brains were obtained at the time points of 0 hours, 24 hours, 1 week, and 2 weeks. In the control group, including T1WI, T2WI, and SWI, no clear abnormal signal changes were observed (**Figure 1**). In the experimental group, there were no significant abnormalities seen at 0 hours after the HIBD procedure. However, after 24 hours, the border area of the brain started to become blurred. After one week, some inhomogeneity of the brain was noted with some hypersignal and hyposiganal appearing in the white matter area of the hippocampus and lateral ventricle. The phase value of the area showed an increasing pattern from time points 0 hours to 24 hours, decreasing over time (**Figure 2**). Phase value means of the two groups were statistically different at P<0.05.

Intracerebroventricular injections of BMSCs labelled with SPIO-PLL.

To determine the effects of route of administration on treatment outcomes, stem cells were directly injected into the neonate rats exposed to hypoxic ischemia via the lateral ventricles side. SWI was used to assess pathological changes because it is more sensitive than conventional MRIs. No changes were observed in the HIBD, control, and tail vein group images.

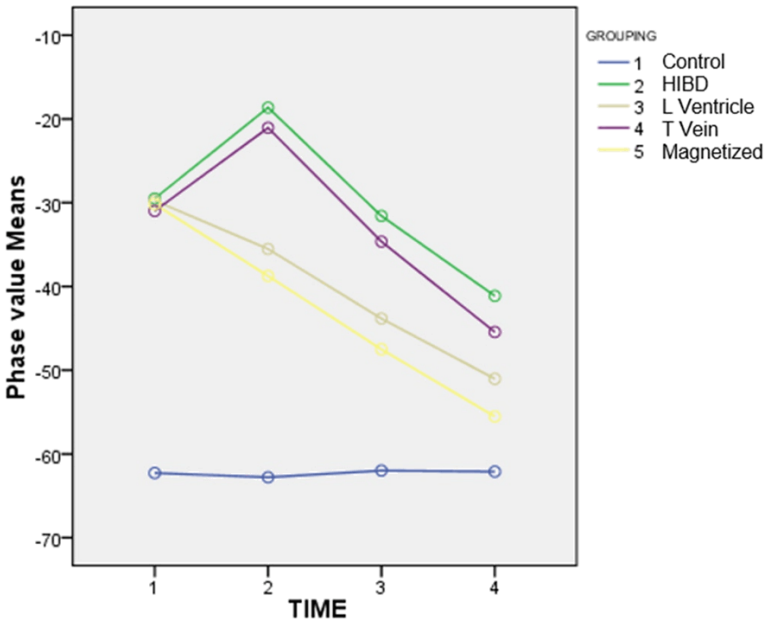

Figure 2. SWI phase value patterns in different groups at various time points. Note that the phase value of the control group was almost constant over time, while the HIBD and Tail vein group rapidly increased for 24 hours before decreasing gradually over time. Lateral ventricle and the Magnetized group decreased gradually over time.

However, the IV group showed a low-density signal around the posterior horn of the lateral ventricle, seen after 24 hours of hypoxic-ischemia (**Figure 3**). SWI phase values in the control group did not change over time. However, in the four groups, it significantly reduced after 24 hours and progressively decreased thereafter (**Figure 2**). SWI phase values between the IV group and the other 4 groups were significantly different (P<0.05) (**Table 1**). There were no significant differences between the HIBD and Tail vein group. SWI phase values at various time points in the IV group were lower than in the control group, suggesting that intracerebroventricular injections of the BMSCs provides good effects in early hypoxic ischemic brain damage. Present results were confirmed by histopathology examinations (**Figure 4**).

Intra-venous injections of BMSCs

Labelled BMSCs with SPIO-PLL were injected through the rat tail veins. No obvious changes were seen on SWI images at the starting point and after 24 hours. However, a spot dot was observed in the white matter of the brain after 1 week. SWI phase values rapidly increased from time points 0 hours to 24 hours, progressively reducing over time. There were statistically significant differences between this group, control group, and LV group (P<0.05). Present results suggest that IV injections could be effective, although slower than LV injections.

