의학박사 학위논문

척수손상 모델에서 MCP-1 단백질로 유도되는 성체신경줄기세포의 치료적 효과 연구

Significant Therapeutic Effects of Adult Human Neural Stem Cells for Spinal Cord Injury Are Mediated by Monocyte Chemoattractant Protein-1 (MCP-1)

2023년 2월

서울대학교 대학원

의학과 해부학전공

안 재 열

척수손상 모델에서 MCP-1 단백질로 유도되는 성체신경줄기세포의 치료적 효과 연구

지도 교수 이 지 연

이 논문을 의학박사 학위논문으로 제출함
2022년 10월

서울대학교 대학원
의학과 해부학전공
안 재 열

안재열의 의학박사 학위논문을 인준함
2023년 1월

위 원 장 _____ (인)

부위원장 _____ (인)

위　　원 _____ (인)

위　　원 _____ (인)

위　　원 _____ (인)

Abstract

Significant Therapeutic Effects of Adult Human Neural Stem Cells for Spinal Cord Injury Are Mediated by Monocyte Chemoattractant Protein-1 (MCP-1)

Jae Yeol, An
Department of Anatomy and Cell Biology
The Graduate School
Seoul National University

The limited capability of regeneration in the human central nervous system leads to severe and permanent disabilities following spinal cord injury (SCI) while patients suffer from no viable treatment option. Adult human neural stem cells (ahNSCs) are unique cells derived from the adult human brain, which have the essential characteristics of NSCs.

NSC is a promising regenerative modality for various neurodegenerative and neurological disorders including SCI. The objective of this study was to characterize therapeutic effects of ahNSCs isolated from temporal lobes of focal cortical dysplasia type IIIa for SCI and to elucidate their treatment mechanisms. Results showed that the recovery of motor functions was significantly improved in groups transplanted with ahNSCs where, in damaged regions of spinal cords, the numbers of both spread and regenerated nerve fibers were observed to be higher than vehicle group.

In addition, the distance between neuronal nuclei in damaged spinal cord tissue was significantly closer in treatment groups than vehicle group. Based on immunohistochemistry analysis, those neuroprotective effects of ahNSCs in SCI were found to be mediated by inhibiting apoptosis of spinal cord neurons. Moreover, the analysis of conditioned medium (CM) of ahNSCs revealed that such neuroprotective effects were

mediated by paracrine effects with various types of cytokines released from ahNSCs, where monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) was identified as a key paracrine mediator. In this study, the therapeutic range of ahNSC injection dose and the route of administration were also identified, though further translational studies should be carried out to guarantee the safety, therapeutic effect through the performance of optimal injection route, dose and period of administration for patients with SCI in the future.

These results of ahNSCs could be utilized further in the preclinical and clinical development of effective and safe cell therapeutics for SCI with no available therapeutic options at present.

Keywords : spinal cord injury, neural stem cell, lateral ventricle, cytokine, monocyte chemo attract protein-1

Student Number : 2020-37944

TABLE of CONTENTS

1. INTRODUCTION

Axonal regeneration from injured neurons hardly occurs in the adult mammalian central nervous system (CNS) [1]. Such a low neuron-intrinsic regenerative capacity in the CNS is mainly attributed by the inhibitory microenvironment of glial scar, which generates chondroitin sulfate proteoglycans (CSPGs) [2]. The limited regenerative potential of the CNS results in severe and permanent disabilities after spinal cord injury (SCI), such as motor paralysis and neuropathic pain, which can lead to social impairments having a huge impact on patient's quality of life [3,4].

SCI, as one of devastating and disabling neurological injuries, is recognized as a top priority of global health issue due to impact on complexity of nature, quality of life for patient and expensive medical cost. In 2016 it was reported that the number of new SCI cases was around 1 million

with a prevalence of more than 27 million cases globally and tends to grow in the absolute number of people living with SCI [5]

Despite the current treatment options including surgical interventions, rehabilitative care, there are no clinically meaningful treatments currently available for SCI to reverse the persistent sequalae, emphasizing the need for novel therapeutic strategies enabling functional recovery in SCI patients [6].

Based on the nature of multipotency with inherent differentiation capabilities committed to the neuronal lineage promoting and reestablishing the damaged neuronal spinal tracts, the transplantation of Neural Stem Cells (NSCs) demonstrated a therapeutic potential in various neurological pathologies as alternative option by providing neurotrophic support and anti-inflammatory condition [7, 8]. Several pre-clinical studies have shown NSC to be safe and effective leading to an increasing number of clinical trials [9-11]. NSC transplantation can

provide feasible benefits through various mechanisms in the damaged CNS such as modulation of inflammatory response, paracrine neuroprotective effects, and regeneration of lost neural tissue [12, 13]. Previously, we transplanted adult human NSCs (ahNSCs) into the lateral ventricle (LV) of SCI animal models and observed significant therapeutic effects [14]. The ahNSCs injected into the LV migrated via cisterna magna towards damaged regions of spinal cord and persisted up to 5 weeks to reduce an excessive glial scar formation. We also suggested their paracrine factors might also provide neuroprotective effects and promoted angiogenesis [14]. It is important to note that, however, the surgical tissue of patients with hemorrhagic stroke is technically difficult to be obtained. Moreover, paracrine factors that mediated beneficial therapeutic effects were poorly identified in the previous study.

To overcome these limitations, recently, we demonstrated that ahNSCs derived from focal cortical dysplasia (FCD) type

IIIa have cellular properties like neural stem/precursor cells including self-renewing and neural-differentiation potency [15 – 19].

In this study, we analyzed therapeutic effects of ahNSCs isolated from temporal lobes of focal cortical dysplasia type IIIa for SCI and to elucidate their treatment mechanisms. Additionally, we identified neuroprotective effects of monocyte chemoattractant protein-1 (MCP-1; also known as CCL2) among various cytokines released from ahNSCs.

2. MATERIALS and METHODS

Study approval and animal care

Informed written consent was obtained from patients according to the guidelines approved by the Institutional Review Board (IRB) of Samsung Medical Center (2016-11-085-003, Seoul, South Korea). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) from Laboratory Animal Research Center (LARC) at Sungkyunkwan University (SKKUI-ACUC2018-05-06-3, Suwon, Gyeonggi-do, South Korea). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from Institute for Laboratory Animal Research (ILAR) [39].

Primary culture of rat spinal cord neurons (SCNs)

Spinal cords were isolated from E15.5 rat embryos and digested with 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) at 37℃ for 2 minutes (mins). The reaction was terminated using Dulbecco's Modified Eagle's Medium (DMEM, Corning, Corning, NY, USA) containing 1% penicillin/streptomycin (Gibco) and 10% fetal bovine serum (FBS, Gibco). Suspended cells were filtrated using 70-μm cell strainers (Corning), and then maintained in neurobasal medium (Gibco) containing 2% B27 supplement (Gibco), 1% penicillin/streptomycin at 37° C in 5% CO_2 humidified atmosphere. Half of medium was replaced with fresh medium twice a week.

Primary culture of ahNSCs

Three batches of ahNSCs (NSC #1, NSC #2, and NSC #3) were primarily cultured from the temporal lobes of donors with FCD type IIIa, as previously described [14]. Briefly, ahNSCs were maintained in DMEM: Nutrient Mixture F-12 Ham's medium (DMEM/F-12, Gibco) containing 0.5% FBS, 2% B27 supplement, 1% penicillin/streptomycin, 20 ng/ml human epidermal growth factor (EGF, R&D, Minneapolis, MN, USA), and 20 ng/ml human basic fibroblast growth factor (bFGF, R&D) at 37° C in 5% CO_2 humidified atmosphere. AhNSCs at in vitro passage 3-9 were used for experiments. Differentiation of ahNSCs was induced in vitro, as previously described [14].

Collection of conditioned medium (CM) of ahNSCs

AhNSCs were seeded with density of 9,000 cells/cm^2 on T75 culture flasks (SPL lifescience, Pocheon, Korea) and were cultured for 48 hours (hrs). AhNSCs were washed 2 times with phosphate-buffered saline (PBS, Gibco), and maintained in DMEM/F-12 containing 1% penicillin/streptomycin for 24 hrs. CM of ahNSCs was collected and centrifuged at 300 RCF for 3 mins at 4℃. The CM was stored at -80℃.

Enzyme-linked immunosorbent assay (ELISA) assay

Concentrations of MCP-1 in the CM of three different batches of ahNSCs (NSC #1, NSC #2, and NSC #3) were analyzed by an ELISA kit for MCP-1 (Quantikine, R&D) according to the manufacturer's instructions.

Cell viability assay

SCNs were incubated in 96- or 24-well culture plates (2×10^5 cells/mL) at 37° C in 5% CO_2 humidified atmosphere for 7 days. SCNs were treated with the CM of ahNSCs for 1 hr, washed with PBS, and then treated with neurobasal medium containing 1% penicillin/streptomycin with/without H_2O_2 for 24 hrs. Anti-MCP-1 neutralizing antibody (10 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and recombinant MCP-1 protein (1 ng/ml, R&D) were applied and followed by adding 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) into each well for 4 hrs. After 100 μl of dimethyl sulfoxide (DMSO, Panreac applichem, Barcelona, Spain) was added, plates were incubated at 37° C for 1 hr. Light absorption was measured at a 595 nm wavelength. Cell viability was also evaluated by trypan blue exclusion method using an automated cell counter (TC10, Bio-Rad, Hercules, CA, USA).

Western blot

Cells were harvested and lysed using RIPA lysis buffer (Santa Cruz Biotechnology) with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amount of proteins was separated by SDS-PAGE and analyzed by immunoblotting with primary antibodies for cleaved caspase3 (Cell Signaling Technology, Danvers, MA, USA), Bax, Bcl2, and actin (Santa Cruz Biotechnology).

Annexin V/propidium iodide (PI) assay

Cells (1 x 10^6 cells/mL) were stained with an Annexin V Apoptosis Detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction, and then evaluated using a flow cytometry.

