The Neuronal Regeneration of Adult Zebrafish After Spinal Cord Injury Is Enhanced by Transplanting Optimized Number of Neural Progenitor Cells

Chih-Wei Zeng¹,², Jin-Chuan Sheu², and Huai-Jen Tsai³

Abstract
Cell transplantation is commonly used to study the regeneration and repair of the nervous system in animals. However, a technical platform used to evaluate the optimum number of transplanted cells in the recipient’s spinal cord is little reported. Therefore, to develop such platform, we used a zebrafish model, which has transparent embryos, and transgenic line huORFZ, which generates green fluorescent protein (GFP)-expressing cells in the central nervous system under hypoxic stress. After GFP-expressing cells, also termed as hypoxia-responsive recovering cells, were obtained from hypoxia-exposed huORFZ embryos, we transplanted these GFP-(+) cells into the site of spinal cord injury (SCI) in adult wild-type zebrafish, followed by assessing the relationship between number of transplanted cells and the survival rate of recipients. When 100, 300, 500, and 1,000 GFP-(+) donor cells were transplanted into the lesion site of SCI-treated recipients, we found that recipient adult zebrafish transplanted with 300 donor cells had the highest survival rate. Those GFP-(+) donor cells could undergo proliferation and differentiation into neuron in recipients. Furthermore, transplantation of GFP-(+) cells into adult zebrafish treated with SCI was able to enhance the neuronal regeneration of recipients. In contrast, those fish transplanted with over 500 cells showed signs of inflammation around the SCI site, resulting in higher mortality. In this study, we developed a technological platform for transplanting cells into the lesion site of SCI-treated adult zebrafish and defined the optimum number of successfully transplanted cells into recipients, as 300, and those GFP-(+) donor cells could enhance recipient’s spinal cord regeneration. Thus, we provided a practical methodology for studying cell transplantation therapy in neuronal regeneration of zebrafish after SCI.

Keywords
cell transplantation, differentiation, hypoxia, proliferation, spinal cord injury, zebrafish

Introduction
The transplantation of hematopoietic stem cells, as well as bone marrow stromal cells, in immunocompromised mice can be a guide toward understanding the function and regeneration of stem cells and the efficacy of tissue engineering in general.⁴⁻⁵. In addition to the study of neurological disorders, cell transplantation may be a rewarding approach toward the study of spinal cord injury (SCI). In fact, stem cell transplantation to the SCI-treated rat demonstrated remyelination of damaged axons and improvement in locomotion.⁶⁻⁹. Such transplanted stem cells could survive, differentiate, integrate, and restore damaged tissue, resulting in recovery of locomotion in the SCI rat.¹⁰⁻¹⁴. These transplanted stem cells could also self-renew, proliferate, and migrate to the lesion site in the central nervous system (CNS), as well as differentiate into oligodendrocytes, astrocytes, and neurons to secrete many neurotrophic factors.¹⁵,¹⁶. The protocols of cell transplantation to SCI-treated mammals commonly involve localized lesion transplantation.
intravenous injection, and subarachnoid block. Stem cell transplantation has, so far, been mainly performed in mice, rats, and rabbits. However, some limitations have been encountered. First, gentamicin must be given intraperitoneally every day until 7 days post-surgery to avoid infection. Second, because these animals cannot urinate by themselves after SCI, enormous researcher effort is required, because they must squeeze the bladder until the urinary reflex is restored. Such obstacles call for an alternative SCI model animal, and we herein report that zebrafish is that candidate model. First, zebrafish require no antibiotics after SCI, and excretion is not affected. Furthermore, zebrafish larvae do not require immunodepleting or multiple genetic modifications before stem cell transplantation, resulting in significantly reducing the impact of graft rejection. Also, zebrafish larvae provide a unique system that allows us to observe the interaction of transplanted cells and the process of regeneration in vivo. These advantages explain why zebrafish are increasingly used for SCI studies of neuronal regeneration.

