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TITLE:

Intraspinal Cavity Injection of Human Mesenchymal Stem Cells and Tracking Their Migration into the Rat Brain

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KEYWORDS:

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SUMMARY:

Several routes of administration can be used to deliver mesenchymal stem cells (MSCs) to the brain. In the present study, MSCs were delivered throughout the neuraxis and brain via intra-spinal cavity injection. MSCs were injected into the spinal cavities of rats, and stem cell migration was tracked and quantified.

ABSTRACT:
Mesenchymal stem cells (MSCs) have been studied for the treatment of various diseases. In neurodegenerative diseases involving defects in both the brain and the spinal cord, the route of administration is very important, because MSCs must migrate to both the brain and the spinal cord. This paper describes a method for administering MSCs into the spinal canal (intraspinal cavity injection) that can target the brain and spinal cord in a rat model. One million MSCs were injected into the spinal canals of rats at the level of lumbar vertebrae 2–3. After administration, the rats were euthanized at 0, 6, and 12 h post-injection. Optical imaging and quantitative real-time polymerase chain reaction (qPCR) were used to track the injected MSCs. The results of the present study demonstrated that MSCs administered via the spinal cavity could be detected subsequently in both the brain and spinal cord at 12 h. Intraspinal cavity injection has the advantage of not requiring general anesthesia and has few side effects. However, the drawback of the low migration rate of MSCs to the brain must be overcome.

INTRODUCTION:

Mesenchymal stem cells

Under disease conditions, MSCs secrete disease-specific therapeutic substances via paracrine actions[1] that have been reported to regulate immune responses, restore damaged tissues, and remove toxic substances[2]. Therefore, MSC therapy is considered more effective than single-target therapy in treating multifactorial diseases such as Alzheimer's disease and sarcopenia[3-6]. Additionally, in contrast to pharmaceuticals, MSCs have a homing effect, moving to the region of the damaged tissue by recognizing inflammatory cytokines or chemokines in the body[7,8]. Unfortunately, only a subset of the cells reach the damaged area, and the viability of MSCs decreases during migration[9-12]. Thus, to maximize the therapeutic efficacy of MSCs, it is necessary to deliver viable cells to the target site. Therefore, when administering MSCs, it is important to choose the proper route of administration, based on the nature of the target disease.

Injection route

There are numerous routes by which therapeutic agents are administered to patients. The most common methods are intravenous injection into the systemic circulation, oral administration, and subcutaneous or intramuscular injection. In neurodegenerative diseases, the main obstacle in delivering therapeutic agents to the brain is the blood-brain barrier (BBB). The BBB protects the brain from external pathogens by means of tight junctions between blood vessels and the brain parenchyma[13,14]. However, the BBB also paradoxically prevents therapeutic agents from entering the brain parenchyma. Therefore, passage through the BBB is the main hurdle in the development of brain disease therapies[15,16]. Intracerebral injection is performed to overcome this drawback by injecting target substances directly into the brain through surgical operation[17-19]. However, the side effects of surgical interventions should be considered, especially as the needle damages neuronal cells during the procedure.

Intraspinal cavity administration

Intrathecal administration—the administration of drugs into the spinal canal or subarachnoid space—delivers drugs to the brain or neuraxis through the cerebrospinal fluid (CSF) and is a viable alternative to intracerebral injection. Intrathecal injections can be subdivided according to the

injection site: lateral ventricle, cisterna magna, and spinal cavity. All three routes allow drugs or cells to disperse throughout the CSF into the brain and spinal cord. Drug delivery to the brain may be more efficient in the case of intracerebroventricular and intra-cisterna magna injections because the agent is injected close to the brain. However, intraspinal cavity injection has the advantages of not requiring general anesthesia or surgery for inserting an intraventricular reservoir, being generally safe[20], and can be repeatedly performed if necessary.

The purpose of this study was to validate intraspinal cavity administration as a means of delivering MSCs to both the brain and spinal cord. First, the intraspinal cavity was established in a rat model. Next, MSCs were labeled with a lipophilic tracer, DiD (DiIC18(5); 1,1-dioctadecyl-3,3,3,3- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt), to evaluate the efficiency of stem cell migration to the spinal cord and brain. Ex vivo optical imaging was performed to assess cell dispersion. This simple protocol can be performed without surgical intervention and may be used for the purpose of not only administering stem cells, but also pharmaceuticals, antibodies, contrast media, and other substances intended to be delivered to the spinal cord or brain.

PROTOCOL:

NOTE: This study was approved by the Institutional Animal Care and Use Committee (Approval number: 20170125001, Date: January 25, 2017) of the Samsung Biomedical Research Institute (SBRI) at Samsung Medical Center. As an accredited facility of the Association for Assessment and Accreditation of Laboratory Animal Care International, the SBRI acts in accordance with the guidelines set forth by the Institute of Laboratory Animal Resources.

1. Preparation of human Wharton's jelly-derived MSCs

1.1 Cultivation of human Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs)

1.1.1 Thaw a vial of human WJ-MSCs quickly in a 37 °C water bath. Transfer the WJ-MSCs to a 50 mL conical tube, and add growth medium at a volume at least 10 x that of the cells (v/v). Pipet up and down to suspend the cells.

1.1.2 Centrifuge at 300 × *g* for 5 min. Carefully discard the supernatant, and resuspend the cells.

NOTE: Be careful not to discard the cell pellet.

1.1.3 Seed WJ-MSCs in T175 flasks at a density of 5,000–6,000 cells/cm^2. Incubate WJ-MSCs in a 37 °C CO_2 incubator. Change the growth medium every 72 h until WJ-MSCs reach 80–90% confluency.

NOTE: Generally, it takes 3–4 days for the MSCs to reach 80–90% confluency.

1.2 Subcultivation of human WJ-MSCs

1.2.1 Discard the growth medium, and wash the cells with 10 mL of phosphate-buffered saline (PBS). Remove the PBS, and add 5 mL of 0.25% trypsin-disodium ethylenediaminetetraacetic acid (EDTA) (see the **Table of Materials**). Incubate the cells at 37 °C in a CO_2 incubator for 3 min until the WJ-MSCs detach from the culture flask.

1.2.2 Add 5 mL of growth medium containing 10% fetal bovine serum to neutralize the 0.25% trypsin-EDTA. Collect the cell mixture and transfer it to a 50 mL conical tube. Wash the cell culture flask with 10 mL of growth medium, and collect the cells in a 50 mL tube using a sterile serological pipet.

1.2.3 Centrifuge the cell mixture at 300 × *g* for 5 min. Discard the supernatant, resuspend the cells in 10 mL of growth media, and count the number of WJ-MSCs.

CAUTION: Be careful not to discard the cell pellet.

1.2.4 Seed WJ-MSCs at a density of 4,000–6,000 cells/cm^2, depending on the experiment.

1.3 Labeling WJ-MSCs with DiD dye and the preparation of WJ-MSCs for intraspinal cavity injection

NOTE: The DiD dye-labeling procedure was performed following manufacturer's instructions.

1.3.1 Detach WJ-MSCs when they reach 80% confluency, using the procedure mentioned above. Suspend WJ-MSCs at a density of 1 × 10^6/mL in phenol-red-free minimum essential medium (MEM) α without serum.

1.3.2 Add 5 µL of DiD labeling solution per 1 mL of cell suspension; mix gently with pipetting.

1.3.3 Incubate for 15 min at 37 °C; centrifuge the cell suspension at 300 × *g* for 5 min.

1.3.4 Remove the supernatant, and resuspend the WJ-MSCs in phenol-red-free MEM α at a density of 1 × 10^6/0.2 mL.

2. Intraspinal cavity injection of WJ-MSCs

2.1 Preparation for intraspinal cavity injection

2.1.1 Anesthetize 6-week-old Sprague-Dawley rats with 5% isoflurane; then, maintain anesthesia with 2% isoflurane throughout the surgical procedure.

NOTE: Optimize the anesthetic conditions before starting the experiment.

2.1.2 Shave the surgical area using an electric shaver for small animals.

NOTE: The electric shaver can be replaced with a manual razor and shaving gel.

2.1.3 Disinfect the surgical area using povidone-iodine. Create a 3 cm incision in the skin with a surgical blade. Resect the remaining skin and muscle tissue using a surgical blade and scissors. Reveal the spinous processes at lumbar 2–3 (L2–3).

2.2 Injection of DiD-labeled WJ-MSCs via the intraspinal cavity

2.2.1 Place the rat in a prone position. Flex the rat's spine appropriately to widen the distance between the adjacent spinous processes, using sufficient amounts of paper tissue or other materials that can aid in maintaining the appropriate position.

