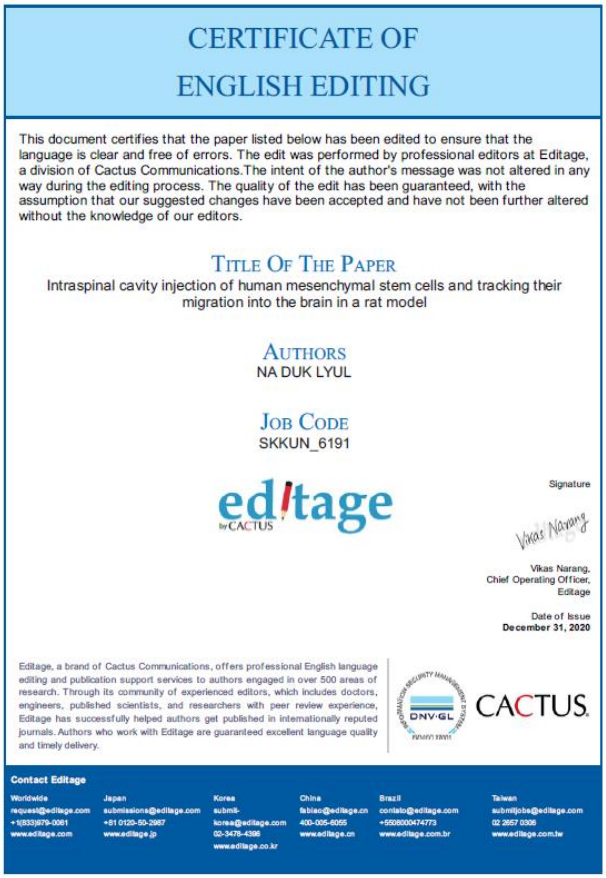
Response to Editor's Comments

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

editage by CACTUS

Signature

Vikas Narang,
Chief Operating Officer,
Editage

Date of Issue
December 31, 2020

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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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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

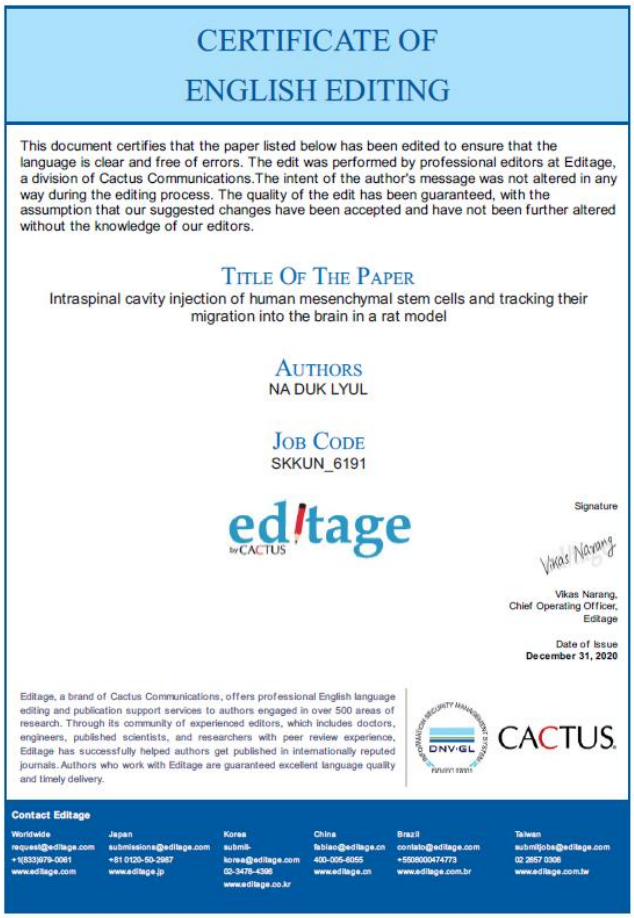
"In the case of intra-spinal cavity injection, the procedure is relatively non-invasive and does not cause neuronal damage in the brain, as can happen with intracerebroventricular injections, and it has low possibility that side effects will occur due to intra-spinal cavity injection[20]."

→ 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.”