However, even though this animal model is a good platform with which to study the nerve repair process, no detailed protocols have been described that define the limitation of cell number transplanted into adult SCI-treated zebrafish without causing any side effects. Therefore, the present study aimed to clarify the optimum number of transplanted neural progenitor cells able to achieve the desired functional result, but without generating any side effects to the recipient zebrafish. To address this issue, we employed a zebrafish transgenic line huORFZ which harbors a human uORFchop (huORFchop) motif to inhibit the translation of GFP reporter located at the downstream coding sequence in the absence of stress. However, green fluorescent protein (GFP) was expressed when embryos were treated with stress. Zeng et al. found a specific subtype of cell population expressing GFP in the brain and spinal cord in the presence of hypoxic stress, termed as hypoxia-responsive recovering cells (HrRCs). Unlike GFP-(-) apoptotic cells, the GFP(+) cells, also termed as HrRCs, can sensitively respond to hypoxia and undergo proliferation, migration, and differentiation into mature functional neurons during neuronal regeneration. These HrRCs, which are composed of subtype cells from neuron stem/progenitor cells (NSPCs), radial glial cells (RGs), and a small proportion oligodendrocyte progenitor cells and oligodendrocytes, play a critical role in neuronal regeneration after hypoxic stress. It is anticipated that this methodology will potentially serve as the technique of choice for scientists performing cell transplantation studies using the zebrafish SCI model.

**Materials and Methods**

**Zebrafish Husbandry**

Zebrafish wild-type (WT) strain AB/TU and transgenic line huORFZ were cultured indoors, and their developmental stages were identified according to standard procedures described by Westerfield. All embryos were cultured in embryo medium (EM) (140 mM NaCl, 5.4 mM KCl, 0.25 mM NaH2PO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4, and 4.2 mM NaHCO3 at pH 7.2) for 1 day, and the medium was replaced by fresh EM containing 0.003% 1-phenyl-2-thiourea (Sigma, St. Louis, MO, USA).

**Fixation and Bone Decalcification of Adult Zebrafish**

We followed the procedures described by Kroehne et al. with some modifications. After we collected the adult zebrafish, they were anesthetized with 100 mg/l Tricaine (the ethylester of 3-aminobenzoic acid; Sigma) and both head and tail were removed. We then fixed trunk samples in a 4% paraformaldehyde (pH 7.2 to 7.4; Sigma) at 25°C for 3 days, followed by treating with 20% of decalcification stock solution (2.5M EDTA, Thermo, Waltham, USA; final concentration of 0.5M EDTA, pH 7.8) at 25°C for 7 days, washing three times with phosphate-buffered saline (PBS) (pH 7.4), keeping samples at 4°C.

**Frozen Section of Adult Zebrafish**

We followed the procedures described by Zeng et al., except we used a 25-μm thick slice. The green fluorescence signal shown on each section was detected by primary polyclonal antibody against GFP (Abcam, Cambridge, UK) and observed by an A1 confocal laser-scanning microscope (Nikon, Nagoya, Japan). The green fluorescence signal shown on each frozen section was detected by primary polyclonal antibody against GFP (Abcam, Cambridge, UK) at 1:150 dilution, anti-phospho-histone 3 (PH3) (Millipore, Bedford, MA, USA) at 1:200, and anti-HuCD (Fausett et al., 2006) (early neuronal marker; Invitrogen, Carlsbad, CA, USA) at 1:250. The secondary antibody was either goat anti-rabbit or anti-mouse Cy3-conjugated fluorescence (Millipore) at 1:500.

**Confocal Microscopy and Imaging Processes**

The fluorescent signal shown on embryos was observed by a Zeiss confocal microscope (LSM 780, Carl Zeiss AG) and an A1 confocal laser-scanning microscope (Nikon). The image processing software with NIS-Elements Confocal and Zeiss LSM 780 (Carl Zeiss AG) Confocal was used for image capture.

**Hypoxic Exposure of Zebrafish Embryos from Transgenic Line huORFZ**

We followed the procedures described by Zeng et al. to carry out hypoxic treatment of zebrafish embryos. Briefly, 80 ml of EM at pH 5.8 was placed in a 100-ml serum bottle capped with a rubber stopper having a glass tube and kept at 28°C for 5 to 10 min, followed by pumping 99% nitrogen for 5 min to make deoxygenated EM. When 100 zebrafish
embryos were developed at 72 hours post-fertilization, the used EM was replaced by fresh deoxygenated EM. The bottle was sealed tightly to avoid bubbles and kept at 28°C for 2 h. After that, the hypoxia-exposed larvae were collected, fresh EM replaced, and kept at 28°C for the following experiment.