2.2.2 Fill a 1 mL syringe with 0.2 mL of DiD-labeled WJ-MSCs. Place a 23 G syringe-needle combination vertically between the spinous processes of L2 and L3, and insert the needle until it touches the vertebral body.

2.2.3 When the needle touches the vertebral body, retract it by approximately 0.5 cm, placing the tip of the needle in the spinal canal. Tilt the syringe, and place the tip of the needle such that it points toward the rostral direction. Inject WJ-MSCs into the spinal cavity over a 1 min period.

NOTE: The speed of injection should be optimized in advance.

2.2.4 After injection, completely remove the syringe from the spinal canal. Suture the incision, and then disinfect the surgical site using povidone-iodine.

2.3 Post-procedure treatment

2.3.1 Stabilize and restrain the rat to prevent any movement, placing it upside-down at a 45° angle for 15 min, while it is still under anesthesia. After 15 min, discontinue anesthesia, and wait for the rat to rouse.

3. Evaluation of intraspinal cavity injection

3.1 Euthanasia of the rats and isolation of the brain and spinal cord at 0, 6, and 12 h post-injection

3.1.1 Anesthetize the experimental animals with 5% isoflurane; maintain anesthesia with 2% isoflurane during PBS perfusion.

3.1.2 Make an incision below the diaphragm using surgical scissors. Open the incision with forceps, and cut the rib cage rostrally to expose the heart.

3.1.3 Make a small hole in the right atrium, and insert a butterfly needle into the left ventricle.

Perfuse 100 mL of cold PBS into the left ventricle for 4–5 min, until the liver is cleared of blood.

3.1.4 After perfusion, make a long incision on the backside from the head to the tail using a surgical blade along the longitudinal plane. Isolate the remaining brain and the whole spine using surgical scissors, forceps, and a bone cutter. Remove the remaining ribs, connected bones, and flesh.

3.2 Ex vivo DiD fluorescent optical imaging

3.2.1 Place the isolated tissues in the chamber of the optical imaging device.

3.2.2 Set the parameters as follows: emission, 700 nm; excitation, 605 nm; and exposure time, 2 seconds, as photons per second per centimeter squared per steradian (p/s/cm^2/sr). Capture the optical images.

NOTE: All images should be acquired with identical illumination settings (lamp voltage, filters, f/stop, field of view, and binning).

3.2.3 Draw three rectangular regions of interest (ROIs) of equivalent size for the spinal cord and one circle ROI for the brain using the drawing tool. Measure the fluorescent intensities of the ROIs .

3.3 Extraction of genomic DNA (gDNA) from the spinal cord and brain tissue

3.3.1 Remove the skull and spine carefully using surgical forceps, scissors, and a rongeur.

3.3.2 Harvest the brain and spinal cord from the skull and spine. Cut the spinal cord into three pieces (cervical, thoracic, and lumbar).

NOTE: The harvested tissues must be stored at -80 °C if they are not analyzed immediately.

3.3.3 Grind the tissues using a pre-chilled mortar, pestle, and liquid nitrogen. Extract gDNA using commercial products, following the manufacturer's instructions.

3.4 Quantitative real-time polymerase chain reaction (qPCR)

3.4.1 Quantify the amount of gDNA in each sample using a spectrophotometer.

3.4.2 Perform qPCR using 10 ng of gDNA per sample and human *Arthrobacter luteus* (*ALU*) primers[12,21].

3.4.3 Calculate the exact number of WJ-MSCs in the samples using the $\Delta\Delta C_T$ method[22].

REPRESENTATIVE RESULTS:

To evaluate the efficacy of intraspinal cavity injection of MSCs, DiD-labeled MSCs were used in the present study. Before injecting MSCs into the spinal cavity, the labeling efficacy was assessed in vitro using optical imaging and fluorescence microscopy (**Figure 1**). After staining the MSCs with the DiD labeling reagent using the procedure described in protocol section 3.1, optical images were taken of the culture plates on which DiD-labeled MSCs were seeded (**Figure 1A**). DiD-labeled MSCs (+DiD) are shown in red; naïve MSCs (-DiD), which were not labeled with DiD dye, did not show a positive signal. This result was confirmed using fluorescence microscopy (**Figure 1B**). MSCs were also stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize their shape. Naïve MSCs (-DiD) appeared blue, indicating staining with DAPI or nuclear staining, but did not show red color, which is associated with DiD staining. In contrast, DiD-labeled MSCs (+DiD) showed both blue and red colors, indicating that the DiD labeling method had been successful.

[Place **Figure 1** here]

Next, the method of intraspinal cavity injection was evaluated. To evaluate and optimize the intraspinal cavity injection procedure, trypan blue dye was used instead of DiD-labeled MSCs, as described in protocol section 2 (**Figure 2**). Trypan blue dye was injected, and the rat was euthanized immediately. The spinal cord tissue harvested from the rat was cut into three pieces transversely, and the brain was cut into coronal sections. It was found that the injected trypan blue dye had stained the spinal cord tissues (lumbar, thoracic, and cervical cords). Moreover, both the inferior and superior sides of the brain were stained blue. However, the trypan blue dye did not penetrate the lateral ventricle of the brain. These results indicated that this method of intraspinal cavity injection was successful.

[Place **Figure 2** here]

Using the protocol successfully optimized as described above, DiD-labeled MSCs were injected via the intraspinal cavity in rats (**Figure 3**). The rats were euthanized 0, 6, and 12 h post-injection, and ex vivo optical imaging was performed (**Figure 3A**). Compared with signals in the control (no-injection) animals, high and condensed positive signals were detected in the lumbar spinal cords of rats euthanized immediately post-injection (0 h). At 6 h post-injection, the positive signal was dispersed throughout the lumbar spinal cord. Finally, high-positive signals were observed in the lumbar and cervical cord regions and even in the brain at 12 h post-injection. The signal intensity of the optical images was quantified at each time-point using image analysis software (**Figure 3B**). A significant increase in signal intensity was identified in the lumbar spinal cord at 0 h post-injection and in the brain at 12 h post-injection.

[Place **Figure 3** here]

A previous study reported that the human *ALU* sequence can be used as a quantitative marker for measuring the number of human origin cells, such as neural stem cells and MSCs, in the context of xenograft transplantation[9,11,12]. Following the guidelines from the previous study, qPCR analysis using a human-specific *ALU* primer was used in the present study to evaluate the

in vivo distribution and migration of DiD-labeled MSCs (**Figure 4**). After amplification of *the ALU* sequence, the PCR product was separated on an agarose gel and visualized (**Figure 4**A). Compared with the control sample (the gDNA extracted from the brains of rats in the no-injection group), the *ALU* sequence was highly amplified in the 12-h sample (gDNA extracted from the brains of rats at 12 h post-injection). However, in both samples, mouse *Gapdh* was highly amplified, indicating that human origin cells, especially the human MSCs used in this study, were present only in the 12-h group sample. Next, the distribution of MSCs in the brain and other organs (heart, lung, liver, spleen, and kidney) was confirmed using the same method (**Figure 4B**). Similar to the results of optical imaging, a significant number of MSCs were detected only in the brain at 12 h post-injection. The other samples showed variance in the ratio of *ALU* to *Gapdh*, but all the signals from the samples, except the brain at 12 h post-injection, were all under the limit of detection, which implies that no significant amplification occurred.

[Place **Figure 4** here]

The results of the present study demonstrate that MSCs delivered via intraspinal cavity injection were distributed in the lumbar region at all time-points and migrated to the brain at 12 h post-injection.

FIGURE AND TABLE LEGENDS:

Figure 1: DiD labeling of MSCs in vitro. (**A**) The DiD-labeled MSCs were seeded in a 6-well culture plate, and optical imaging was performed. (**B**) Images were taken using fluorescence microscopy. The MSC nuclei are indicated by blue (DAPI), and the incorporated DiD is localized in the cytosol of +DiD cells (red). Scale bars = 500 µm. This figure has been modified from Kim et al.[12]. Abbreviations: -DiD = naïve MSCs, +DiD = MSCs labeled with the DiD reagent; MSC = mesenchymal stem cell; DiD = DiIC18(5); 1,1-dioctadecyl-3,3,3,3- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt; DAPI = 4',6-diamidino-2-phenylindole.

Figure 2: Confirmation of intraspinal cavity injection in a rat model. The trypan blue dye was injected into the spinal cavity of a test rat. After injection, the rat was euthanized, and a necropsy was performed. The trypan blue dye injected via the spinal canal migrated to and stained both the spinal cord (lumbar, thoracic, and cervical) and the brain blue. Scale bars = 1 cm. This figure has been modified from Kim et al.[12].