Reserve transcription-polymerase chain reaction (RT-PCR)

RNA was isolated using a Takara MiniBEST Universal RNA Extraction Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The DNA-free RNA was reverse transcribed with a cDNA synthesis kit (Takara) according to the manufacturer's protocol. Amplification was performed by denaturation at 95° C for 5 mins, followed by 35 three-step cycles of 95° C for 30 seconds (secs), 52° C for 30 secs and 72° C for 30 secs. After amplification, PCR products were subjected to a 0.8-1.5% agarose gel electrophoresis and visualized by ethidium bromide. Primers used for amplification were as follows: BAX, forward 5'-GTGGCAGCTGACATGTTTG-3' and reverse 5'-ATCAGCTCGGGCACTTTAG-3'; Bcl2, forward 5'-CTGAACCGGCATCTGCAC AC-3' and reverse 5'-GCAGGTCTGCTGACCTCACT-3'; Actin, forward 5'-

AGCCATGTACG TAGCCATCC-3' and reverse 5'-CTCTCAGCTGTGGTGGTGAA-3'.

Immunofluorescence analysis and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Cells cultured on coverslips were fixed with 4% paraformaldehyde at 4° C for 30 mins. After washing with PBS, cells were permeabilized in PBS containing 1% Triton X-100 and 0.5% NP-40 (Sigma-Aldrich), and then blocked with 3% bovine serum albumin (BSA) and 1% normal horse serum (Santa Cruz Biotechnology). Cells were incubated with antibodies against Tuj1 (1:100, Abcam, Cambridge, UK), cleaved caspase3 (1:100, Cell Signaling Technology), and then appropriate secondary antibodies (Invitrogen). Cells were stained with a TUNEL kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. 4',6-diamidino-2-phenylindole

(DAPI) (Vector Laboratories, Burlingame, CA, USA) was used for nuclei. After mounting, the cells were examined using confocal laser scanning microscopy (BIORP, Leica, Wetzlar, Germany).

Spinal cord injury animal model

10-week-old Sprague-Dawley rats (female, Orient Bio., Sungnam, South Korea) were anaesthetized with 2.5-5% isofluorane (Hana Pharm, Seoul, South Korea). A 2 cm-long incision made in the level of T9-T10 and following laminectomy exposed the thoracic spinal cord. Moderate SCI was induced by an Infinite Horizon (IH) impactor (200-kKdyn; Precision System Instrumentation, Fairfax, VA, USA). Cefazolin (33.3 mg/kg, Chong Kun Dang, Seoul, Republic of Korea) and ketoprofen (10 mg/kg, Uni Biotech, Seoul, South Korea) were intramuscularly administered daily for 2 days to prevent infection and reduce pain. The urinary bladder was compressed

twice daily until spontaneous micturition was observed. Motor function was measured by the Basso-Beatti-Bresnahan (BBB) locomotor rating test [40], weekly.

Cell Transplantation

Animals with BBB score from 4 to 6 on the seventh day after SCI were randomly divided into four groups: the vehicle, low, medium, and high group (n = 8 for vehicle group and n = 10 for other groups). On the seventh day after SCI, the transplantation into the lateral ventricle (LV) was performed according to the previous report [41]. Briefly, rats were fixed in a rodent stereotactic device (Model 900 small animal stereotaxic instrument, KOPF stereotaxic, Tujunga, CA, USA) to make Burr holes [anterior-posterior (AP): −0.8 mm, medial-lateral (ML): 1.4 mm, dorsal-ventral (DV): 3.8]. 3, 10, or 30 × 10^5 ahNSCs in 30 μl HBSS (Gibco) (low, medium, or high group, respectively) were injected into the

LV for 10 mins using a 26G Hamilton syringe (Hamilton Company, Reno, NV, USA) and a syringe pump (LEGATO™111, KD scientific, Holliston, MA, USA). 30 μl HBSS was used for vehicle group. AhNSCs (NSC #1) at in vitro passage 6 (P6) were used. After transplantation, the injection needle was maintained for 5 mins to prevent leakage, and then removed at a speed of 1 mm/min. 10 mg/kg ketoprofen was administrated intramuscularly to reduce pain after the surgery. For immunosuppression, 10 mg/kg cyclosporine A (Chong Kun Dang Pharmaceutical Corp., Seoul, South Korea) was subcutaneously administered daily from 24 hrs before transplantation to sacrifice.

Immunohistochemistry and TUNEL assay

Spinal cords of animals with SCI were embedded in paraffin as described previously [16]. Formalin-fixed, paraffin-embedded tissues were sectioned (4μm) and then placed on silane-

coated slides (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). After heating on a slide warmer (Lab-line Instruments USA, Dubuque, IA, USA) at 65 ° C for 30 mins, slides were deparaffinized and rehydrated. Antigen retrieval was carried out by boiling in the target retrieval solution (Dako, Carpentaria, CA, USA) (4 times, 5 mins for each). Slides were incubated in 0.3% ammonia in 70% methanol for 1 hr, washed in 50% methanol for 10 mins, and then incubated in 3% hydrogen peroxide in methanol for 12 mins to quench endogenous peroxidase activity. Slides were incubated in a peroxidase-blocking solution including 5% BSA and 2% normal goat serum (GenDEPOT, Katy, TX, USA) in a peroxidase-blocking solution (Dako) for 1 hr at RT. Primary antibodies were then added and incubated at 4° C overnight: NeuN (1:500, Millipore, Temecula, CA, USA), Tuj1 (1:1000, Abcam), and cleaved caspase 3 (1:50, Cell Signaling Technology). Sections of spinal cord were incubated with an appropriate

Horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) for 1 hr at RT, visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako), and then counterstained with hematoxylin (Sigma-Aldrich). For immunofluorescence, sections were treated with appropriate secondary antibodies and then counterstained with DAPI. Paraffin sections were stained with a TUNEL kit (Roche) according to the manufacturer's protocol.

Image analysis

For DAB staining, colors in scanned images were separated into purple and brown using QuPath image analysis software (Queen's University Belfast, Northern Ireland, UK) as described previously [13,42]. Computational color deconvolution was applied to separate DAB staining from the hematoxylin. The brown immunohistochemical staining was analyzed using ImageJ software (NIH Image, Bethesda, Maryland, USA). For immunofluorescence, nuclei with positive signals were counted in

each section. In the western blot results, bands were quantified using image J software and normalized to actin.

Statistical analysis

In vivo and in vitro data were shown as average ± standard error (SEM) and average ± standard deviation (SD), respectively. The p-value < 0.05 in two-tailed Student's t test was interpreted as statistically significant.

3. RESULTS

3.1. Therapeutic effect of ahNSCs for SCI

To examine therapeutic effects of ahNSCs for SCI, 3 × 10^5 (low), 1 × 10^6 (medium), or 3 × 10^6 (high) ahNSCs in 30 μl Hanks' Balanced Salt solution (HBSS) were transplanted into the LV of SCI animal models at 1 week after injury (n =8 for each group). When recovery of motor functions was measured by the Basso, Beattie and Bresnahan (BBB) score (**Table 1**), the medium and high groups showed significantly higher scores compared to the vehicle group (30 μl HBSS, n = 10) from 1 week to 5 weeks after the treatment (**Figure 1A and B**). However, significant differences were not observed among three groups transplanted with ahNSCs. The low group had statistically significant functional recovery only at 5 weeks after the transplantation of ahNSCs.

When the area of cavity was measured in the damaged spinal cords at 5 weeks after treatment (**Figure 1C**), the medium and high groups showed significantly smaller cavity areas than that of the vehicle group (**Figure 1D**). In contrast, there was no significant difference between the low and vehicle group. The tissue loss of the medium group was significantly less than that of the low group. Significant negative correlation between the BBB score at 6 weeks post SCI and the tissue loss in the spinal cord was observed in the analysis of cavity size (**Figure 1E**).

These results indicated that ahNSCs derived from the temporal lobe of FCD type IIIa surgical samples had significant therapeutic effects for SCI in a dose dependent manner, which are similar with those of ahNSCs derived from the surgical samples of hemorrhagic stroke [14].

3.2. In vivo neuroprotective effects of ahNSCs

To find *in vivo* treatment mechanism of ahNSCs, immunohistochemistry against NeuN and Tuj1 was performed 5 weeks after transplantation. Since NeuN and Tuj1 are the specific markers of viable neuronal cell body and nerve fiber of neurons, respectively, distance between rostral and caudal NeuN-positive neurons in damaged spinal cord would indicate the degree of tissue degeneration by SCI. We observed that the distance of the vehicle group was significantly farther than those of the medium and high groups (**Figure 2A** and **Figure 8A**). Moreover, the distance showed significant negative correlation with the BBB score at 6 weeks post SCI (Figure 2B)

Next, immunoreactivity against Tuj1 showed viable axons and dendrites at five points near the lesion (dorsal, epicenter, rostral, ventral, and caudal). Tuj1-positive nerve fibers at the

dorsal and epicenter points would indicate the regeneration of fibers given that spinal cord tissue of those areas was directly destroyed by traumatic damage (regenerated axon) (**Figure 8B**). On the other side, degree of tissue damage could be indirectly evaluated by Immunoreactivity against Tuj1 at the rostral, ventral, and caudal areas since physical force was delivered to spinal cord dorsally (spared axon) (**Figure 8C**). At both analyses, Tuj1 expression of the medium group was significantly higher than the vehicle group (**Figure 2C and E**).

Meanwhile, the high groups showed significantly less degeneration of neuronal nuclei compared to the vehicle group (**Figure 2B**), a significant difference of Tuj1 expression between the high and vehicle group was not observed (**Figure 2C and E**). High Tuj1 expression was significantly correlated with high BBB score at 6 weeks post SCI in both analyses (**Figure 2D and F**).

Taken together, these results indicated that ahNSCs mediated neuroprotective effects in the injured spinal cords and showed significant therapeutic effects. To validate the neuroprotective effects of ahNSCs further, an apoptosis of neural cells in damaged spinal cords was analyzed at 7 days after treatment of 1×10^6 ahNSCs (n =5). It was observed that the transplantation of ahNSCs significantly reduced terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive apoptotic cells (**Figure 3A and B**).