**SCI of Adult Zebrafish**

To prepare mechanical SCI for adult zebrafish, we followed the procedures described by Fang et al.25 with some modification.23 After adult fish were anesthetized in 0.02% Tricaine (Millipore, Bedford, MA, USA), we employed fine tips (Proserv Instruments, New Taipei City, Taiwan) to remove squamae 6 mm in length, starting from the brain-stem junction toward the caudal part, using a sterilized surgical blade (Proserv Instruments) to cut off skin and muscle, and a sterilized microsurgical blade to transect the spinal cord. After SCI, the lesion site was treated with vetbond tissue adhesive gel (St. Paul, MN, USA). Finally, the treated zebrafish were kept at 28°C in water with a 0.1% antifungal drug (Universal Medikament, Hamburg, Germany).

**Fluorescence-activated Cell Sorting (FACS) Cell Sorter**

First, zebrafish embryos were washed with 1× E3 medium (60× stock preparation: 34.8 g NaCl, 1.6 g KCl, 5.8 g CaCl₂·2H₂O, and 9.78 g MgCl₂·6H₂O, pH 7.2), followed by immersion in Ringer’s solution (116 mM NaCl, 2.6 mM KCl, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0) to gently remove the yolk. Second, embryos underwent cell dissociation using protease solution (0.25% trypsin, 1 mM EDTA, pH 8.0, in PBS) at 28°C for 10 min, followed by homogenization for 5 min at least three times. Third, 200 μl of 1× stop solution (6× stock preparation: 30% fetal bovine serum, 6 mM CaCl₂·2H₂O, in PBS) was added to stop protease reaction. Finally, we saved the cell pellet after centrifugation, added 1 ml of suspension media (1% fetal bovine serum (FBS; Stem Cell Technology, Vancouver, Canada), 0.8 mM CaCl₂, 50 U/ml penicillin, 0.05 mg/ml streptomycin, Dulbecco’s modified Eagle’s medium (GIBCO, InvitrogenTM, Carlsbad, CA, USA)), took 200 μl aliquots to pass through a 40-μm cell strainer and dispensed into a falcon FACS strainer (STEMCELL, Cambridge, USA) for cell sorting. We always kept cell on ice.

**Cell Transplantation**

All utensils needed to be disinfected with 70% alcohol to avoid infection. We cleaned the lesion site with sterilized swabs and used a stainless-steel pinnette to open the wound site.25 A 1.5-μl PBS solution containing 100, 300, 500, and 1,000 GFP- (+) cells was individually transplanted into the recipient’s lesion site using Fisher-brand aerosol barrier pipette tips (Thermo, Waltham, USA). After transplantation, tissue adhesive gel was used to seal the lesion site to prevent infection. Each cell transplantation process did not exceed 10 s to reduce lethality. Afterwards, DNA-free swabs with cotton tip and wooden handle (Thomas Scientific, Phoenix, AZ, USA) were used to gently remove surface water that covered the lesion site. The treated zebrafish were kept in 28°C water containing a 0.1% antifungal drug, one fish per tank. Water and reagents were replaced daily, and each experiment was carried out on 10 adult zebrafish.

**Mortality of SCI-treated Adult Zebrafish After Cell Transplantation**

After cell transplantation, the condition of lesion site and the symptom of each SCI-treated adult zebrafish were examined every 24 h for 5 days. Simultaneously, the mortality of recipients in each group was recorded daily and counted in accumulation. Ten adult zebrafish were used in each experimental group. The mortality rate was averaged from three independent experiments, presenting as mean ± SD, using the light-stimulated response approach to examine the effect of transplanting neural progenitor cells on enhancing neuronal regeneration in adult zebrafish after SCI. The swimming pattern of each individual fish was tracked in line for 30 s after light stimulation for 1 s.

**Adult Zebrafish Behavioral Assay**

The behavioral assay of adult zebrafish followed the protocol described by Shimmura et al.26 with some modifications. All fish were kept in a test tank with fixed size (15×15×15 cm³) containing water at 28°C. The locomotion of each fish sample was recorded for 3 min (Dipp-AAM, Ditect, Japan), and two-dimensional data were analyzed by Microsoft Excel.

**Quantifications and Statistics**

Unless otherwise indicated, each experiment was repeated at least three times or more. Animals were randomly assigned to different experimental groups, but no formal method of randomization was used. We used one-way analysis of variance (ANOVA), followed by Dunn’s multiple comparison test, as well as two-way ANOVA, followed by Student’s t-test (normally distributed data) for comparisons. Error bars indicate the standard error of the mean. A P-value of <0.05 was considered significant.