Using a magnet to attract labelled cells to the brain

A small magnet was placed on the heads, 2 hours prior to magnetic resonance imaging scans, to attract the bone marrow stem cells labelled with SPIO-PLL. There were statistically significant differences between this group and others (P<0.05). SWI results showed a round homogenous loss of signal of SWI, which was very intense at the time points 0 hours and 24 hours. However, it reduced significantly after 1 week and was almost invisible after 2 weeks (**Figure 3**). The same effects were seen on phase value measurements at various time points, decreasing significantly over time (**Figure 2**).

Evaluation of neurological behavior

To determine if BMSCs improve outcomes after hypoxic-ischemic exposure, the neurological behavior of neonate rats was evaluated using a modified neurological severity score test (**Table 2**). Parameters included reflexes, walking tests, sensory tests, balance tests, and abnormal movement. The test was classified into three categories, mild injury (1-4), moderate (5-8), and severe damage (9-12). Results show that the rats reacted slightly to a pinch of their tails after an hour of hypoxic-ischemic induction.

Discussion

Histopathological examinations of the control group showed light edema occurring over time with SWI phase values that were almost constant. This phenomenon can be attributed to injuries of the common carotid artery that do not progress to HIBD. In this case, blood flow and oxygen to the brain was restored by sutur-

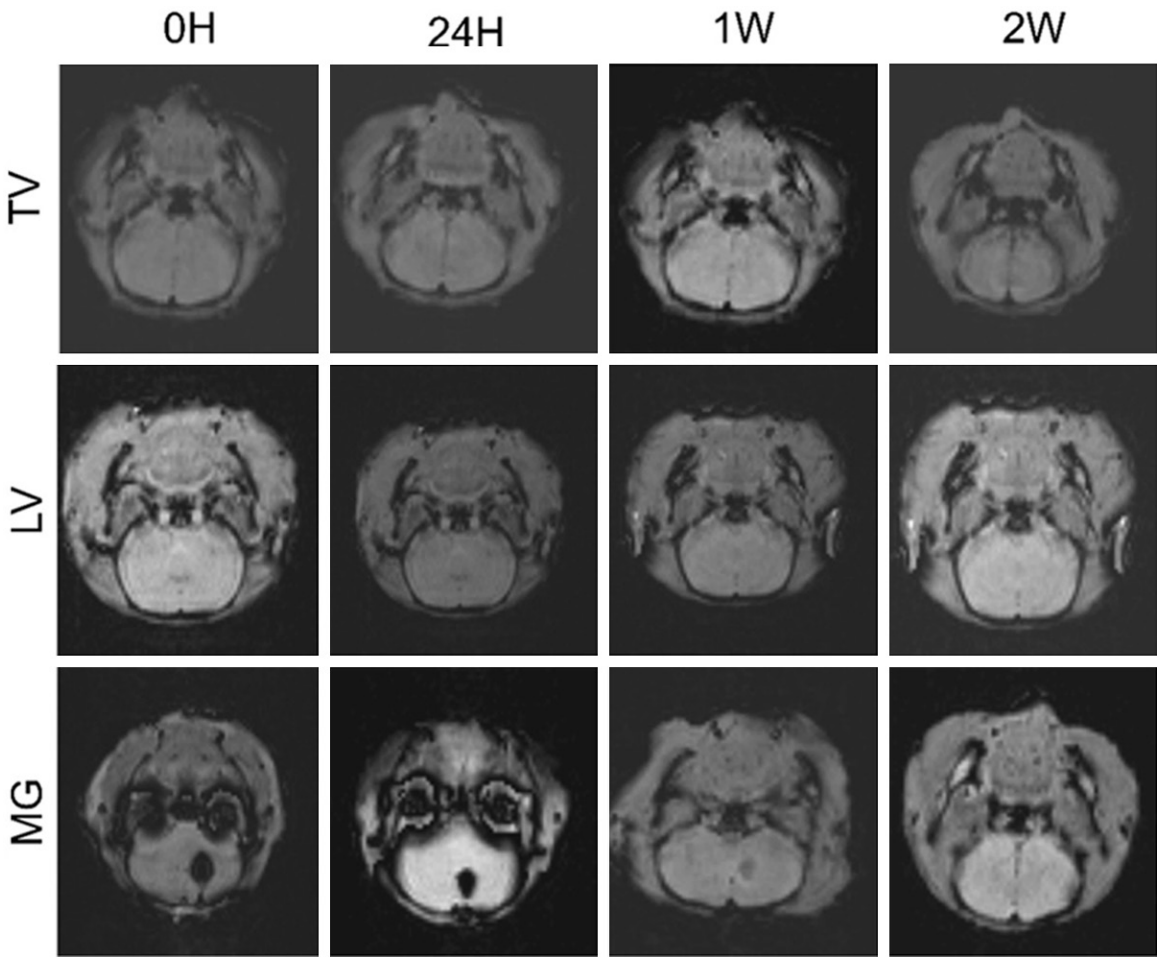