Figure 3: Optical imaging analysis to evaluate the efficacy of intraspinal cavity injection. (**A**) After injecting the MSCs into the spinal cavity, the rats were euthanized at the following time points: 0, 6, and 12 h post-injection, after which ex vivo optical imaging was performed. The positive signal is shown as yellow-red color. (**B**) From the images, the signal intensities were quantified using software. Equivalent regions of interest were drawn for each experimental group. Data are presented as the mean ± standard error of mean (SEM). $*p < 0.05$. This figure has been modified from Kim et al.[12]. Abbreviations: MSCs= mesenchymal stem cells; CTL = control.

Figure 4: Quantitative real-time polymerase chain reaction to confirm the distribution and

migration of MSCs. (**A**) The amplified PCR products of brain genomic DNA extracted from the control and 12-h groups were visualized on an agarose gel. *ALU* is a human-specific primer, and mouse *Gapdh* primer was used for normalization. The amplified PCR products are shown as intense white bands. (**B**) qPCR analysis was performed, and the ratio of *ALU* to *Gapdh* in each sample was calculated. The red dashed line indicates the limit of detection in real-time PCR analysis. Data are presented as the mean ± standard error of mean (SEM). **p* < 0.05. This figure has been modified from Kim et al.[12]. Abbreviations: *ALU* = *Arthrobacter luteus;* PCR = polymerase chain reaction; qPCR = quantitative real-time PCR; MSCs = mesenchymal stem cells; Gapdh = glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION:

The optimal route of administration for treatment with MSCs should be chosen depending on the target disease, the patient's condition, and the type of drug to be delivered. In cell therapies, including MSC therapy, direct injection of stem cells into the brain or intrathecally via the CSF must be considered as the cells cannot pass through the BBB[19]. Intraspinal cavity injection is relatively non-invasive and does not cause neuronal damage in the brain, unlike intracerebroventricular injections, and is associated with a low risk of side effects[20]. Accessing CSF via a lumbar puncture is a procedure that can be performed very easily at clinical sites. Therefore, it is not difficult to administer stem cells, drugs, contrast agents, or other substances to patients via intraspinal cavity injections[23-25]. In contrast, intracerebroventricular injections, which require surgical intervention, are more complicated. However, for experimental animals, injection into the intraspinal cavity is more difficult than administering agents directly into the brain. This is because rodents, including mice and rats, are very small compared to humans[26].

In the case of intraspinal cavity administration, the needle must be inserted between the spinous processes. Compared with the gaps in humans, the gaps between the bones in a rat are very narrow, making access difficult. To overcome this, the smallest needle, ideally a 23 G needle, should be used. Although a 26 G needle can be used, such a thin needle can bend easily. The size of the needle can be adjusted based on the age of the experimental animal. Additionally, to facilitate intraspinal cavity administration in a rat model, it is necessary to flex the spine, widening the gap between spinous processes, making it easier for the needle to access the space between the spinous processes. The injection site and direction of the needle can also be adjusted as needed. The gaps between cervical spinous processes are relatively wider than those between lumbar spinous processes. However, if the injection is performed near the cervical or thoracic portions of the spinal cord, incorrect placement of the needle may cause serious spinal cord damage, including paralysis of the lower limbs of the experimental animal or patient.

Therefore, when selecting an upper spinal cord region, care must be taken to prevent damage to the spinal cord and nerves. In humans, the spinal cord ends at L1–2, where the cauda equina starts. The cauda equina is a bundle of lumbar and sacral nerves; therefore, the lumbar spine is a relatively safer location for injection than the cervical or thoracic spine. Therefore, only the lumbar spine, particularly the region under L2 where the cauda equina starts, is recommended for intraspinal cavity injection. Based on this consideration, the lumbar spine under L2 was selected for the present study to minimize spinal nerve damage. To track the stem cells delivered

to the spinal cord and brain, a DiD reagent was used to label the WJ-MSCs, which were visible under a fluorescence microscope and in in vitro and ex vivo optical imaging experiments (**Figure 1** and **Figure 3**). Unlabeled WJ-MSCs did not show any positive DiD fluorescence in vivo in the control group (no-injection). These results indicate that the lipophilic DiD dye can be used as a tracking agent for stem cell therapy. Currently, many different agents have been developed to track transplanted stem cells[27-29]. These tracking reagents can be adjusted based on the equipment used for evaluation, such as magnetic resonance imaging, computed tomography, and optical imaging. A previous study reported the use of iron nanoparticles to track MSCs delivered via intracerebroventricular injection into the brain[9,29]. Thus, various metallic nanoparticles and lipophilic agents, such as DiD, can be used for in vivo and ex vivo stem cell tracking.

To evaluate the migration and distribution of WJ-MSCs delivered via intraspinal cavity injection, qPCR analysis was performed with an *ALU* primer. The primary objective of the present study was to optimize the method of intraspinal cavity administration and evaluate its efficacy. Therefore, analysis methods were selected for tracking and quantifying the overall distribution and migration of WJ-MSCs throughout the brain and spinal cord at various time-points. For this reason, optical imaging was performed with the brain and spinal cord still connected. The whole brain or spinal cord (cervical, thoracic, and lumbar) was ground up, and the exact numbers of WJ-MSCs in those tissues were calculated via qPCR analysis with an *ALU* primer. The human-specific primer *ALU* has been reported to have high sensitivity and specificity for detecting human origin cells among rodent cells[21]. Additionally, the Ministry of Food and Drug Safety in Korea recommends using human *ALU* primers to evaluate the biodistribution of stem cells as part of the preclinical data collected for investigational new drug approval. To identify the exact location of WJ-MSCs migrating toward the brain and spinal cord at different time-points, immunohistochemical staining (IHC) should be performed. However, IHC was not performed here, which is a limitation of this study.

The euthanasia time-points should also be appropriately selected. The speed of stem cell migration toward the brain and the distribution pattern throughout the neuraxis depend on the delivered substances and the state of the experimental animals or patients. It is important to determine the physical and chemical characteristics of injected materials. Various factors such as size, mass, lipophilicity, and half-life can affect the time required to migrate to the brain and disperse throughout the entire central nervous system (CNS). Therefore, an appropriate euthanasia time-point must be established in accordance with the properties of the substance being administered. Moreover, the physical state of the test subject is also important. In both patients and diseased animal models, there are many substances, such as inflammatory cytokines and target epitopes, that can attract therapeutic agents (stem cells, immune cells, and antibody drugs) toward lesion sites. Therefore, it will take less time for WJ-MSCs to reach the brain if a CNS disease model is used. In the present study using a wild-type rat model, three different time-points (0, 6, and 12 h) were selected. The experimental animals in the 0 h group were euthanized immediately after stem cell injection, and WJ-MSCs were detected only in the lumbar spinal cord around the injection site. In contrast, WJ-MSCs were observed in the brains and cervical spinal cords of rats in the 12 h group, indicating that it took a minimum of 12 h for the WJ-MSCs to

migrate to the brain and cervical cord in a wild-type rat model. Theoretically, additional WJ-MSCs can migrate to the brain as time progresses, but this was not evaluated or proven in the present study.

The intraspinal cavity administration of MSCs has the disadvantage of low efficiency of delivery to the brain compared with that of intracerebroventricular or intraparenchymal administration[12]. The first reason for this is the distance from the administration site to the brain, and the second reason pertains to CSF flow. As CSF is produced in the choroid plexus located in the lateral ventricle of the brain, CSF flows from the lateral ventricle to the spinal cord[30]. Therefore, in this study, the rats were placed in an upside-down position at a 45° angle for 15 min to aid the migration of MSCs to the brain. A greater angle or longer wait time may promote increased migration of MSCs to the brain. Additionally, the volume, speed, and dosage of the injection can be modified to achieve more efficient delivery to the brain and spinal cord. The present study introduces a process by which WJ-MSCs can be administered via the intraspinal cavity at L2–3 and evaluated the migration and distribution patterns of the stem cells at 0, 6, and 12 h post-injection in a rat model. Although only a small number of WJ-MSCs delivered via the intraspinal cavity route moved to the rat brain in the present study, this number can be increased by adjusting several variables. The preclinical data provided in the present study can be considered as scientific basis for the clinical use of intraspinal cavity injection of stem cell therapy, immunotherapy, and other curative substances.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