**Results**

**Optimizing the Number of Transplanted Cells**

Taking advantage of a zebrafish transgenic line huORFZ in the present study, we first exposed huORFZ embryos to hypoxia to induce GFP-expressing cells (Fig. 1A and B).
Second, we used the FACS sorter to isolate GFP- (+) cells, which served as donor cells (Fig. 1C). Meanwhile, we administered mechanical SCI to adult WT zebrafish (Fig. 1D). The GFP- (+) cells were then transplanted into the lesion site of SCI-treated adult zebrafish.

At two days post-transplantation (dpt), we determined the presence of GFP- (+) cells in the lesion site of adult zebrafish. To accomplish this, we collected the recipients, performed frozen sections, and observed them under the fluorescent microscope. No green fluorescence signals were observed in the nontransplanted control group (Fig. 2A, upper panels). However, GFP- (+) cells were detected at the lesion site of SCI (Fig. 2A, lower panels), suggesting that GFP- (+) cells had been successfully transplanted into recipients. Next, to determine the growth condition of recipients relative to the number of transplanted cells, we divided the experiment into four groups, i.e. those transplanted with 100, 300, 500, and 1,000 cells individually into the SCI lesion site. As shown in Table 1, no obvious effect on morphological appearance was observed in recipients having both 100 and 300 transplanted cells. However, recipients transplanted with 500 cells resulted in inflammation and reddish appearance around the lesion site, and those transplanted with 1,000 cells resulted in seriously swollen and reddish appearance with tissue fluid seepage. Therefore, the recipient adult zebrafish transplanted with 300 cells exhibited better spinal cord regeneration.

We also calculated the mortality rate of adult SCI-treated zebrafish transplanted with different numbers of cells. As shown in Fig. 2B, zebrafish transplanted with 100 cells had only 6.67% ± 5.77% mortality on the fourth day after transplantation. The 300-cell transplantation group had a 13.33% ± 5.77% mortality rate on the third day and 23.33% ± 5.77% mortality rate on the fourth day. The 500-cell transplantation group had a 20.00% ± 5.77% mortality rate on the first day and up to 75.00% ± 5.77% mortality rate on the fourth day. The 1,000-cell transplantation group had a 53.33% ± 5.77% mortality rate on the first day and 96.66% ± 5.77% mortality rate on the fourth day. These data suggested that the transplantation of different numbers of cells in SCI-treated adult zebrafish had different consequences. Most importantly, disputing “the higher the number the better the results” hypothesis, herein the highest number of transplanted cells did not result in the best physiological results for the recipients.
Figure 2. GFP-(+) cells were shown in the lesion site of SCI-treated adult zebrafish and induced the lethality of recipients after transplantation. (A) After the frozen section, the tissue at SCI site was observed under bright-field microscopy and GFP fluorescence microscopy. Upper panels: nontransplantation control; lower panels: transplanted cells. The yellow arrows indicate that the GFP signal originated from the GFP-expressing cells transplanted into the lesion site of SCI-adult zebrafish. Dotted lines indicate the location of the spinal cord. All images were obtained from the cross section. (B) The accumulation of mortality rates of SCI-adult zebrafish. Ten adult zebrafish were used in each experimental group. Each value was averaged from three independent experiments. Mortality rate (%) was accumulated and presented as mean ± SD on every day after cell transplantation. GFP: green fluorescent protein; SCI: spinal cord injury.

Table 1. Effect of the Number of Transferred Cells on Lesion Site in the Context of Symptoms Observed.

| Number of transplanted cells | SCI only | 100 | 300 | 500 | 1,000 |
|-----------------------------|---------|-----|-----|-----|-------|
| Symptoms of spinal cord injuries | No effect | No effect | No effect | Inflammation and swelling | Inflammation, swelling, and tissue fluid exudation |