Figure 3. SWI images of each group and various time points. Results show that, in the tail vein group, no obvious changes occurred over time. The Lateral ventricle group shows a low-density signal around the posterior horn of the lateral ventricle. The Magnetized group shows a round homogenous loss of signal, which was very intense at the time points 0 hours and 24 hours, but reduced significantly after 1 week. Abbreviations: TV: Tail vein. LV: Lateral ventricle. MG: Magnetized.

Table 1. Phase values in the various groups

	Control	Tail Vein	HIBD	L- Ventricle	Magnetized
0 h	-62.27±0.36	-30.98±0.46	-29.50±0.23	-29.75±0.27	-30.16±0.49
24 h	-62.78±0.65	-21.05±0.74	-18.63±0.36	-35.52±0.26	-38.74±0.57
1 w	-61.97±0.44	-34.64±0.80	-31.58±052	-43.82±0.39	-47.50±0.60
2 w	-62.18±0.83	-45.44±0.69	-41.12±0.26	-51.02±0.67	-47.50±0.60
F value	1.26	2.50	3.52	4.03	4.45
P value	<0.001	<0.001	<0.001	<0.001	<0.001

Abbreviations: HIBD: Hypoxic-ischemic Brain Damage; TV: Tail vein; LV: Lateral ventricle; MG: Magnetized.

ing the wounds. An incision of the right common carotid artery in seven-day old newborn rats resulted in edema of the perivascular cells, not causing severe damage to the brain [21]. In rats, cerebral blood prevents severe brain damage since injury to one side of the carotid artery is compensated by the Willis ring, formed by the internal carotid and vertebral arteries beneath the brain [22].

There were significant differences between the development of HIBD experimental mice models and the control group ($p<0.05$). However, no significant changes were observed in SWI