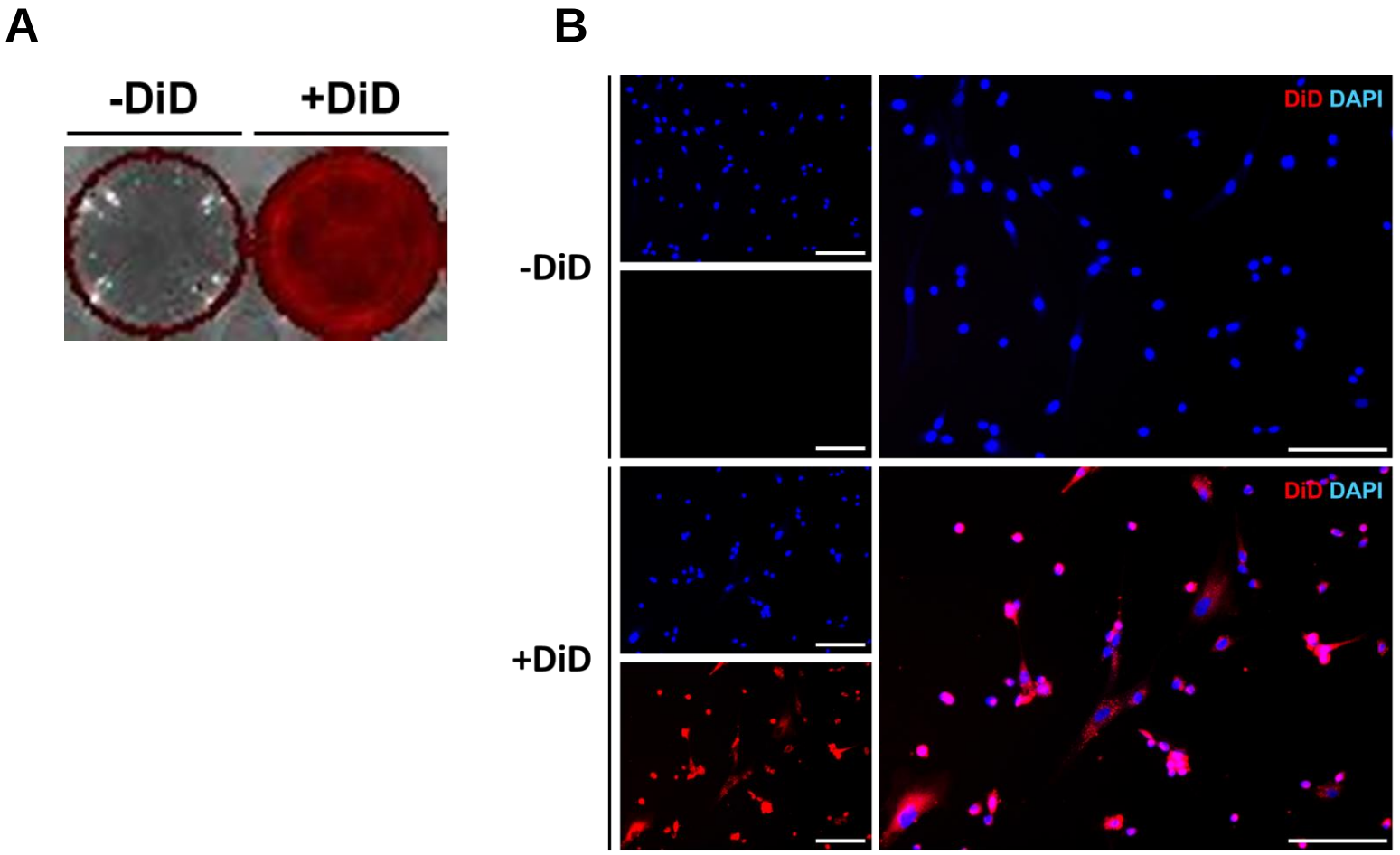


Figure 2

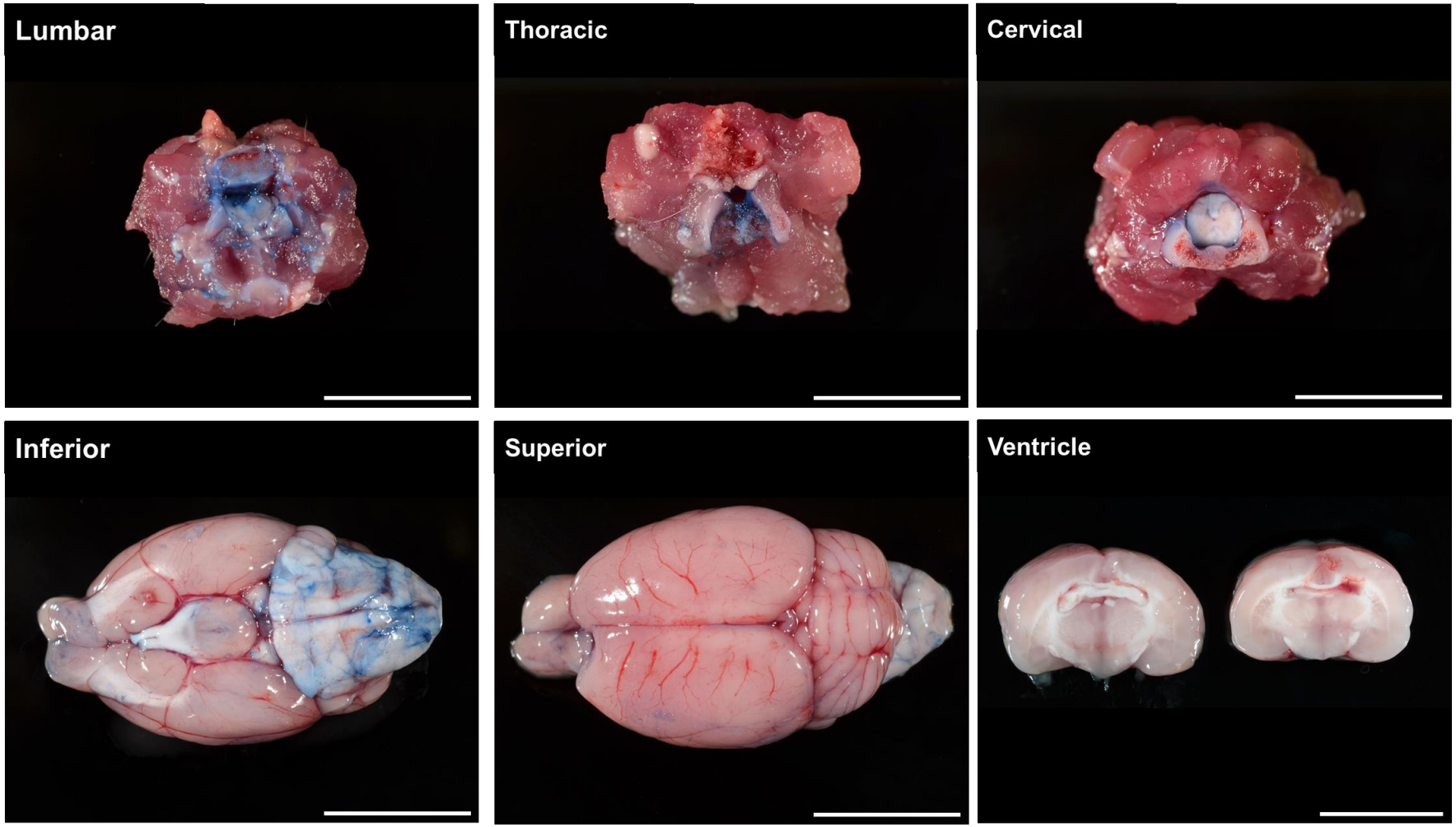
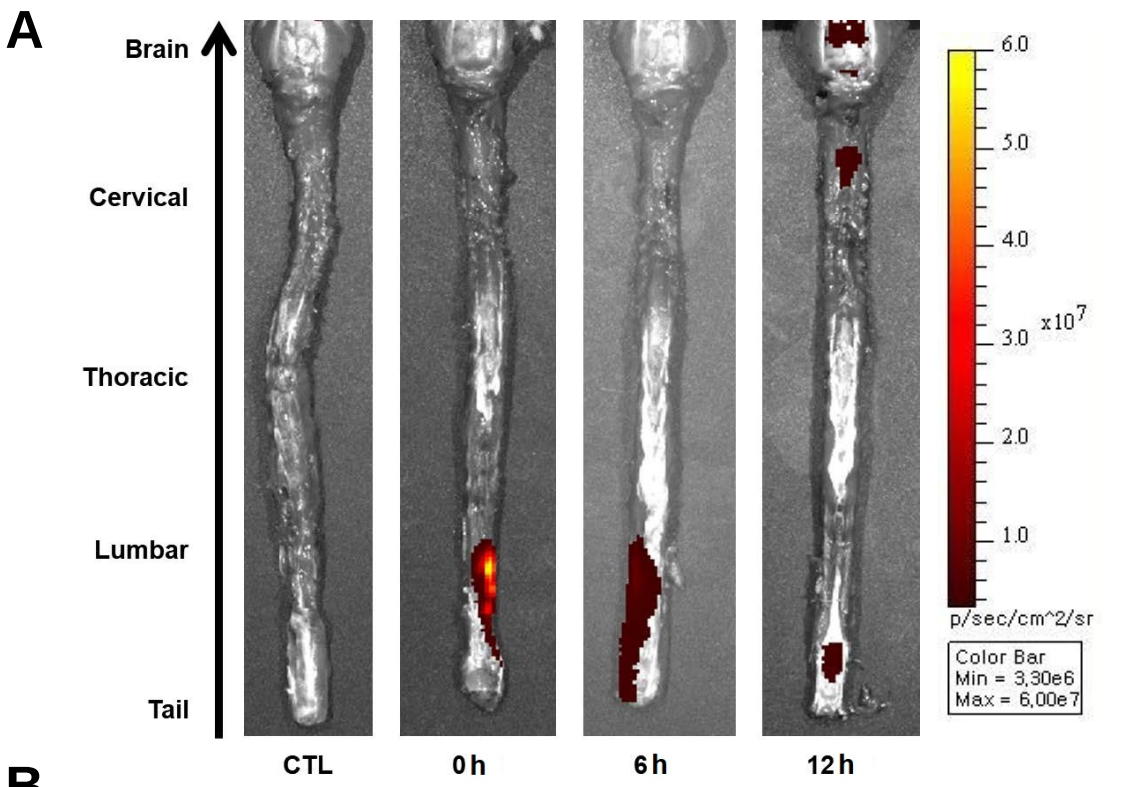