SCI: spinal cord injury; No effect: inflammation was not observed at the lesion site.
Furthermore, to determine whether the 300 donor GFP- (+) cells were correctly transplanted to the lesion site in zebrafish after SCI, we performed nine series cross sections to detect donor GFP- (+) cells (Fig. 3A). The results shown on each section were 8.67 ± 2.52, 11.67 ± 3.79, 33.33 ± 2.52, 36.67 ± 4.04, 46 ± 20, 28 ± 2.65, 17.67 ± 3.06, 13.33 ± 2.08, and 1.00 ± 1.00 GFP- (+) cells counted on sections B, C, D, E, F, G, H, I, and J, respectively.
respectively, at 1 dpt (Fig. 3B–J). In total, there were 196.33 ± 5.03 GFP- (+) cells counted from nine sections (Fig. 3K). We noticed that, since there was a 0.3 cm distance between each section and each section was only 25 μm thick, the number of transplanted cells counted from total frozen sections, such as 196.33 ± 5.03 cells shown here, should be underestimated. Additionally, not all GFP- (+) cells transplanted into host could entirely survive after 1 dpt.

GFP-expressing Donor Cells Could Improve Neuronal Regeneration in Adult Zebrafish After SCI

Zeng et al. 20 found that the GFP-expressing cells are hypoxia-responsive and -resistant, but play a role in neuronal recovery. Specifically, only GFP-expressing-NSPC and GFP-expressing-RG subtypes can proliferate, migrate, and differentiate into functionally mature neurons during recovery. Finally, most mature neurons are differentiating unipolar
Figure 5. Transplantation of GFP-(+) cells into adult zebrafish treated with SCI enhances its neuronal regeneration. (A) Schematic diagram illustrates the outline of the experimental flowchart regarding how we performed cell transplantation in adult SCI-treated zebrafish. Zebrafish embryos from transgenic line huORFZ at 72 hpf were exposed to hypoxic stress. After these embryos developed 96 hpf were sacrificed, tissues were dissociated, and then employed on FACS to sort out the GFP-(+) cells, followed by transplanting GFP-(+) cells into SCI-treated adult zebrafish. After transplantation, fish samples were cultured in a tank sized 15 × 15 × 15 cm³ for behavior analysis. The swimming distance from X-Y axis (in cm) of each fish was recorded every 2 days during recovery using the touch-evoked response approach. (B) The locomotion pattern of each examined fish from three experimental groups, as indicated, was tracked in line. (C) The swimming distance was averaged from nine fish samples obtained from the SCI-treated zebrafish (served as a control) and SCI-treated zebrafish with transplantation. The t-test was used to perform statistical analysis (*** p < 0.001; error bars indicate mean ± SD). dpt: day post-transplantation; FACS: fluorescence-activated cell sorting; GFP: green fluorescent protein; hpi: hours post-injury; hpf: hours post-fertilization; SCI: spinal cord injury; WT: wild type.
neurons. In this study, the transplanted cells we used were those GFP-expressing cells able to differentiate into functional and mature neurons in hypoxia-exposed huORF2 larvae. Therefore, it is reasonable for us to hypothesize that the transplanted GFP-expressing cells at the lesion site of SCI-treated adult zebrafish are not only able to undergo proliferation but also able to differentiate into functional neurons. To confirm this hypothesis, we collected SCI-treated WT adult zebrafish after transplantation for 5 and 7 days and performed frozen cross sections to detect the proliferation and differentiation of cells transplanted into recipients using proliferation marker PH3 and early neuron marker HuCD, respectively. Thereafter, we examined whether the transplanted GFP-expressing donor cells could also express PH3 and HuCD markers labeled with red fluorescent protein (RFP) at 5 and 7 dpt. The results demonstrated that the GFP-expressing cells transplanted into recipients did exhibit yellow fluorescence signal, which was due to the colocalization of two signals, GFP and RFP (Fig. 4). Besides, 53.33% ± 8.50% GFP-expressing cells could express HuCD at 7 dpt. The line of evidence suggested that the cells transplanted in SCI-treated recipients were, to some extent, able to undergo proliferation and differentiation into neurons.

Finally, we determined if swimming distance would improve even more in fish transplanted with GFP-expressing cells. We performed behavioral analysis in which we compared the swimming distance between the transplanted and nontransplanted adult zebrafish after SCI. Compared to the nontransplanted zebrafish, results showed that the swimming distance of transplanted zebrafish with GFP-expressing cells increased 1.54-fold (Fig. 5), suggesting that GFP-expressing donor cells transplanted into host fish did help recipients to improve neuronal regeneration in adult zebrafish after SCI.