Moreover, NeuN- and cleaved caspase 3-double positives significantly decreased in the ahNSCs transplanted group compared to the vehicle group (30 μl HBSS, n =5) (**Figure 3C and D**), indicating that the neuroprotective effects of ahNSCs on spinal cord neurons might be mediated by inhibiting apoptosis.

3.3. In vitro neuroprotective effects of ahNSCs

The *in vivo* neuroprotective effects of ahNSCs were confirmed *in vitro*. When primary cultured rat spinal cord neurons (SCNs) were treated by various concentrations of H_2O_2 for 24 hrs, it was observed that the survival of SCNs was significantly reduced (**Figure 4A**). Moreover, the length of neurites of SCNs decreased dramatically by applying the H_2O_2 at 7 days *in vitro* (DIV 7) (**Figure 4A**).

In contrast, conditioned media (CM) of two independent batches of ahNSCs (NSC #1 and #2) did not cause similar toxic effects mediated by H_2O_2 on the survival and neurite length of SCNs (**Figure 4B and D**). When SCNs were maintained in the CM of ahNSCs for 1 hr before H_2O_2 treatment, the toxic effects mediated by H_2O_2 were significantly reduced and two batches of the CM of ahNSCs showed similar *in vitro* neuroprotective effects (**Figure 4C and D**).

These results indicated that ahNSCs exert their neuroprotective effects in the paracrine manner.

When *in vitro* apoptosis of SCNs was analyzed by TUNEL assay (**Figure 5A and B**), immunocytochemistry against cleaved caspase 3 (**Figure 5C and D**), and flow cytometry using propidium iodide (PI) and Annexin V (**Figure 5E and F**), it was observed that the treatment of H_2O_2 induced the apoptosis of SCNs *in vitro*. Pretreatment of CM of ahNSCs (NSC #1) for 1 hr before applying H_2O_2 significantly reduced the apoptosis of SCNs. It was observed that results of Western blot (**Figure 6A and B**) and quantitative reverse transcription-polymerase chain reaction (RT-PCR) (**Figure 6C and D**) showing an increase in pro-apoptotic factor, Bcl-2-assocated X protein (BAX), and a decrease in anti-apoptotic factor, B-cell lymphoma 2 (Bcl-2), by H_2O_2 treatment, were revered by the pretreatment of CM of ahNSCs (NSC #1).

These results demonstrated that paracrine mediators contained in the CM of ahNSCs could inhibit neuronal apoptosis by mediating neuroprotective effects of ahNSCs.

3.4. Neuroprotective effects of ahNSCs mediated by MCP-1

To identify the paracrine mediator that shows neuroprotective effects of ahNSCs, the analysis on various cytokines produced by ahNSCs was conducted [20]. The concentration of MCP-1 was found to be highest among various types of paracrine factors in the CM of ahNSCs and was reported to have prominent neuroprotective effects [20].

High concentration of MCP-1 in the CM of ahNSCs was confirmed by ELISA assay in three different batches of ahNSCs (NSC #1, #2, and #3) (**Figure 7A**). Moreover, *in vivo* production of MCP-1 in ahNSCs were confirmed in the rat brains by immunohistochemistry (data not shown).

Based on the analysis, it was found that the presence of MCP-1 neutralizing antibody to the CM of ahNSCs (NSC #1) offset and reversed the neuroprotective effects of ahNSCs

(Figure 7B). In contrast, the presence of recombinant protein of human MCP-1 (rhMCP1) showed neuroprotective effects similar to that of the CM of ahNSCs (NSC #1) (Figure 7C).

In the western blot analysis, treatment of the CM of ahNSCs also showed a significant decrease in the expression of cleaved caspase 3, which was reversed in the presence of anti-MCP-1 neutralizing antibody in the CM of ahNSCs (Figure 7D and E).

4. DISCUSSION

Since the first report by McDonald et al. in 1999 utilizing mouse neural progenitor cells (NPC) transplanted into SCI animal models to show the capacity of survival of grafted NPCs and of differentiation capability into neurons, astrocytes, and oligodendrocytes and more importantly of migration along the rostral/caudal axis from the lesion epicenter [49], various studies have been followed to unpuzzle the working mechanism behind NSCs for SCI and other neurological disorders and to demonstrate the therapeutic potential of NSCs in SCI in both preclinical and clinical settings.

In the previous study, we demonstrated *in vivo* therapeutic effects of ahNSCs in SCI animal models, which were primarily isolated and cultured from surgical samples of cerebral cortex with hemorrhagic stroke [14]. These samples

were obtained by the surgical process of removing some portion of brain tissue of cerebral cortex to lower the intracranial pressure of patients with stroke. In contrast, ahNSCs originated from the temporal cortex of FCD type IIIa were obtained and used in this study. FCD type IIIa is a type of FCD which have combined abnormality hippocampal sclerosis (HS) in the hippocampus and FCD in the cerebral cortex [21, 22]. Although patients with FCD type IIIa suffer from epilepsy, the symptom has been reported to derive from pathologies of the hippocampus [23]. Histological analysis of the temporal cortex of FCD type IIIa could show abnormal cortical layering, such as microcolumns and dyslamination [24]. However, the abnormalities are localized and might be results of epileptic seizure [25].

During surgery for hemorrhagic stroke and FCD type IIIa, cerebral cortex samples were obtained by decompression and approach to the hippocampus, respectively, rather than for

elimination of disease focus [26, 27], Since these surgical samples harbor relatively normal histology, ahNSCs primarily cultured from the hemorrhagic stroke and FCD type IIIa shared very similar patterns of ahNSC specific marker-expression, differentiation potential to neural cells, and proliferation properties [15, 16], Moreover, when ahNSCs were transplanted into the brains of rodents, they have not induced any abnormal symptoms including intracranial hemorrhage and seizure [14].

In SCI animal models, ahNSCs from both conditions demonstrated identical relationship between injection dose and treatment effects. There were differences in the treatment effects among the treated groups. Particularly, it was found from our study that ahNSC medium dosing group was most effective among therapeutic groups in aspects of the tissue loss or regenerated including a relative comparison of tissue loss area, a distance between rostral/caudal NeuN+ cells, a percentage of

rostral/ventral/caudal Tuj1+ regenerated axon, eventually correlating the results of BBB behavioral test.

The differences might result from the numbers of ahNSCs that migrated into the lesions of spinal cords. Given the differentiation potential and paracrine factors of ahNSCs, ahNSCs might exert therapeutic effects for SCI via differentiation into functional neural cells and excretion of neuroprotective paracrine factors [28–30].

Despite those inquiries including the number of transplanted cells, the number of migrated cells in the lesion site of SCI, the optimal route of administration and time-window of intervention, our results indicated that ahNSCs from neurological disorders might be utilized to develop cell therapeutics for SCI.

In addition, our previous study on the evaluation of the survival and distribution of transplanted ahNSCs, demonstrated the safety and efficacy of transplanted ahNSCs in

rodent SCI models as ahNSCs were found to migrate from Lateral Ventricle of Brain through cisterna magna to the lesion site of injured spinal cord and persist in this niche for up to 5 weeks since the presence of human DNA of ahNSCs was confirmed by RT-PCR using human-specific primers. The amount of human DNA was found to be decreased gradually at the site of transplantation [14].

Regarding that the functional recovery by ahNSCs transplantation in animal models of SCI had not been reduced as times goes on, neuroprotective effects of ahNSCs for SCI could be mainly mediated by paracrine factors of ahNSCs. Our study demonstrated that the CM of ahNSCs as well as transplantation of ahNSCs inhibit apoptosis of neural cells in the oxidative stress and exert neuroprotective effects *in vitro* and *in vivo*, respectively. Various types of cytokines were detected in the CM of ahNSCs [44, 45]. Especially, the level of MCP-1 (CCL2) was relatively higher in the CM of

ahNSCs. Since the concentration of MCP-1 was similar in three independent batches of CMs of ahNSCs, the high expression of MCP-1 might be one of key features of ahNSCs. Although MCP-1 is a well-known pro-inflammatory cytokine, its receptor, CCR2, is expressed by neurons in various locations of CNS including spinal cord [31, 32]. In neurons, MCP-1 reduced the ability of neuronal N-methyl-D-aspartate (NMDA) receptors to respond to ligand, thereby reducing glutamate release and subsequent neurotoxic effects [33]. In this study, the neuroprotective effects of the CM of ahNSCs on the survival of primarily cultured spinal cord neurons under the oxidative condition were offset and reversed by the presence of MCP-1 neutralizing antibody. Since oxidative and excitatory stress are involved in the neuronal apoptosis in a damaged spinal cord [34, 35], MCP-1 could be the one of major paracrine factors that mediate the *in vitro* and *in vivo* neuroprotective effects of ahNSCs. *In vitro*

experiments in this study are not enough to explain the *in vivo* roles of MCP-1 in the treatment effects of ahNSCs. *In vivo* experiments hiring neutralizing antibodies against MCP-1 and/or specific shRNAs for MCP-1 should be required in future studies.

The presence of MCP-1 in a lesion of damaged spinal cord attracts monocyte, which in turn could provoke local inflammatory reactions [36, 37]. Conventionally, the inflammation in damaged neural tissue has been reported to worsen the destruction of tissue by diseases. However, recent studies have demonstrated that monocytes recruited in the lesion of SCI can differentiate anti-inflammatory/resolving phenotype macrophages and degrade glial scar that prevent axons and dendrite from regrowth [38, 39]. Although results were not shown, a significant reduction of active astrocytes was observed in the high and medium group compared to the vehicle group in this study. A decrease in the glial scar

formation by transplanting ahNSCs were reported in our previous study using ahNSCs from hemorrhagic stroke in SCI animal models [14]. Effects of MCP-1 released by ahNSCs on the inflammatory reaction and glial scar formation in SCI animal models need to be elucidated further.