Figure 3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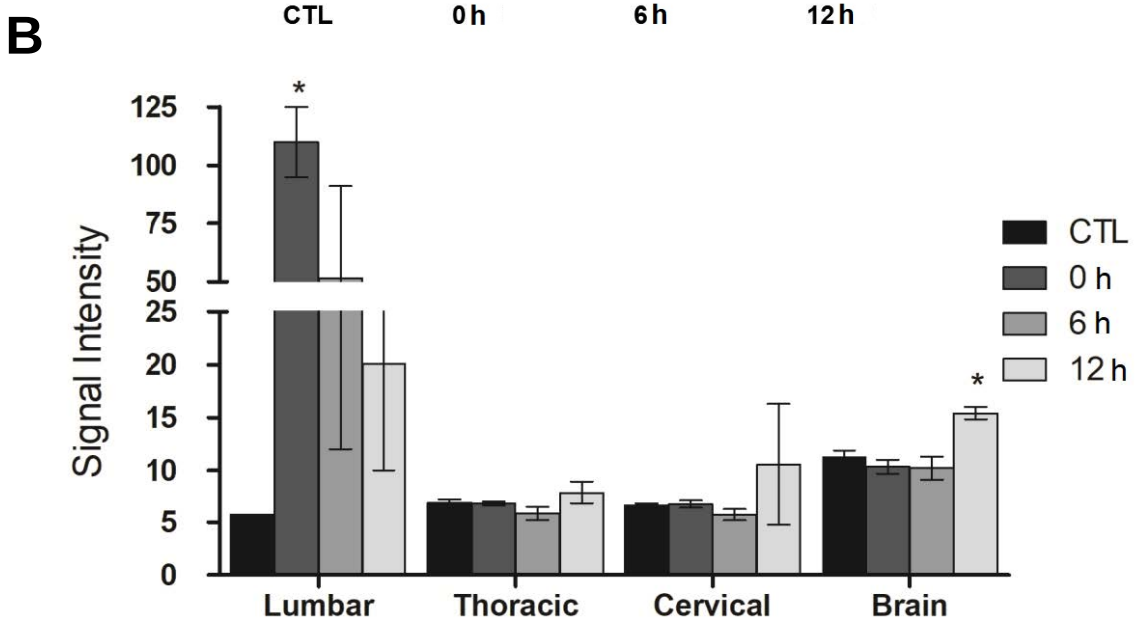
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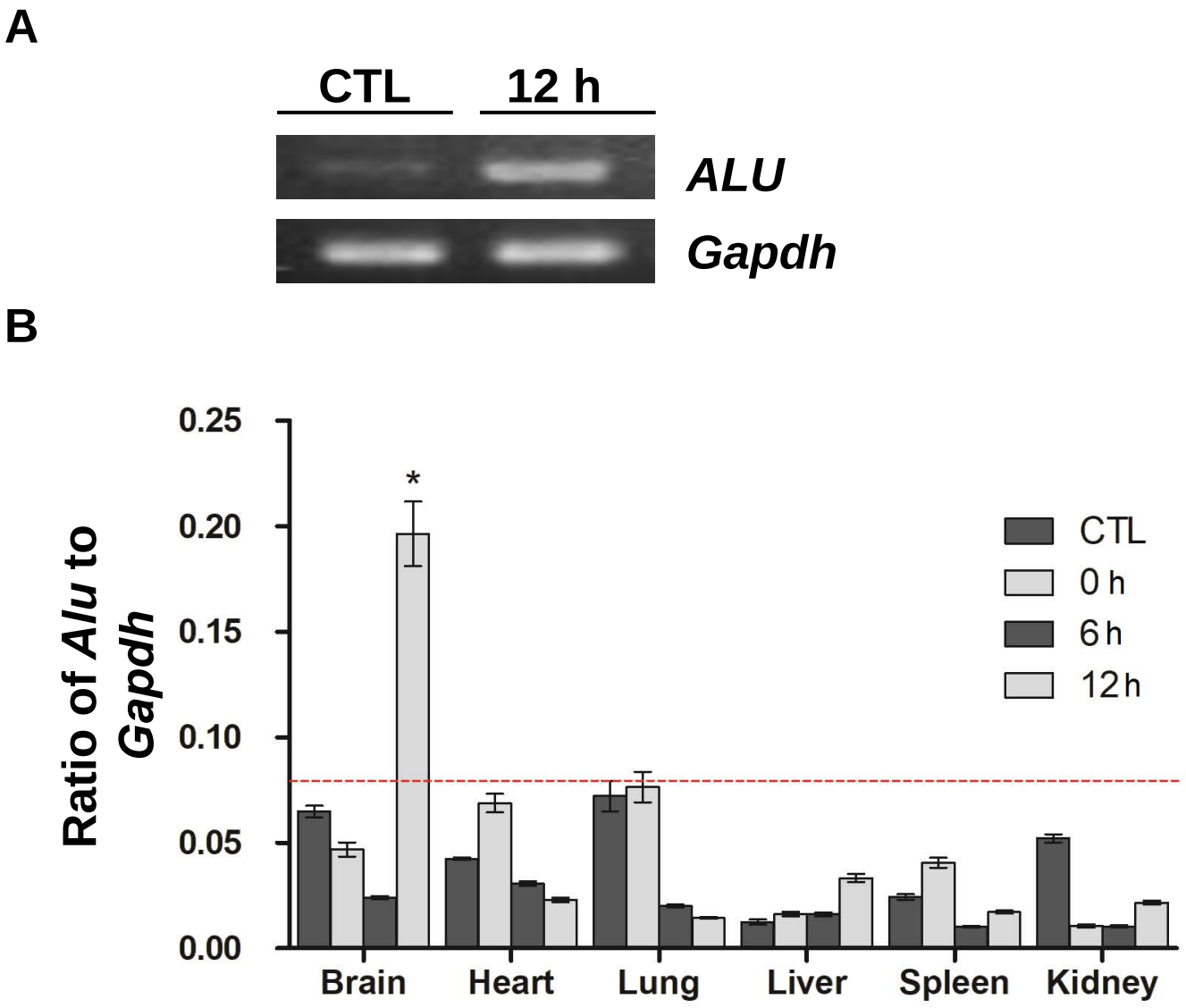
Figure 4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Spleen
Kidney