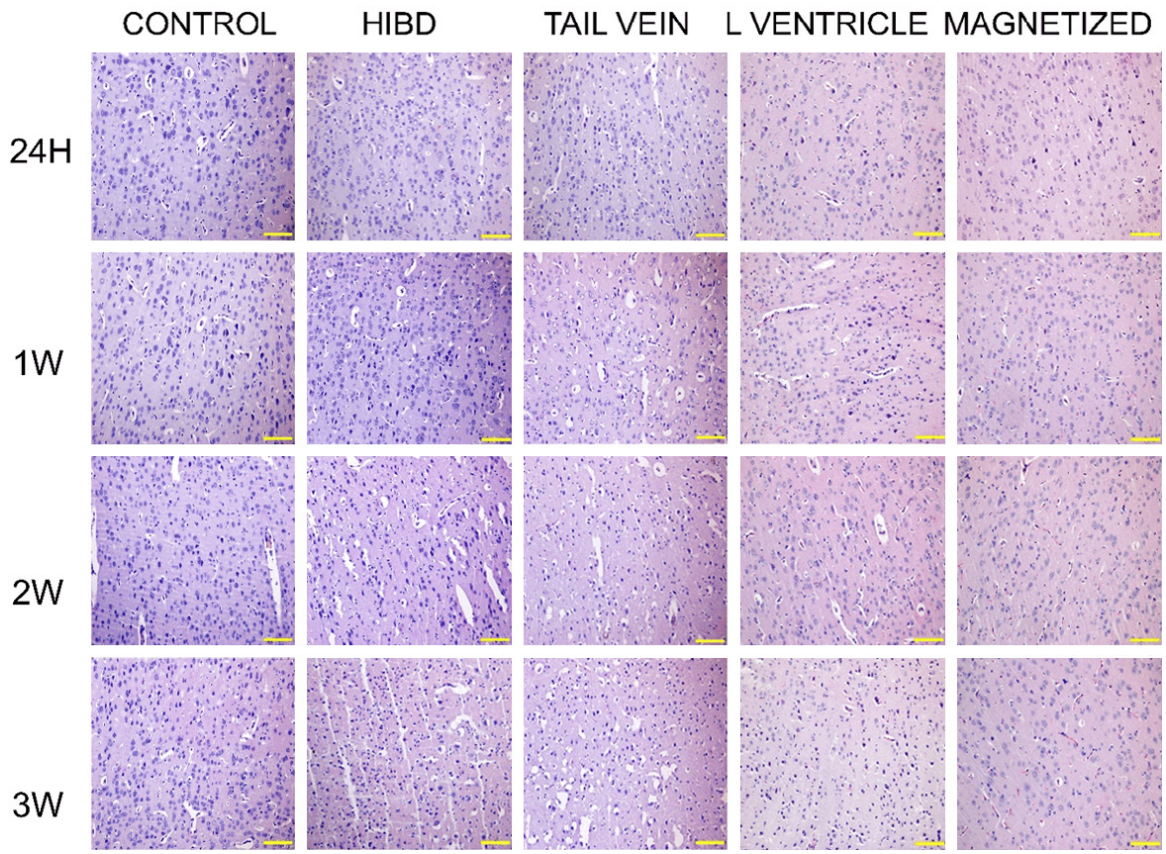

Figure 4. Histopathology results of each group and various time points. Note that, in the control and magnetized group, only a mild brain edema was noted at each time point, while in the HIBD group. After 24 hours: Brain edema, neuronal edema, and a small amount of microglia infiltration was noted. After 1 week: Brain edema, a minor neuronal degeneration, and small glial cell infiltration are seen. After 2 weeks: Brain edema, neuronal degeneration and necrosis, red neurons, reduced neuronal cells, and focal hyperplasia of microglia. After 3 weeks: Brain edema, neuronal degeneration and necrosis, neuronal red change, and small glial cells flake hyperplasia. For the lateral ventricle group, 24 hours after the injection: Mild brain edema, small amount of neuronal degeneration, and small amount of microglia infiltration was noted; 1 week after: Mild brain edema, small amount of neuronal degeneration, and a small amount of microglia infiltration; 2 weeks after: Mild brain edema, neuronal degeneration, microglia infiltration was visible; 3 weeks later: Mild edema of the brain, partial neuronal degeneration, small glial cell focal infiltration.

phase values in this group. This is because the mice did not experience hypoxia that resulted in changes in MRI signals and values [23]. Variations in phase values, observed in this study, increased over time. HIBD is a progressive disease that occurs over time, following brain injuries caused by a combination of hypoxia and ischemia. Progressive brain damage results in the production of microglia, which removes damaged cells [24].

In the HIBD group, hypo-intensity and more brain damage was noted. After 24 hours, white matter residue was observed. This occurred due to the excessive release of cellular contents, such as calcium. Additionally, features, such as neuronal degeneration, cell death (necrosis), and generalized edema, were manifested. Present results concur with those reported by Zille et al. (2012). They suggested that acute neuronal injury occurs when hypoxic or ischemic insult is not reversed [25]. Therefore, failure to reverse HIBD leads to further manifestation of pronounced signs, such as neuronal degeneration and increased glial cells, as observed after one week.