Furthermore, as mentioned above the route of administration is significant subject to be considered regarding cell transplantation. There were a number studies with different routes of administration reported previously including intraspinal, intrathecal and intravenous [46]. Despite the results of comparative study showing no significant differences in functional recovery in behavioral experiments of SCI animals NSCs injected locally at the lesion or distally [47], more investigations to demonstrate an optimal administration method with minimally invasive and efficacious procedures should be conducted with regard to the route of administration for cell transplantation in clinical settings Factors related to translational

medicine such as the compliance of both clinicians and patients are important since it's directly correlated with the clinical outcomes, thus well studied and cleared in preclinical settings in prior to clinical trials.

Many reports of clinical trials have described the benefits of NSCs in thoracic SCI patients. For example, StemCells, Inc. conducted a series of Phase II clinical trial using human CNS stem cells from fetal brain in T2-T11 SCI Cohorts. Particularly, phase I/II clinical trial (NCT01321333) was conducted in 2011 at the University Hospital Balgrist, Zurich, Swiss showed a safe and well-tolerable profile and interestingly, showed that 7 out of 12 patients experienced sensory improvements after receiving a total of 20million cells directly into the spinal cord, 2 injects rostral and 2 caudally to the lesion site [48]. As a follow up the positive results, a second clinical trial (NCT01725880) was conducted in 2012 but discontinued based on business decision after confirming

the sensory gains were lost once the immunosuppression agents were removed after 9 months during clinical trials. Consequently, the cell source used for cell transplantation in clinical settings should be carefully considered as a real problem using non-patient cells that could cause immune rejection by host immune system. Nevertheless, there are a number of clinical trials (NCT03069404, NCT02688049) have been conducted to confirm the safety and therapeutic efficacy of NSC transplantation for SCI patients.

In conclusion, non-clinical results evidently showed that ahNSCs from temporal cortex of FCD type IIIa surgical samples could exert significant therapeutic effects for SCI, which were mediated by neuroprotective paracrine factors of ahNSCs. Particularly, MCP-1 released by ahNSCs was considered to play an important role in mechanisms of neuroprotection and anti-apoptosis for SCI animal models. In this study we set up steppingstones by providing promising

non-clinical data which could encourage the future development of cell therapeutics for SCI whereas there are no available therapeutic options at present. Nevertheless, further translational studies should be carried out to guarantee safety, therapeutic effect through the performance of optimal injection route, dose and period of administration for patients with SCI in the future. Lastly, since previous clinical trials using stem cells as single agent have been showing some modest improvements for SCI repair, it is important topic to consider the combinatory therapy with other agents rather than a stand-alone of cell transplantation.

5. References

1. Palmisano, I.; Di Giovanni, S., Advances and Limitations of Current Epigenetic Studies Investigating Mammalian Axonal Regeneration. *Neurotherapeutics* **2018,** 15, (3), 529-540.
2. Bradbury, E. J.; Burnside, E. R., Moving beyond the glial scar for spinal cord repair. *Nat Commun* **2019,** 10, (1), 3879.
3. Kim, G. U.; Sung, S. E.; Kang, K. K.; Choi, J. H.; Lee, S.; Sung, M.; Yang, S. Y.; Kim, S. K.; Kim, Y. I.; Lim, J. H.; Seo, M. S.; Lee, G. W., Therapeutic Potential of Mesenchymal Stem Cells (MSCs) and MSC-Derived Extracellular Vesicles for the Treatment of Spinal Cord Injury. *Int J Mol Sci* **2021,** 22, (24).

4. Shiao, R.; Lee-Kubli, C. A., Neuropathic Pain After Spinal Cord Injury: Challenges and Research Perspectives. *Neurotherapeutics* **2018,** 15, (3), 635-653.

5. James, S.L.; Theadom, A.; Ellenbogen, R.G.; Bannick, M.S.; Montjoy-Venning, W.; Lucchesi, L.R.; Abbasi, N.; bdulkader, R.; Abraha, H.N.; Adsuar, J.C.; et al. Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. **2019,** 18, 56-87.

6. James, N. D.; McMahon, S. B.; Field-Fote, E. C.; Bradbury, E. J., Neuromodulation in the restoration of function after spinal cord injury. *Lancet Neurol* **2018,** 17, (10), 905-917.

7. Liao, L. Y.; Lau, B. W.; Sanchez-Vidana, D. I.; Gao, Q., Exogenous neural stem cell transplantation for cerebral ischemia. *Neural Regen Res* **2019,** 14, (7), 1129-1137.

8. Levi, A. D.; Anderson, K. D.; Okonkwo, D. O.; Park, P.; Bryce, T. N.; Kurpad, S. N.; Aarabi, B.; Hsieh, J.; Gant, K., Clinical Outcomes from a Multi-Center Study of Human Neural Stem Cell Transplantation in Chronic Cervical Spinal Cord Injury. *J Neurotrauma* **2019,** 36, (6), 891-902.

9. Gupta, N.; Henry, R. G.; Kang, S. M.; Strober, J.; Lim, D. A.; Ryan, T.; Perry, R.; Farrell, J.; Ulman, M.; Rajalingam, R.; Gage, A.; Huhn, S. L.; Barkovich, A. J.; Rowitch, D. H., Long-Term Safety, Immunologic Response, and Imaging Outcomes following Neural Stem Cell Transplantation for Pelizaeus-Merzbacher Disease. *Stem Cell Reports* **2019,** 13, (2), 254-261.

10. Curtis, E.; Martin, J. R.; Gabel, B.; Sidhu, N.; Rzesiewicz, T. K.; Mandeville, R.; Van Gorp, S.; Leerink, M.; Tadokoro, T.; Marsala, S.; Jamieson, C.; Marsala, M.; Ciacci, J. D., A First-in-Human, Phase I Study of

Neural Stem Cell Transplantation for Chronic Spinal Cord Injury. *Cell Stem Cell* **2018,** 22, (6), 941-950 e6.

11. Kalladka, D.; Sinden, J.; Pollock, K.; Haig, C.; McLean, J.; Smith, W.; McConnachie, A.; Santosh, C.; Bath, P. M.; Dunn, L.; Muir, K. W., Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet* **2016,** 388, (10046), 787-96.
12. Bonilla, P.; Hernandez, J.; Giraldo, E.; Gonzalez-Perez, M. A.; Alastrue-Agudo, A.; Elkhenany, H.; Vicent, M. J.; Navarro, X.; Edel, M.; Moreno-Manzano, V., Human-Induced Neural and Mesenchymal Stem Cell Therapy Combined with a Curcumin Nanoconjugate as a Spinal Cord Injury Treatment. *Int J Mol Sci* **2021,** 22, (11).
13. Ahuja, C. S.; Mothe, A.; Khazaei, M.; Badhiwala, J. H.; Gilbert, E. A.; van der Kooy, D.; Morshead, C. M.; Tator, C.; Fehlings, M. G., The leading edge: Emerging neuroprotective and neuroregenerative cell-based therapies for

spinal cord injury. *Stem Cells Transl Med* **2020,** 9, (12), 1509-1530.

14. Won, J. S.; Yeon, J. Y.; Pyeon, H. J.; Noh, Y. J.; Hwang, J. Y.; Kim, C. K.; Nam, H.; Lee, K. H.; Lee, S. H.; Joo, K. M., Optimal Preclinical Conditions for Using Adult Human Multipotent Neural Cells in the Treatment of Spinal Cord Injury. *Int J Mol Sci* **2021,** 22, (5).
15. Joo, K. M.; Kang, B. G.; Yeon, J. Y.; Cho, Y. J.; An, J. Y.; Song, H. S.; Won, J. H.; Kim, S. J.; Hong, S. C.; Nam, D. H., Experimental and clinical factors influencing long-term stable in vitro expansion of multipotent neural cells from human adult temporal lobes. *Exp Neurol* **2013,** 240, 168-77.
16. Yeon, J. Y.; Hwang, J. Y.; Lee, H. W.; Pyeon, H. J.; Won, J. S.; Noh, Y. J.; Nam, H.; Joo, K. M.,

Optimized Clump Culture Methods for Adult Human Multipotent Neural Cells. *Int J Mol Sci* **2018,** 19, (11).

17. Lee, K. H.; Pyeon, H. J.; Nam, H.; Won, J. S.; Hwang, J. Y.; Lee, K. A.; Yeon, J. Y.; Hong, S. C.; Nam, D. H.; Lee, K.; Lee, S. H.; Joo, K. M., Significant therapeutic effects of adult human multipotent neural cells on spinal cord injury. *Stem Cell Res* **2018,** 31, 71-78.
18. Lee, K. H.; Nam, H.; Jeong da, E.; Kim, S. S.; Song, H. J.; Pyeon, H. J.; Kang, K.; Hong, S. C.; Nam, D. H.; Joo, K. M., Sensitive Tumorigenic Potential Evaluation of Adult Human Multipotent Neural Cells Immortalized by hTERT Gene Transduction. *PLoS One* **2016,** 11, (7), e0158639.
19. Nam, H.; Lee, I. H.; Sa, J. K.; Kim, S. S.; Pyeon, H. J.; Lee, K. H.; Lee, K.; Lee, S. H.; Joo, K. M., Effects of Long-Term In Vitro Expansion on Genetic

Stability and Tumor Formation Capacity of Stem Cells. *Stem Cell Rev Rep* **2022,** 18, (1), 241-257.

20. Kim, S. S.; Pyeon, H. J.; Bae, Y. K.; Nam, H.; Kim, C. K.; Lee, S. H.; Joo, K. M., Adult Human Multipotent Neural Cells Could Be Distinguished from Other Cell Types by Proangiogenic Paracrine Effects via MCP-1 and GRO. *Stem Cells Int* **2021,** 2021, 6737288.
21. Miyata, H.; Hori, T.; Vinters, H. V., Surgical pathology of epilepsy-associated non-neoplastic cerebral lesions: a brief introduction with special reference to hippocampal sclerosis and focal cortical dysplasia. *Neuropathology* **2013,** 33, (4), 442-58.
22. Fauser, S.; Essang, C.; Altenmuller, D. M.; Staack, A.; Steinhoff, B. J.; Strobl, K.; Bast, T.; Schubert-Bast, S.; Doostkam, S.; Zentner, J.; Schulze-Bonhage, A., Is there evidence for clinical differences related to the new

classification of temporal lobe cortical dysplasia? *Epilepsia* **2013,** 54, (5), 909-17.