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% Trypsin-EDTA	Gibco-invitrogen	25200114	Cell culture
Fetal bovine serum	biowest	S1520	Culture medium supplement
gentamicin	Gibco-invitrogen	15710-072	Culture medium supplement
Gentra Puregene Tissue Kit	QIAGEN	158689	gDNA isolation
MEM, no glutamine, no phenol red	Gibco	51200038	WJ-MSC fomulation for injection
Miminum Essential Medium alpha	Gibco-invitrogen	12571063	WJ-MSC culture medium
Power SYBR Green PCR Master Mix	Applied Biosystems	4368577	quantitative real time PCR reagent
QuantStudio 6 Flex Real-Time PCR System	Thermo fisher	4485694	quantitative real time PCR
trypan blue	Gibco	15250061	Injection
Vybrant DiD Cell-Labeling Solution	invitrogen	V22887	Stem cell labeling solution
Xenogen IVIS Spectrum system	Perkin Elmer	124262	Optical imaging device

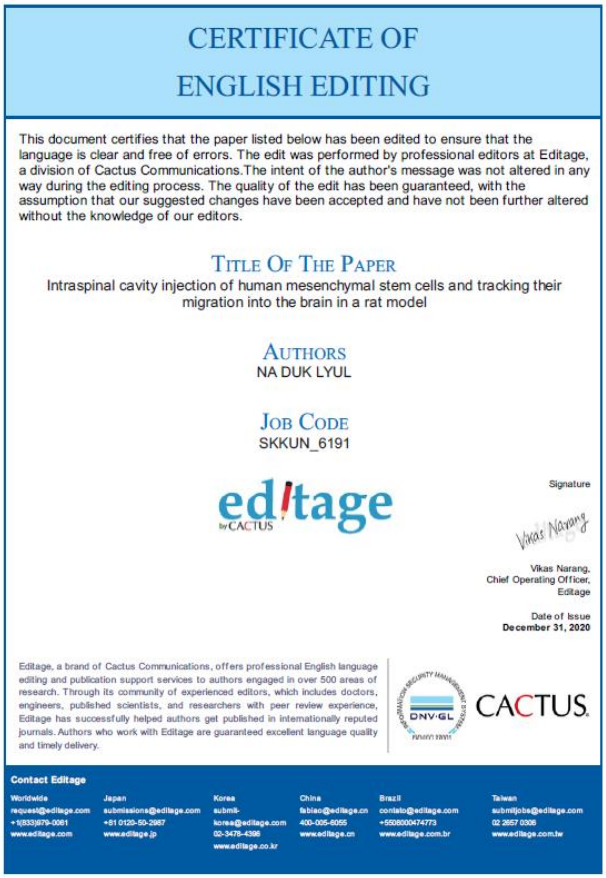
Response to Editor's Comments

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

→ To address the editor's concerns, we revised our manuscript, and also the English language editing was performed by Editage.



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TITLE OF THE PAPER
Intraspinal cavity injection of human mesenchymal stem cells and tracking their migration into the brain in a rat model

AUTHORS
NA DUK LYUL

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SKKUN_6191

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Vikas Narang,
Chief Operating Officer,
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Date of Issue
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2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

→ To address the editor's concerns, we edited our manuscript and especially the protocol section.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to

your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

→ To address the editor's concerns, we added the details to the protocol section.

4. Please do not highlight anesthesia and euthanasia steps and notes.

→ To address the editor's concerns, we revised the manuscript.

5. Please do not abbreviate journal names in the reference list.

→ Actually, we downloaded and used the Endnote style from the link in Instructions for Authors: JoVE EndNote style file. According to the editor's comment, we edited our reference style.

Response to Reviewer 1 Comments

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Thank you for the opportunity to review this manuscript. In this manuscript, the authors showed a new route to inject MSCs into the CNS, and tracing shows that injected MSCs are able to migrate.

Major Concerns:

Although the articles introduced a new route for injecting MSCs, they have not rationalized it properly. Moreover, the reason for using Trypan blue is not clear as it implies different concept than migrating the cells. The penetration of Trypan blue in the CNS needs to be shown by tissue sectioning. Also, the evidence for MSCs migration is not convincing as they only put optical imaging and it is surprising that even "0 hour" post-injection, they have detected the cells. Naturally, they should need more time to be able to migrate within the CNS. The detected signal can be only cells in the CSF and central canal. The authors need to quantify the number of migrated cells by histological methods and provide conclusive data. Regarding to PCR, I am not sure if it is the proper way to look at their migration as they did not verify it by immunohistochemical methods. There are very nice markers to detect xenograft cells in the host tissue such as STEM101, STEM121, and Ku80. Overall, I think this manuscript needs a substantial improvement to be qualified for publication.

→ Thank you for your comments. The purpose of using trypan blue dye in present study was not for identifying stem cell dispersion or distribution throughout the brain and spinal cord, but for optimizing the procedure of intra-spinal cavity administration. As a way to ensure that the needle tip is located within (do not insert the spinal cord) and that the desired substance (WJ-MSC in this study) is injected into the spinal cavity, trypan blue dye was used which is not bio-toxic and can be identified in blue. As a result, when the trypan blue dye was administered in an established way (intra-spinal cavity), it was confirmed that the dye stained the spinal cord and the brain in blue. From that result, we concluded that the needle tip of the syringe was well located in the spinal cavity of a rat model and the dye was successfully administered into the CSF.

→ Thank you for your kind suggestion about the analysis method. We agreed to your opinion that Immunohistochemical staining (IHC) is a very effective way to determine where the stem cells have migrated to and engrafted in the brain. However in our opinion, it is thought that method is not an effective way to determine how many cells have moved throughout the tissue

especially the brain and spinal cord in this study. This is because the stem cells are not distributed at the same density throughout the brain and spinal cord and observing through a microscope can contain interpretation error depends on which slides, and areas of the slide were selected. Moreover, it was impossible to detect all of the stem cells that are transplanted in the brain parenchyma. In fact, our team has used IHC method using STEM121 and anti-human mitochondria antiodies to confirm whether the stem cells are well translated in the brain parenchyma in several previous studies (doi:10.1016/j.bbrc.2018.09.012, doi:10.1016/j.bbrc.2017.08.115, doi:10.1016/j.neurobiolaging.2016.08.002, doi:10.1007/s12015-016-9694-0) and also we have Ku80 antibodty as well. We have tried to use appropriate analysis methods according to the purpose of the paper and concluded that optical imaging and qRT-PCR are more appropriate than the IHC in the present study. We stated in the Discussion section Line 376-390. Please understand the statements mentioned in Line 376-390.

Line 376-390:

"To evaluate the migration and distribution of WJ-MSCs delivered via intra-spinal cavity injection, qRT-PCR analysis using an ALU primer was performed. The primary objective of the present study was to optimize the method of intra-spinal cavity administration and evaluate its efficacy. Therefore, we chose analysis methods for tracking and quantifying the overall distribution and migration of WJ-MSCs throughout the brain and spinal cord at various time points. For this reason, optical imaging was conducted with the brain and spinal cord still connected. Moreover, the whole brain or spinal cord (cervical, thoracic, and lumbar) were ground up, and the exact numbers of WJ-MSCs in those tissues were calculated via qRT-PCR analysis using an ALU primer. The human-specific primer ALU has been reported to have high sensitivity and specificity for detecting human origin cells among rodent cells[20]. Additionally, the Ministry of Food and Drug Safety in Korea recommends using human ALU primers to evaluate the bio-distribution of stem cells in the collection of preclinical data for investigational new drug approval. To identify the exact location of WJ-MSCs migrating toward the brain and spinal cord at different time points, immunohistochemical staining (IHC) should be performed. However, IHC was not performed in the present study, which remains a limitation to our results."

Minor Concerns:

The manuscript lacks clarity and coherence. The language needs major improvement, and it should be re-written based on academic writing principles. Also, scientific terminologies have not used properly in this manuscript.

→ To address the reviewer's concern, the English language editing was performed by Editage.

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Authors

NA DUK LYUL

Job Code

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Chief Operating Officer,
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Response to Reviewer 2 Comments

Reviewer #2:

Manuscript Summary:

This is a moderately interesting technique but the reasoning behind the need for the technique is based on a flawed premise. I would not mind seeing a video about how to perform an intrathecal injection into a rat spinal cord, but not for the reasons the authors suggest. Intrathecal injections are not without significant risk to patients and introducing cells into the intrathecal compartment or ventricles rarely results in cell engrafting into the CNS parenchyma. Intraspinal injections carry the risk of causing paralysis or introducing an infection directly into the central nervous system of the patient, not to mention the extreme headache pain that patients routinely experience as a result of any changes in cerebrospinal fluid volume. Scar tissue formation at the site of injection could cause back pain and reduced mobility and require multiple injections to be performed at different levels. The cerebrospinal fluid does not bypass the blood-brain barrier. Introducing cells into the spinal cavity would allow trophic factors and exosomes from MSCs to more readily reach the brain and spinal cord at higher concentrations than IV infusion for example, but it is unlikely that cells would migrate into the tissue and engraft there. This is just one of many examples of jarringly inaccurate statements made by the authors. Also, the grammar is poor throughout. I have listed some examples of grammar errors or inaccurately worded statement below, but I quit before I got to the representative results section. I just got tired of dealing with the language problems.