Discussion
The capacity of neuronal regeneration in mammals is very limited. Thus, employing stem cell transplantation technology to study neuronal regeneration after SCI in mammals has been commonly attempted. However, this process is quite complicated, laborious, and time-consuming. Therefore, zebrafish not only have a higher capability of neuronal regeneration, but as a model animal, it is easier to manipulate in vivo in terms of studying optimization, quantification, assessment, and efficiency of transplanted cells in recipients. Gadani et al. reported that repair mechanisms rely on beneficial aspects of inflammation in mammals. For example, NSPC transplantation improves ankle movement and hindlimb placement in association with a slight increase in the abundance of T cells, decrease of B cells, and reduced M1-like macrophages following SCI. Actually, the zebrafish model can be used to simulate the mammalian system after SCI in order to understand the repair process and molecular mechanisms underlying zebrafish regeneration. It is plausible that the knowledge obtained from fish might be applied to mammals. Another important issue is inflammatory response. The level of inflammatory response is dependent on the number of transplanted cells. In zebrafish, several chemokines and cytokines are upregulated after SCI, such as interleukin-4 receptor, interferon-1, and transforming growth factor beta 1. These factors are also upregulated in mammals after CNS injury. Similar to other vertebrates, zebrafish microglia express typical vertebrate macrophage genes. Furthermore, the expression of many transcriptional regulators, immune pathogen receptors, and pruning-associated genes, which are also found in mammals, suggests functional conservation between mammals and fish. Taken together, the similarities between adult zebrafish and mammalian CNS regeneration in fundamental respects give us insights to better understand how a permissive niche can be created to achieve successful axonal regeneration in the mammalian CNS and to adopt successful therapeutic strategies and final use in clinical study. This study showed that these similarities might be highly potential to serve as the technological platform for investigators performing cell transplantation approach to study neuronal regeneration of spinal cord using the SCI-treated adult zebrafish model.

Specifically, we demonstrated the optimum number of cells transplanted in zebrafish spinal cord lesion site to be around 300, as determined by the survival rate of SCI-treated adult zebrafish. We noticed that although both the 100-cell- and 300-cell-transplanted adult zebrafish showed good survival after transplantation, mortality of the 300-cell-transplanted fish was higher, indicating that the 100-cell-transplanted fish had the highest survival rate. However, 100-cell-transplanted fish did not completely regenerate the spinal cord (data not shown). In contrast, the adult zebrafish transplanted with 300 cells exhibited better spinal cord regeneration because the surging distance of the 300-cell-transplanted fish was longer than that of the 100-cell-transplanted fish (data not shown). We also demonstrated that the 300-cell-transplanted SCI-treated adult zebrafish exhibited GFP-expressing cells, which were able to proliferate and differentiate into neurons with extended axons (Fig. 4). Additionally, we compared the swimming distance between the 300-cell-transplanted and nontransplanted zebrafish after SCI. Results showed that the swimming distance of the 300-cell-transplanted zebrafish increased over that of the nontransplanted zebrafish (Fig. 5), suggesting that the transplantation of 300 GFP-expressing cells was the optimal number to improve nerve repair capacity in adult zebrafish after SCI, even though the 300-cell-transplanted zebrafish was not representative of the highest survival.

Acknowledgments
This work was partially supported by the Ministry of Science and Technology, Taiwan (108-2811-B-715-500), partially supported by the Liver Disease Prevention and Treatment Research Foundation, Taipei, Taiwan, and partially supported by Mackay Medical College under grant of RD1080130. We also thank National Institute
for Basic Biology, Spectrograph and Bioimaging Facility, Japan, for supporting microscopy and adult zebrafish behavioral assay experiments.

**Authors’ Contributions**

CWZ contributed to the study design, carrying out experiments, data analysis and interpretation, and writing draft. JCS and HJT conceived and designed the research and data interpretation, and contributed to editing the manuscript, and serving as PIs to apply for funding.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

This study was approved by the Institute of Biomedical Science, Mackay Medical College, New Taipei City, Taiwan.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**ORCID iD**

Huai-Jen Tsai https://orcid.org/0000-0001-8242-4939

**Statement of Animal Rights**

All of the experimental procedures involving animals were conducted and approved by the Institute of Biomedical Science, Mackay Medical College, New Taipei City, Taiwan.

**Statement of Informed Consent**

There are no human subjects involved in this article.

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