23. Cossu, M.; d'Orio, P.; Barba, C.; Asioli, S.; Cardinale, F.; Casciato, S.; Caulo, M.; Colicchio, G.; Consales, A.; D'Aniello, A.; De Benedictis, A.; De Palma, L.; Didato, G.; Di Gennaro, G.; Di Giacomo, R.; Esposito, V.; Guerrini, R.; Nichelatti, M.; Revay, M.; Rizzi, M.; Vatti, G.; Villani, F.; Zamponi, N.; Tassi, L.; Marras, C. E., Focal Cortical Dysplasia IIIa in Hippocampal Sclerosis-Associated Epilepsy: Anatomo-Electro-Clinical Profile and Surgical Results From a Multicentric Retrospective Study. *Neurosurgery* **2021,** 88, (2), 384-393.
24. Kim, S. H.; Choi, J., Pathological Classification of Focal Cortical Dysplasia (FCD) : Personal Comments for Well Understanding FCD Classification. *J Korean Neurosurg Soc* **2019,** 62, (3), 288-295.

25. Thom, M.; Eriksson, S.; Martinian, L.; Caboclo, L. O.; McEvoy, A. W.; Duncan, J. S.; Sisodiya, S. M., Temporal lobe sclerosis associated with hippocampal sclerosis in temporal lobe epilepsy: neuropathological features. *J Neuropathol Exp Neurol* **2009,** 68, (8), 928-38.

26. Beez, T.; Munoz-Bendix, C.; Steiger, H. J.; Beseoglu, K., Decompressive craniectomy for acute ischemic stroke. *Crit Care* **2019,** 23, (1), 209.

27. Chong, S.; Phi, J. H.; Lee, J. Y.; Kim, S. K., Surgical Treatment of Lesional Mesial Temporal Lobe Epilepsy. *J Epilepsy Res* **2018,** 8, (1), 6-11.

28. Cummings, B. J.; Uchida, N.; Tamaki, S. J.; Salazar, D. L.; Hooshmand, M.; Summers, R.; Gage, F. H.; Anderson, A. J., Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* **2005,** 102, (39), 14069-74.

29. Rong, Y.; Liu, W.; Wang, J.; Fan, J.; Luo, Y.; Li, L.; Kong, F.; Chen, J.; Tang, P.; Cai, W., Neural stem cell-derived small extracellular vesicles attenuate apoptosis and neuroinflammation after traumatic spinal cord injury by activating autophagy. *Cell Death Dis* **2019,** 10, (5), 340.
30. Veneruso, V.; Rossi, F.; Villella, A.; Bena, A.; Forloni, G.; Veglianese, P., Stem cell paracrine effect and delivery strategies for spinal cord injury regeneration. *J Control Release* **2019,** 300, 141-153.
31. Gosselin, R. D.; Varela, C.; Banisadr, G.; Mechighel, P.; Rostene, W.; Kitabgi, P.; Melik-Parsadaniantz, S., Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. *J Neurochem* **2005,** 95, (4), 1023-34.
32. Dansereau, M. A.; Gosselin, R. D.; Pohl, M.; Pommier, B.; Mechighel, P.; Mauborgne, A.; Rostene, W.; Kitabgi, P.; Beaudet, N.; Sarret, P.; Melik-Parsadaniantz, S., Spinal

CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem* **2008,** 106, (2), 757-69.

33. Madrigal, J. L.; Leza, J. C.; Polak, P.; Kalinin, S.; Feinstein, D. L., Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline. *J Neurosci* **2009,** 29, (1), 263-7.

34. Hou, Y.; Luan, J.; Huang, T.; Deng, T.; Li, X.; Xiao, Z.; Zhan, J.; Luo, D.; Hou, Y.; Xu, L.; Lin, D., Tauroursodeoxycholic acid alleviates secondary injury in spinal cord injury mice by reducing oxidative stress, apoptosis, and inflammatory response. *J Neuroinflammation* **2021,** 18, (1), 216.

35. Gu, C.; Li, L.; Huang, Y.; Qian, D.; Liu, W.; Zhang, C.; Luo, Y.; Zhou, Z.; Kong, F.; Zhao, X.; Liu, H.; Gao, P.; Chen, J.; Yin, G., Salidroside Ameliorates Mitochondria-Dependent Neuronal Apoptosis after Spinal

Cord Ischemia-Reperfusion Injury Partially through Inhibiting Oxidative Stress and Promoting Mitophagy. *Oxid Med Cell Longev* **2020,** 2020, 3549704.

36. Mukhamedshina, Y. O.; Akhmetzyanova, E. R.; Martynova, E. V.; Khaiboullina, S. F.; Galieva, L. R.; Rizvanov, A. A., Systemic and Local Cytokine Profile following Spinal Cord Injury in Rats: A Multiplex Analysis. *Front Neurol* **2017,** 8, 581.
37. Farfara, D.; Lifshitz, V.; Frenkel, D., Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. *J Cell Mol Med* **2008,** 12, (3), 762-80.
38. Shechter, R.; Raposo, C.; London, A.; Sagi, I.; Schwartz, M., The glial scar-monocyte interplay: a pivotal resolution phase in spinal cord repair. *PLoS One* **2011,** 6, (12), e27969.

39. Raposo, C.; Schwartz, M., Glial scar and immune cell involvement in tissue remodeling and repair following acute CNS injuries. *Glia* **2014,** 62, (11), 1895-904.

40. In *Guide for the Care and Use of Laboratory Animals*, th, Ed. Washington (DC), **2011.**

41. Basso, D. M.; Beattie, M. S.; Bresnahan, J. C., A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* **1995,** 12, (1), 1-21.

42. Won, J. S.; Nam, H.; Lee, H. W.; Hwang, J. Y.; Noh, Y. J.; Nam, D. H.; Lee, S. H.; Joo, K. M., In vivo distribution of U87MG cells injected into the lateral ventricle of rats with spinal cord injury. *PLoS One* **2018,** 13, (8), e0202307.

43. Bankhead, P.; Loughrey, M. B.; Fernandez, J. A.; Dombrowski, Y.; McArt, D. G.; Dunne, P. D.; McQuaid, S.; Gray, R. T.; Murray, L. J.; Coleman, H. G.; James, J. A.; Salto-Tellez, M.; Hamilton, P. W., QuPath: Open-

source software for digital pathology image analysis. *Sci Rep* **2017,** 7, (1), 16878.

44. Cheng, Z.; Bosco, D. B.; Sun, L.; Chen, X.; Xu, Y.; Tai, W.; Didier, R.; Li, J.; Fan, J.; He, X.; Ren, Y., Neural Stem Cell-Conditioned Medium Suppresses Inflammation and Promotes Spinal Cord Injury Recovery. Cell Transplant **2017**, 26, (3), 469-482.
45. Pawitan, J. A., Prospect of stem cell conditioned medium in regenerative medicine. Biomed Res Int **2014**, 2014, 965849.
46. Amemori, T.; Ruzicka, J.; Romanyuk, N.; Jhanwar-Uniyal, M.; Sykova, E.; Jendelova, P. Comparison ofintraspinal and intrathecal implantation of induced pluripotent stem cell-derived neural precursors for the treatment of spinal cord injury in rats. Stem Cell Res. Ther. **2015**, 6, 257.
47. Cheng, Z.; Zhu, W.; Cao, K.; Wu, F.; Li, J.; Wang, G.; Li, H.; Lu, M.; Ren, Y.; He, X. Anti-Inflammatory

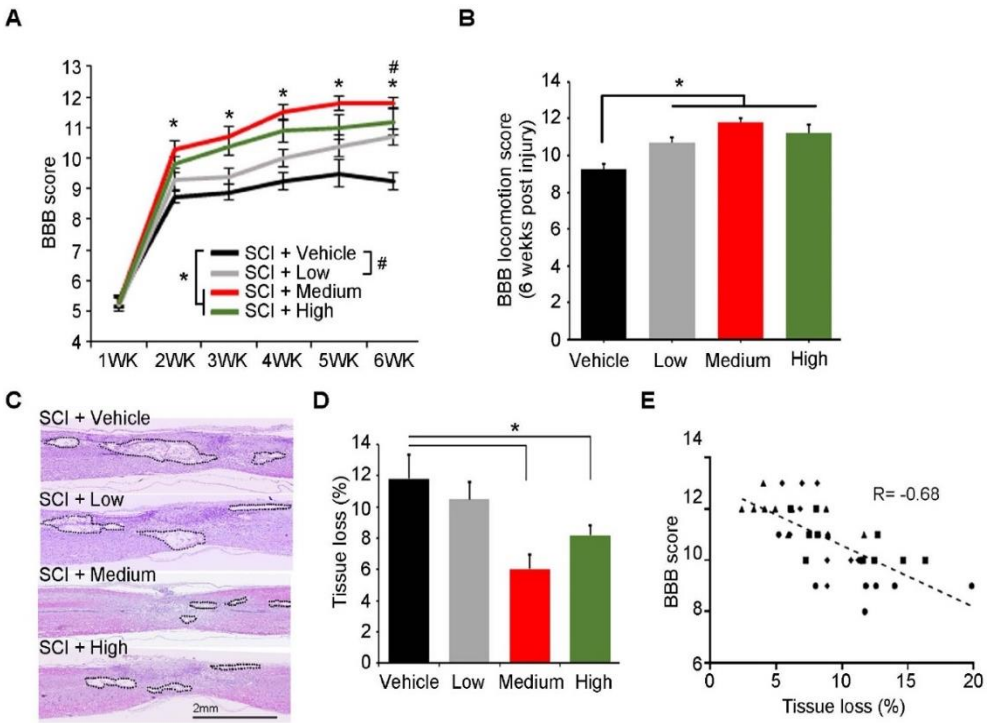
Mechanism of Neural Stem Cell Transplantation in Spinal Cord Injury. Int. J. Mol. Sci. **2016**, 17, 1380.