→ Thank you for this valuable comment. We considered that direct injection to the subarachnoid space is the best way for MSC to target the brain. Among two representative injection methods such as intraventricular injection through intraventricular reservoir (which is widely known as ommaya reservoir) and intra-spinal injection, we considered that intra-spinal cavity injection has advantages in that it does not require general anesthesia or surgery for ommaya reservoir transplantation, it is known to generally safe without significant complications (https://www.ajronline.org/doi/10.2214/ajr.185.3.01850768) and can be repeatedly performed if necessary. In fact, intrathecal injection for chemotherapy, pain control, or myelography are not rarely used in the clinic. Nevertheless, we agree that we have underestimated the complications of intraspinal injection saying "few side effects". Therefore, we revised our manuscript as following.

Line 93-96:

"Intra-spinal cavity injection has advantages in that it does not require general anesthesia or surgery for inserting intra-ventricular reservoir, is known to generally safe[20], and can be repeatedly performed if necessary."

Line 336-339

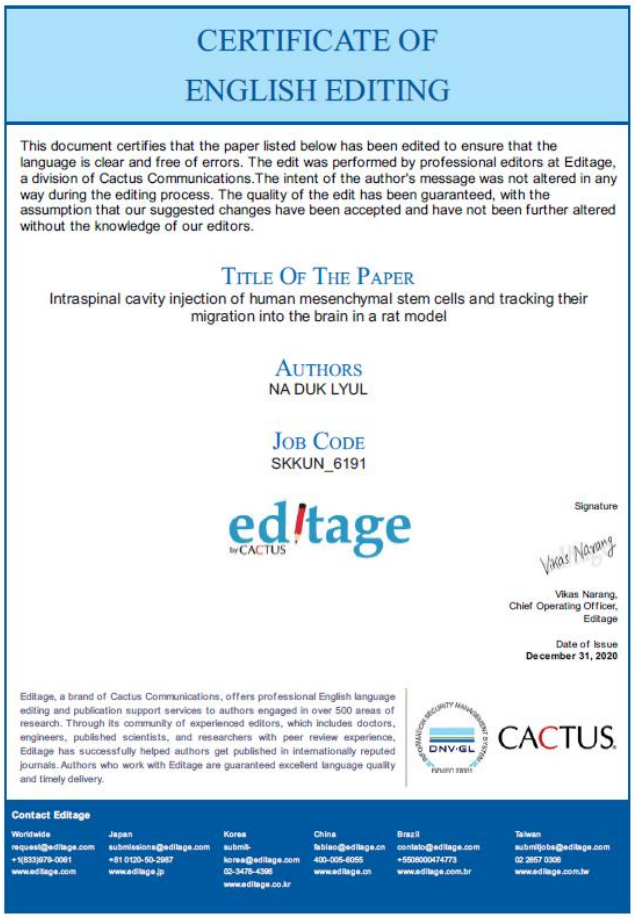
"In the case of intra-spinal cavity injection, the procedure is relatively non-invasive and does not cause neuronal damage in the brain, as can happen with intracerebroventricular injections, and it has low possibility that side effects will occur due to intra-spinal cavity injection[20]."

→ Thank you for comments from the deep understanding about the paracrine action of MSCs. According to the paper previously published by our research team, MSCs can move to the brain parenchyma when administered via cisterna magna in a mouse model (DOI: 10.1089/scd.2014.0487). In that paper, MSCs were migrated to the thalamus, subventricular zone, dentate gyrus, and cortex in the mouse model two weeks after the intra-cisterna magna injection. Therefore, we inferred that introducing MSCs to the CSF can facilitate the migration to the brain parenchyma synergically combined with the homing effect which is a tendency to move toward where balancing homeostasis and repair are needed.

Major Concerns:

Poor grammar. Multiple misstatements.

→ To address the reviewer's concern, we performed English language editing by Editage



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Minor Concerns:

See examples below

SUMMARY:

Line 39: "There is a variety of administration routes used to deliver MSC to the brain." Grammar error. "There are a variety of routes of administration ...

→ To address the reviewer's concern, we revised the manuscript.

Line 40:

"There are several routes of administration used to deliver mesenchymal stem cells to the brain."

ABSTRACT

Line 45-46: "Mesenchymal stem cells (MSCs) are expected to be effective in treating intractable and rare diseases." This sentence is both overly vague and accurate. Mesenchymal stem cells (MSCs) have shown promise for treating some diseases. Most things that look promising in animal models do not actually work in clinical practice. The conditions for which MSCs look most promising (such as stroke, kidney disease and graft versus host disease) are far from rare.

→ To address the reviewer's concern, we revised the manuscript.

Line 45-46:

"Mesenchymal stem cells (MSCs) have been studied with respect to the treatment of various diseases."

Line 46-47: "Especially for neurodegenerative where defection occurs in both the brain and spinal cord, the administration route is very important because MSCs must migrate to both the brain and the spinal cord. " This sentence in grammatically incorrect.

→ To address the reviewer's concern, we revised the manuscript.

Line 46-48:

"In neurodegenerative diseases wherein defection occurs in both the brain and spinal cord, the route of administration is very important, because MSCs must migrate to both the brain and the spinal cord."

Line 48-49: "To achieve these, we established a method for administering MSCs into

the spinal cavity (intra-spinal cavity injection)." I believe that the author means To achieve this goal. Even then though it is not clear how injecting cells into the spinal cavity would achieve the desired goal.

→ To address the reviewer's concern, we revised the manuscript.

Line 48-49:

"Herein, we established a method for administering MSCs into the spinal canal (intra-spinal cavity injection) that can target the brain and spinal cord in a rat model"

Line 49-51: "In addition, optical imaging and the real-time PCR 50 analysis were used to determine whether injected MSCs were migrated to the brain and spinal 51 cord." Grammar error : "..whether injected MSCs migrated to the brain and spinal cord."

→ To address the reviewer's concern, we revised the manuscript.

Line 52-53:

"Optical imaging and real-time polymerase chain reaction (PCR) were used to track the injected MSCs."

Line 51-52: "The results demonstrated that MSCs administered via intra-spinal cavity were successfully moved to both the brain and the spinal cord." Poor choice of words. "The results demonstrated that MSCs administered via intra-spinal cavity could be detected subsequently in both the brain and the spinal cord."

→ To address the reviewer's concern, we revised the manuscript.

Line 53-54:

"The results of the present study demonstrated that MSCs administered via the spinal cavity were detected subsequently in both the brain and spinal cord at 12 hours."

Line 52-53: "Intrathecal injection is composed of Intra-spinal cavity, intra-cerebroventricular and intra-cisterna magna injection." Inaccurately worded statement. An intrathecal injection is an intrathecal injection. The author's protocol may have involved injections in three different places, but that does not change the definition of the term intrathecal. I believe that the authors intended to say that their research explored 3

different injection sites.

→ To address the reviewer's concern, we deleted the statement.

Line 53-55: "Among them intra-spinal cavity injection has advantages in that it does not require general anesthesia, has few side effects, and can be repeatedly performed." I agree that the intraspinal cavity injection does not require general anesthesia, but dispute that it has few side effects and can be performed repeatedly. Rats cannot object to having this procedure performed repeatedly, but humans may be much less willing to oblige.

→ With regard to the side effect of intra-spinal cavity injection, we mentioned above that intra-spinal cavity injection is relatively safer than intra-ventricular injection. Although the gap between repeat intra-spinal cavity injection is important, lumbar puncture (CSF tapping, same method) can be performed repeatedly in a clinic.

Line 56-58: "To overcome this, if implanted medical device that allows repeated administration of stem cells is developed and installed, intra-spinal cavity injection can be effective to brain disease and widely used clinically as well." Bad grammar and involves unnecessary speculation about the use of a devise that does not exist to treat unspecified conditions.

→ To address the reviewer's concern, we deleted that statement.

INTRODUCTION:

Mesenchymal stem cell

Line 63-64: "MSCs secrete various substances with therapeutic effects when exposed to disease environments through paracrine action1" This sentence is inaccurate as MSCs secrete these substances in vitro and in vitro in both healthy and diseased environments. The composition of trophic factors, cytokines, exosomes, etc. released by MSCs changes with the environment, but MSCs do not need to be exposed to disease environments to secrete substances with therapeutic effects.