The most effective route for stem cell therapy of BMSCs labelled with SPIO-PLL, according to the current study, is the lateral left ventricle. These findings correspond with Slavc et al. (2018). They concluded that the lateral left ventricle (intraventricular route) is ideal for treatment for conditions with limited blood-brain

Table 2. Neurological behavior test results

Exercise test	
Pinch the rat's tail (up to 3 points)	
Forelegs flexion	1
Limb flexion	1
Active deviation from the central axis in 30 seconds	1
Put the rat on the floor (normal = 0, highest 3)	
Normal movement	0
Can't go straight	1
Circle to the side of the hemiplegia	2
Body down to the side of the hemiplegia	3
Test on balance (normal = 0, highest = 6)	
Maintain a balanced and stable state	0
Grab the edge of the wood	1
Grab the wood and one foot fall off the wood	2
Grab the wood and two feet fall off or rotate on wood (> 60 s)	3
Trying to keep the balance and fall (> the 40 s)	4
Trying to keep the balance and fall (> the 20 s)	5
Trying to keep the balance and fall (<the 20 s)	6

barrier (BBB) permeability [26]. Degeneration and necrosis of some neurons and slight edema of the brain tissues indicate net amelioration of HIBD over time (**Figure 4**), which conforms with the decrease of SWI phase values from -29.5 to -42.9. However, HIBD was not effective relative to magnet use, since they are redistributed into other tissues by blood circulation. For this reason, less concentrations of the injected cells move to the brain. TUNNEL results remained positive in both early and late phases, due to continued cell death (**Figure 5**). Conversely, the control group did not show any significant changes, according to histopathological tests and MRI imaging. However, slight brain edema and minor neuronal degeneration was manifested.

BMSCs therapy can be administered through the venous route, although intravenous administration results in amelioration of HIBD conditions. Present findings suggest that it cannot be used for quick reversal of brain damage caused by hypoxic/ischemic injury. Injection of cells into other sites, rather than directly into the brain ventricles, is not very effective because it requires transportation of the cells to the affected brain tissue [27]. SWI phase values of the Tail vein group slightly increased. However, it started to decrease after 24 hours, indicating the presence of SPIO-PLL in the brain tissue microenvironment. Distribution takes longer when administered through the tail because blood must flow back to the heart before being transported to the brain through the carotid artery. For this reason, the number of visible neurons decreased in the 3rd and 4th weeks, but brain tissue edema and neuronal degeneration and necrosis, as well as glial cell proliferation, remained evident (**Figure 4**). Necrosis was confirmed by positive TUNNEL results in both early and late evaluations. However, the late one was strong. This may be attributed to the redistribution of BMSCs into the bloodstream (**Figure 5**).

HIBD conditions continued to deteriorate in the HIBD group not receiving BMSCs therapy. These findings suggest the need for immediate treatment, preventing further brain damage that may lead to sequelae of neurological manifestations, such as epilepsy. Untreated HIBD conditions lead to more adverse effects, such as neuron degeneration and necrosis. Presence of red neurons, massive dissolving of the neuron body, and disappearance of the axons, as manifested in this case, are characteristic features of diffuse axonal injury that precedes brain insults [28]. Glial cell hyperplasia is also an accompanying feature of progressing HIBD involving brain macrophages, such as microglia, astrocytes, and oligodendrocytes [29].

SWI phase values, along with immunohistochemical and TUNEL assay analysis, confirmed that BMSCs labelled with SPIO-PLL significantly reduced brain ischemia over time. It was also noted that the speed of regeneration of the brain tissue depended on the location or the route of treatment administration. Distribution is faster through the lateral ventricle than the rat tails. Current results indicate that BMSCs labelled with SPIO-PLL play an important role in the treatment of early stage HIBD, especially via intracerebroventricular injections.