48. Curtis, E.; Martin, J.R.; Gabel, B.; Sidhu, N.; Rzesiewicz, T.K.; Mandeville, R.; Van Gorp, S.; Leerink, M.; Tadokoro, T.; Marsala, S.; et al. A First-in-Human, Phase I Study of Neural Stem Cell Transplantation for Chronic Spinal Cord Injury. Cell Stem Cell **2018**, 22, 941–950.

49. McDonald, J.W.; Liu, X.-Z.; Qu, Y.; Liu, S.; Mickey, S.K.; Turetsky, D.; Gottlieb, D.I.; Choi, D.W. Transplanted embryonic stem cells survive, differentiate, and promote recovery in injured rat spinal cord. Nat. Med. **1999**, 5, 1410–1412.

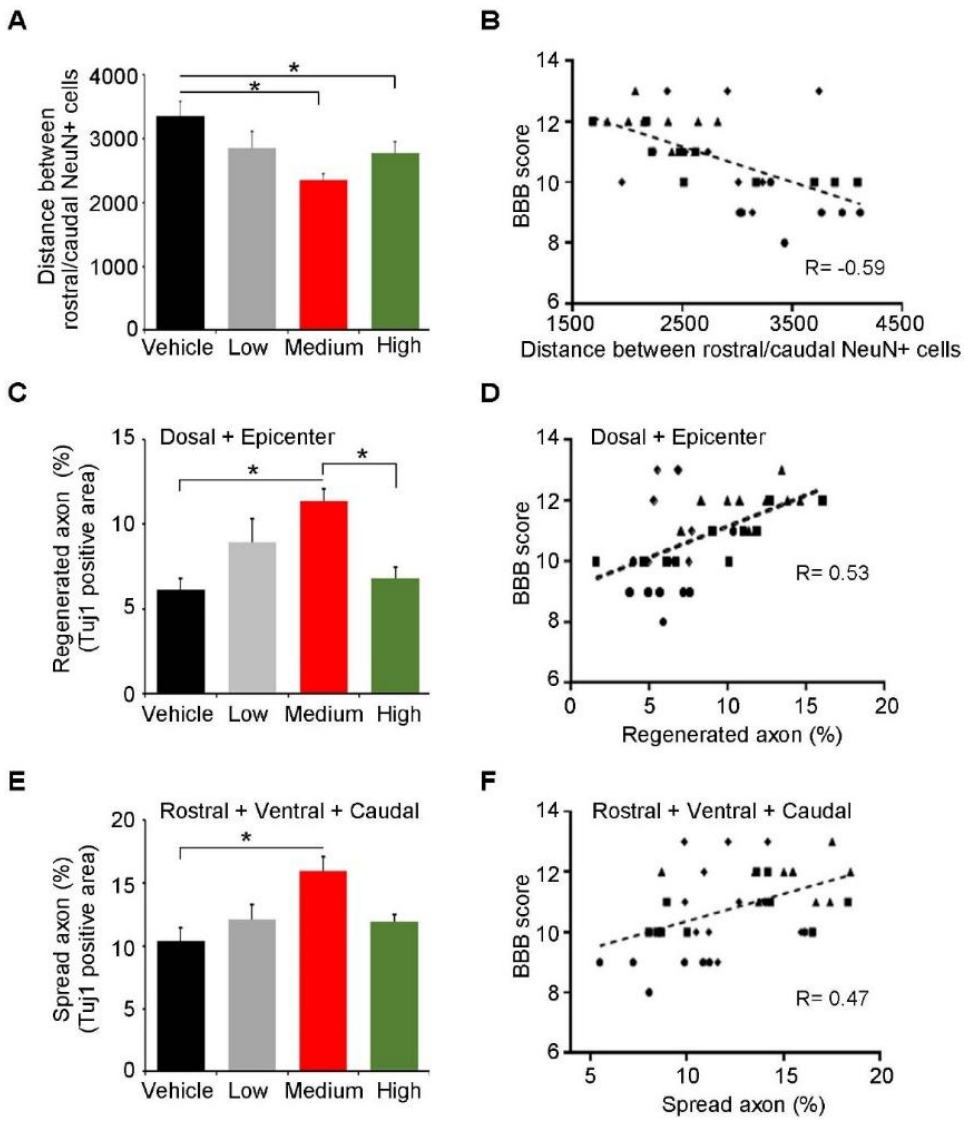
6. Figures

Figure 1. Preclinical therapeutic effects of ahNSCs for SCI



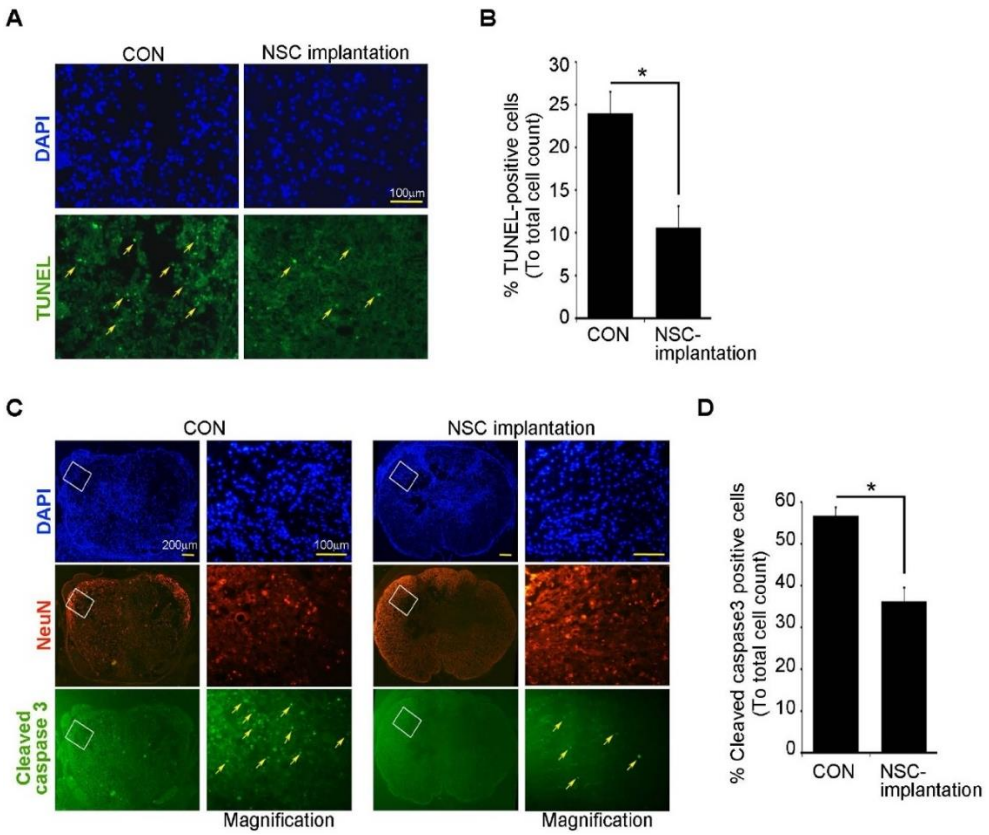
(A) BBB scores were measured and compared once a week until 6 weeks after SCI. *, #, $p < 0.05$. (B) BBB scores were measured and compared at 6 weeks after SCI. *, $p < 0.05$. (C) Tissue loss at injury site was evaluated at 6 weeks post SCI. Black lines delineate the contours of cavities and shrunken tissue. Scale bar = 2 mm. (D) Relative areas of tissue loss were quantified and compared. *, $p < 0.05$. (E) Correlation analysis revealed a significant negative relationship between the degree of tissue loss and the BBB score at 6 weeks post SCI.

Figure 2. In vivo neuroprotective activities of ahNSCs



(A) NeuN-positive neurons adjacent to damaged lesions both rostrally and caudally were identified. The distance between those neurons were quantified and compared. (B) Correlation between the distance and the BBB score at 6 weeks post SCI was analyzed. (C) Tuj1 immunoreactivity was evaluated in five areas of damaged spinal cord: Dorsal, Epicenter, Rostral, Ventral, and Caudal. Tuj1 immunoreactive areas of Dorsal and Epicenter areas were quantified and compared. (D) Correlation between the Tuj1 immunoreactivity of Dorsal and Epicenter area and the BBB score at 6 weeks post SCI was analyzed. (E) Tuj1 immunoreactive areas of Rostral, Ventral, and Caudal areas were quantified and compared among groups. (F) Correlation between the immunoreactivity of Tuj1 in Rostral, Ventral, and Caudal area and the BBB score at 6 weeks post SCI was analyzed. *, $p < 0.05$.