→ Mesenchymal stem cell can show paracrine action even in healthy environments. However, when exposed to diseased environment, various substances with special therapeutic effects are secreted to make the environment healthy. Various studies have reported that the disease specific paracrine factors are screened and selected when MSCs are exposed to the disease environment in vitro or in vivo. Therefore, it will be effective way that exposing MSCs to the disease environment in order to expect disease-specific treatment effects. To address the reviewer's concern, we revised the statement in Line 60-61.

Line 60-61:

“MSCs secrete disease-specific therapeutic substances through paracrine actions in the presence of diseases[1]”

Line 66-68: "Diverse mechanisms through paracrine action of MSCs can be expected to have good therapeutic effects in multifactorial diseases, such as Alzheimer's disease and sarcopenia." This sentence is basically meaningless. The authors may mean that MSCs have diverse paracrine actions which may have therapeutic effects on multifactorial diseases.

→ To address the reviewer’s concern and clarify the meaning, we deleted that statement.

Line 68-69: "Besides, differently from chemical or antibody drug, MSC therapy uses living cells." Grammar and wording errors. I believe the authors mean that unlike many traditional chemical or antibody therapies, MSC based therapies involve administering living cells.

→ To address the reviewer’s concern and clarify the meaning, we deleted that statement.

Line 69-70: "Since MSCs have a chemoattractant property, they can move to damaged tissue region by recognizing inflammatory cytokine or chemokine in the body." Wrong word. Having chemoattractant properties would mean that MSCs attract other cells. Having chemotaxic properties would mean that the cells were themselves attracted to cytokines or other chamicals.

→ To address the reviewer’s concern and clarify the meaning, we deleted that statement and rewrited.

Line 68-70:

“Thus, to maximize the therapeutic efficacy of MSCs, it is necessary to deliver viable cells to the target site. Therefore, it is important to choose the proper route of administration, based on the nature of the target disease, when administering MSCs”

Injection route

Line 77-78" "The most common method is intravenous which is using systemic circulation." This is not true. The most common method of delivering therapeutic agents is orally.

→ To address the reviewer’s concern, we revised the manuscript.

Line 74-75: “The most common methods are intravenous injection into the systemic circulation,

oral administration, and subcutaneous or intramuscular injection."

Line 79-80" "However, the above administration route is not effective in neurodegenerative disease..." Actually, this does not appear to be true for at least some neurodegenerative diseases, where IV delivered MSCs fail to reach the site of CNS injury or disease, but factors released by MSCs transiently lodging in the lungs, do have therapeutic actions. MSCs reaching the site of neurodegeneration may not be necessary for a therapeutic effect.

→ Thank you for your opinion. We agreed to your comment, so deleted the statement.

Intra-spinal cavity administration

Line 93-94" "All three routes have the advantage of being able to cover the entire central nervous system because the drug is spread along the CSF to the brain and spinal cord." Poor wording. All three routes of administration allow drugs or cells to disperse throughout the CSF in the brain and spinal cord.

→ To address the reviewer's concern, we revised the manuscript.

Line 90-91:

"All three routes allow drugs or cells to disperse throughout the CSF into the brain and spinal cord"

Line 99-100: "Based on the above statements, the purpose of this study is to validate intra-spinal cavity administration as a proper route to migrate MSCs to the brain as well as the spinal cord." Delete the first phase, correct to the past tense and clarify your meaning. The purpose of this study was to validate the intraspinal cavity route of administration as a means of delivering MSCs to both the brain and spinal cord.

→ To address the reviewer's concern, we revised the manuscript.

Line 97-98:

"The purpose of the present study was to validate the intra-spinal cavity administration as a means of delivering MSCs to both the brain and spinal cord."

Line 101-103: "Next, a lipophilic tracers, DiD reagent, was labelled on MSCs to measure the efficiency of migration to the spinal cord and the brain." Grammar. Next, MSCs were labeld with a lipophilic tracer, DiD, to evaluate the efficiency of cell migration to the spinal cord and brain,

→ To address the reviewer's concern, we revised the manuscript.

Line 99-101:

"Next, MSCs were labeled with a lipophilic tracer, DiIC18(5); 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD), to evaluate the efficiency of stem cell migration to the spinal cord and brain."

Line 103: "Moreover, ex vivo optical imaging analysis was conducted." Get rid of moreover. Ex vivo optical image analysis was used to assess cell dispersion.

→ To address the reviewer's concern, we revised the manuscript.

Line 101-102:

"Ex vivo optical imaging was performed to assess cell dispersion."

PROTOCOL:

2. Intra-spinal cavity Injection of WJ-MSCs

Line 191: "2.2 Injection of DiD labeled WJ-MSCs via intra-spinal cavity" This will require a lot more detailed description of how to do this than is indicated below as it is the whole point of this article. What type of needle and syringe work best? How do you control the speed of injection? Do you tape down the legs or otherwise stabilize the animal to prevent movement?

→ To address the reviewer's concern, we added the details of the protocol section. Moreover we discussion about the needle size in Line 395-398.

Line 395-398:

"To overcome this, we recommend using the smallest needle possible, ideally a 23-gauge needle. A 26-gauge needle can be used, but a needle this thin can bend easily. The size of the needle can be adjusted based on the age of the experimental animal."

Line 193-196: "2.2.1 Before injection, roll up the paper tissue approximately 6-7cm diameter.

2.2.2 Let the experimental animal prone position. Using the paper tissue rolled up, set the experimental animal roll back position. I have no idea what the authors mean.

→ To address the reviewer's concern, we revised the manuscript.

Line 191-194:

“2.2.1 Place the rat in a prone position.

2.2.2 Flex the rat’s spine appropriately to widen the distance between the adjacent spinous processes, using sufficient amounts of paper tissue or other materials that can aid in maintaining the appropriate position.”

Line 208: "2.2.7 Slowly inject 0.2 mL of WJ-MSCs into the spinal cavity." See above. Define slowly. Should this take 30 seconds or two minutes?

→ To address the reviewer’s concern, we revised the manuscript.

Line 209:

“ 2.2.7 Inject WJ-MSCs into the spinal cavity over a 1-minute period.”

Line 222: 3. Evaluation of intra-spinal cavity injection The authors have given no indication when this was performed. Was cell dispersion evaluated immediately after cell transplantation or 1 week post-transplantation. If the former, the results would say nothing about cell viability after transplantation. The authors show evidence of dye in the brain after 12 hours, but that is not long enough to know if MSCs survived and engrafted.

→ In this study, the experimental animals were euthanized 0, 6, and 12 hours post stem cell administration. Sacrifice time points should be appropriately designed according to the experimental conditions. The result demonstrated that WJ-MSCs need at least 12 hours to migrat up to the brain in the wild-type rat model, and longer follow-up was not carried out in present study. It is expected that more than 12 hours will be required for the engraftment of MSCs to the brain parenchyma, which may later be verified in further study, but has not been identified because it is far from establishing a method of intra-spinal cavity injection, the purpose of this study. We stated the designated time point in Line 225-226 and also discuss about the appropriate analysis time point in the Line 439-457 of discussion section.

Line 225-226:

“3.1 Euthanasia of the rats and isolation of the brain and spinal cord at designated time points: 0, 6, and 12 hours post-injection.”

Line 439-457:

“The euthanasia time points should also be appropriately designated. The speed of stem cell migration toward the brain and the distribution pattern throughout the neuraxis depend on the

delivered substances and the state of the experimental animals or patients. When it comes to injected materials, it is important to determine their physical and chemical characteristics. Various factors such as size, mass, lipophilicity, and half-life can affect the time required to migrate to the brain and disperse throughout the entire central nervous system (CNS). Therefore, an appropriate euthanasia time point must be established in accordance with the properties of the substance being administered. Moreover, the physical state of the test subject is also important. In the case of patients or diseased animal models, there are many substances that can attract therapeutic agents (stem cells, immune cells, and antibody drugs) toward lesion sites, such as inflammatory cytokines and target epitopes. Therefore, it will take less time for WJ-MSCs to reach the brain if a CNS disease model is used. In the present study using a wild-type rat model, three different time points (0, 6, and 12 hours) were selected. The experimental animals in the 0-hour group were euthanized immediately after stem cell injection, and WJ-MSCs were detected only in the lumbar spinal cord around the injection site. In contrast, WJ-MSCs were observed in the brains and cervical spinal cords of rats in the 12-hour group, indicating that it takes a minimum of 12 hours for WJ-MSCs to migrate to the brain and cervical cord in a wild-type rat model. Theoretically, additional WJ-MSCs can migrate to the brain as time progresses, but this was not evaluated or proven in the present study.”