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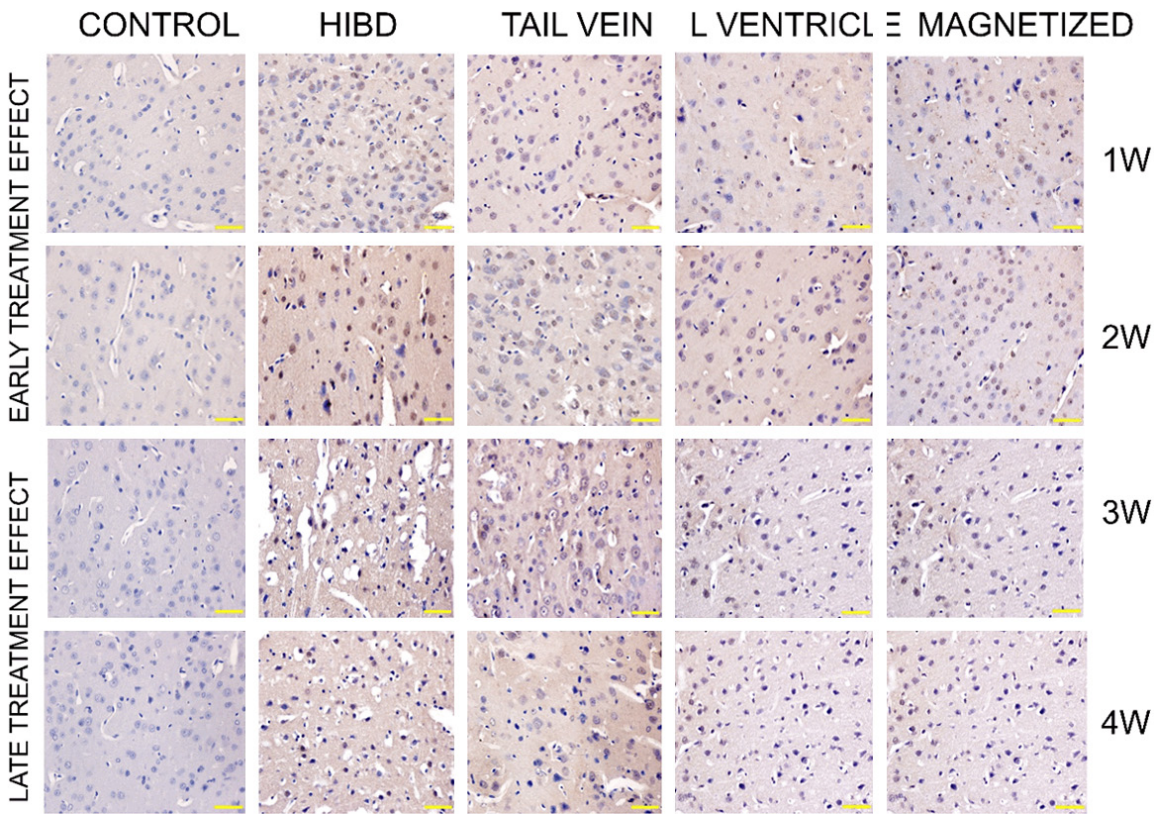

Figure 5. TUNEL Assay Results show: Control group: early and late treatment was negative (-); Magnetized group: early treatment was positive (+) and late treatment was negative (-); HIBD group: early treatment was positive (++) and late treatment was strongly positive (+++); Lateral ventricle group: early treatment was strongly positive (+++) and late treatment was positive (++); Tail vein group: early treatment was strongly positive (+++) and late treatment was positive (++).

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bian Jie, Radiology and Nuclear Medicine Department, Second Affiliated Hospital of Dalian Medical University, No.467 Zhongshan Road, Shahekou District, Dalian City 116027, China. Tel: +86 17709875088; Fax: +86 4672130; E-mail: drbianjie@hotmail.com

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