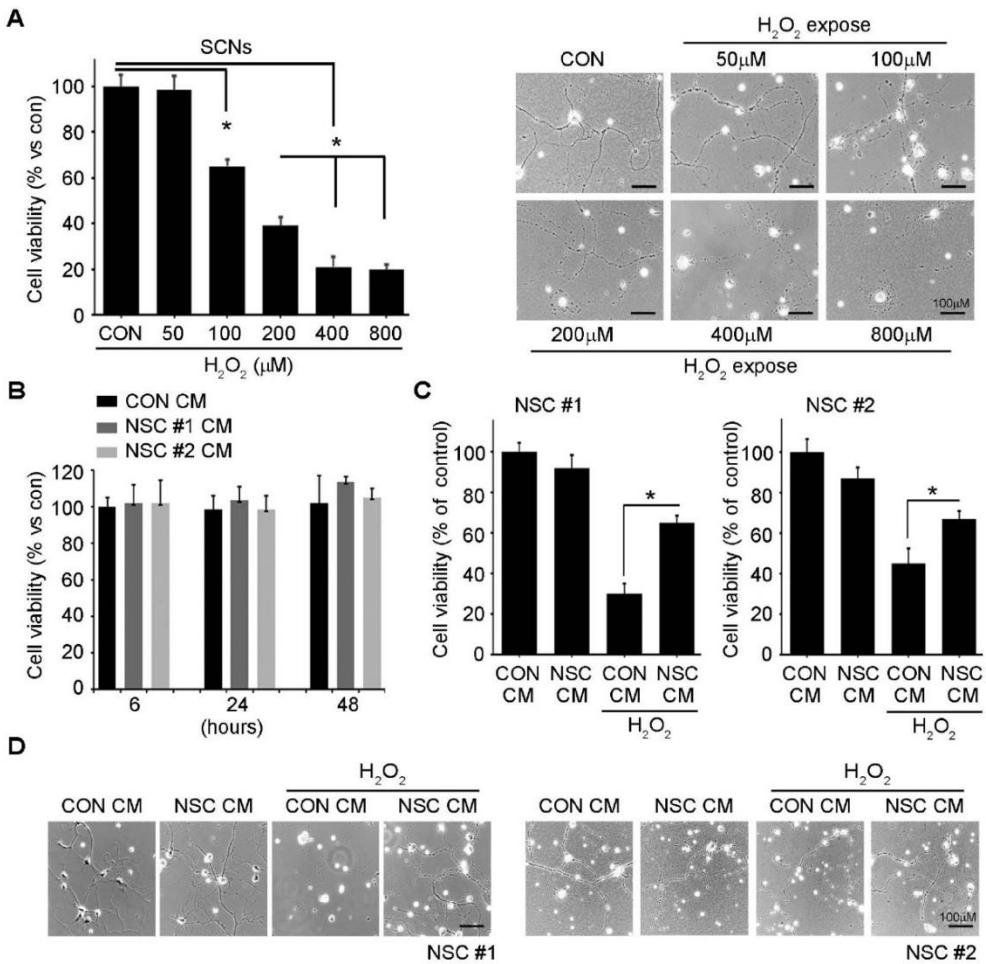
Figure 3. In vivo neuroprotective effects of ahNSCs



(A) Representative images of TUNEL assay in the spinal cords of SCI animal models (B) TUNEL positive cells were counted in each section and compared among groups (n = 5 per

group). *, $p < 0.05$. (C) Representative images of cleaved caspase 3-positive neurons ($NeuN^+$) in spinal cords of SCI animal models (D) The number of cleaved caspase 3-positive cells was counted in each section and compared among groups (n = 5 per group). *, $p < 0.05$.

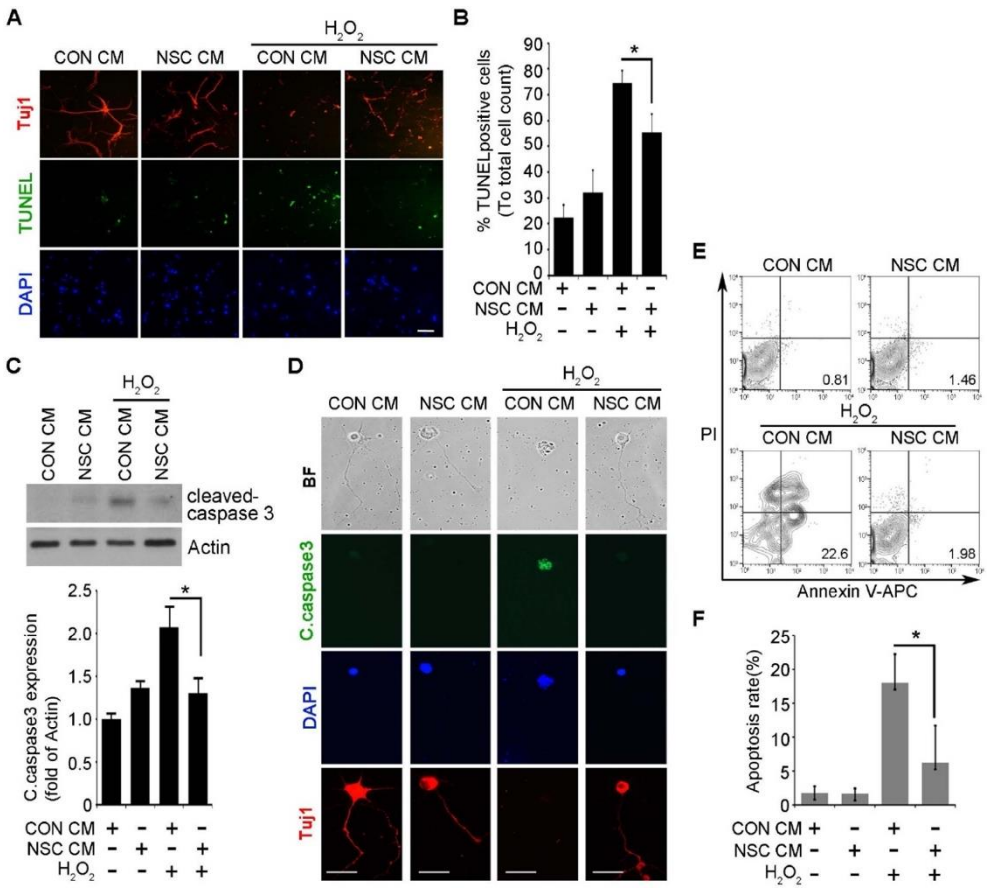
Figure 4. In vitro neuroprotective effects of ahNSCs



(A) The survival rate of SCNs treated with various concentrations of H_2O_2 for 24 hrs was measured by MTT

assay ($n = 5$ for each group). *, $p < 0.05$. Representative images of SCNs were illustrated. Scale bar = 100 μm. (B) MTT assay was performed to estimate the viability of SCNs treated with the CM of NSC #1 or NSC#2. (C) SCNs were treated with the CM of NSC #1 or NSC #2 for 1 hr and then with 200 μM of H_2O_2 for 24 hrs. The viability of SCNs was evaluated by MTT assay ($n = 5$ for each group). *, $p < 0.05$. (D) Representative images of SCNs were illustrated. Scale bars = 100 μm.

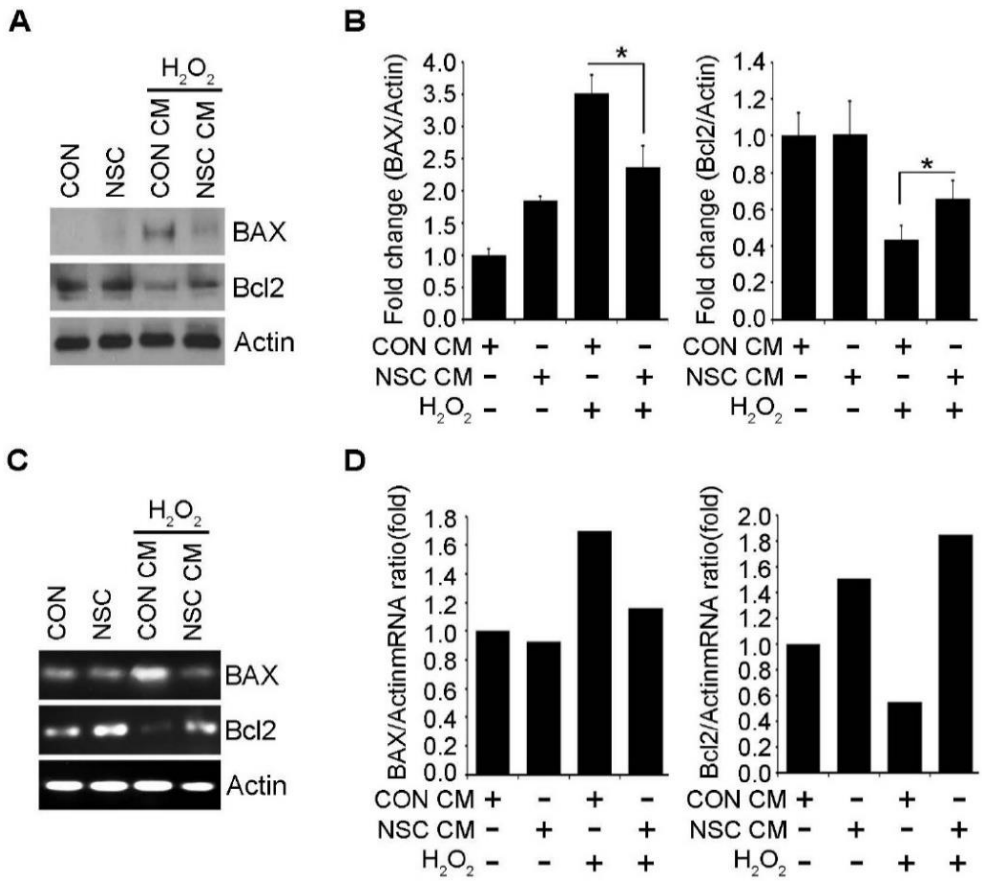
Figure 5. In vitro anti-apoptotic effects of ahNSCs' conditioned medium



(A) SCNs were treated with the CM of NSC #1 for 1 hr and then with 200 μM of H_2O_2 for 24 hrs. $TUNEL^+$

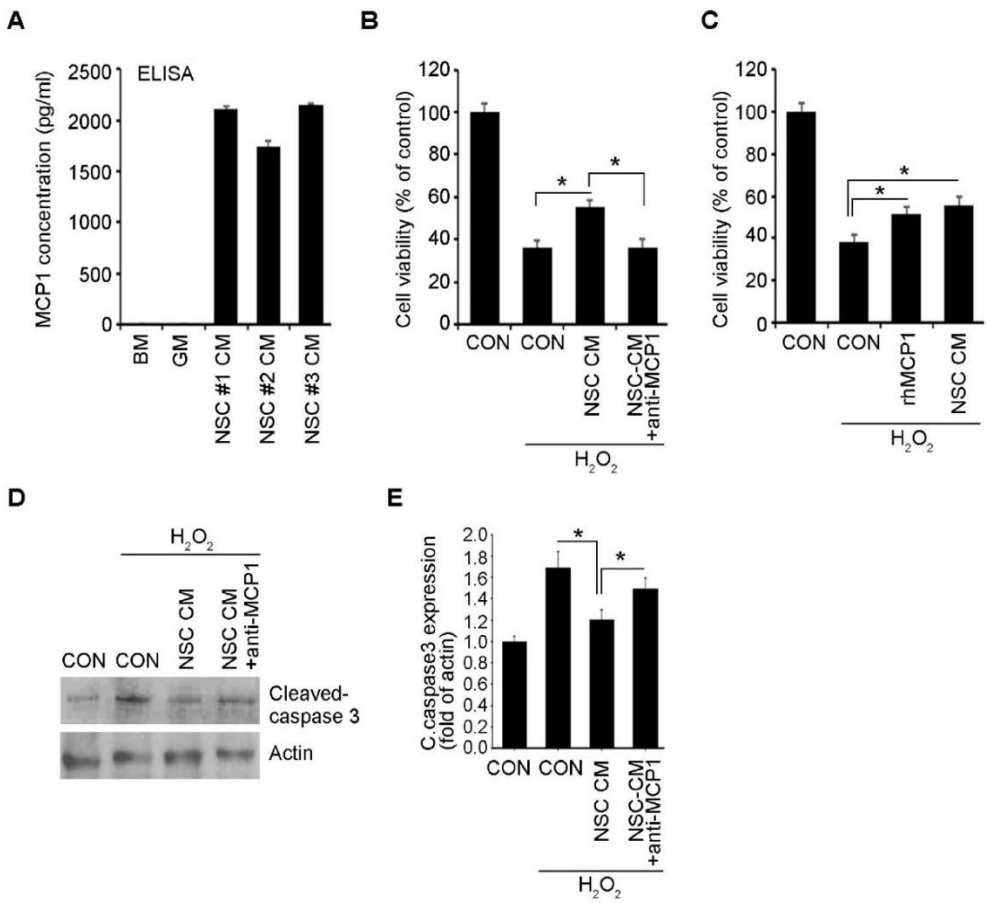
and/or Tuj1^{+} SCNs were visualized in each group. Scale bars = 100 μm. (B) TUNEL^{+} apoptotic SCNs were counted and compared ($n = 5$ per group). *, $p < 0.05$. (C) Expression of cleaved caspase 3 in each group was analyzed by western blot analysis. Actin = loading control. Amount of cleaved caspase was quantified by densitometry analysis (normalized to actin) and compared ($n = 3$ per group). *, $p < 0.05$. (D) Tuj1 or cleaved caspase 3-postive SCNs were visualized in each group. Scale bars = 50 μm. (E) SCN cells were analyzed by Annexin-V/PI. (F) Apoptosis ratio was calculated from the percentage of early apoptosis among different experimental groups ($n = 5$ per group). *, $p < 0.05$.

Figure 6. Apoptosis-related gene expression in SCN cells treated with ahNSCs' conditioned medium



(A) SCNs were treated with the CM of ahNSC #1 for 1 hr and with 200 μM of H_2O_2 for 24 hrs. Expression of BAX and Bcl2 in each group was analyzed by western blot analysis. Actin = loading control. (B) Amount of Bax and Bcl2 was quantified by densitometry analysis (normalized to actin) and compared ($n = 3$ per group). *, $p < 0.05$. (C) Expression of BAX and Bcl2 in each group was analyzed by RT-PCR. Actin = internal control. (D) Amount of mRNA of Bax and Bcl2 was quantified by densitometry analysis (normalized to actin) and compared.

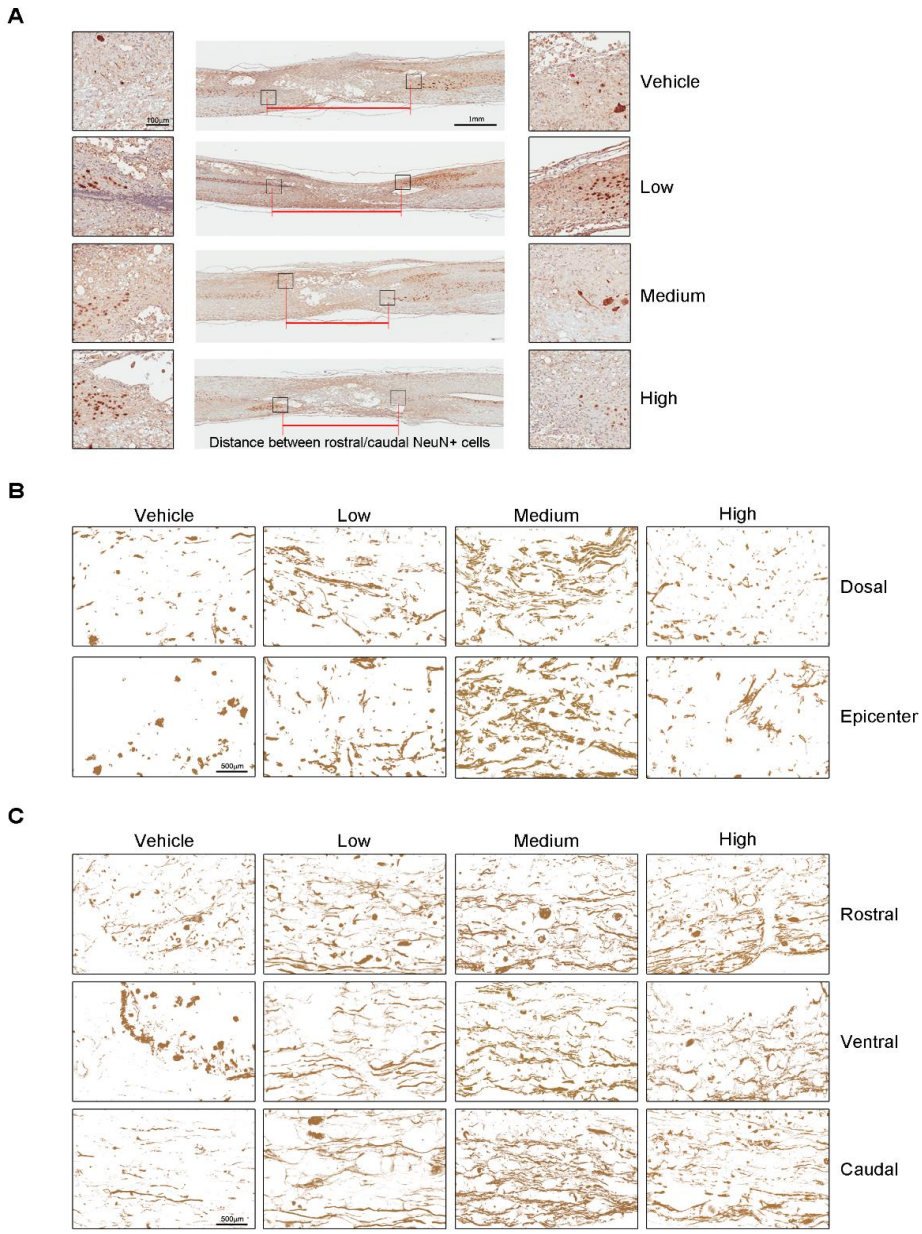
Figure 7. Neuroprotection effects of ahNSCs mediated by MCP-1



(A) Concentration of MCP-1 in the CM of three independent batches of ahNSCs was analyzed by ELISA. All experiments

were performed in triplicate and repeated three times. (B) SCNs were treated with the CM of ahNSC #1 with/without anti-MCP-1 neutralizing antibody (10 μg/ml) for 1 hr and then with 200 μM of H_2O_2 for 6 hrs. Cell viability was analyzed by MTT assay ($n = 5$ for each group). *, $p < 0.05$. (C) SCNs were treated with the CM of ahNSC #1 or recombinant MCP-1 protein (1 ng/ml) with 200 μM of H_2O_2 for 24 hrs. Cell viability was analyzed by MTT assay ($n = 8$ for each group). *, $p < 0.05$. (D) SCNs were treated with the CM of ahNSC #1 with/without anti-MCP-1 neutralizing antibody (10 μg/ml) for 1 hr and then with 200 μM of H_2O_2 for 24 hrs. Expression of cleaved caspase 3 was analyzed by western blot analysis. Actin = loading ve. (E) Amount of cleaved caspase 3 was quantified by densitometry analysis (normalized to actin) and compared ($n = 3$ for each group). *, $p < 0.05$.

Figure 8. Immunohistochemistry against and Tuj1



(A) NeuN-positive neurons adjacent to damaged lesions both rostrally and caudally were identified. The distance between

those neurons were measured (read lines). (B) Tuj1 immunoreactivity was evaluated in the areas of damaged spinal cord: Dorsal and Epicenter. (C) Tuj1 immunoreactivity was evaluated in the areas of damaged spinal cord: Rostral, Ventral, and Caudal.

7. Tables

Table 1. BBB Score[1]

Group	No. of animal	1WK	2WK	3WK	4WK	5WK	6WK
Vehicle	8	5.4 ± 0.52	8.8 ± 0.71	8.9 ± 0.83	9.3 ± 0.87	9.5 ± 1.41	9.3 ± 0.89
Low	10	5.2 ± 0.67	9.3 ± 1.16	9.4 ± 0.84	10 ± 0.94	10.4 ± 1.07	10.7 ± 0.82*
Middle	10	5.3 ± 0.54	10.3 ± 0.82*	10.7 ± 1.06*	11.5 ± 0.85*	11.8 ± 0.79*	11.8 ± 0.63*
High	10	5.3 ± 0.63	9.8 ± 0.79*	10.4 ± 0.97*	10.9 ± 1.20*	11 ± 1.25*	11.2 ± 1.48*

[1]Average ± Standard Error (SEM), *p < 0.05 vs. Vehicle.

국문초록

척수손상 모델에서 MCP-1 단백질로 유도되는 성체신경줄기세포의 치료적 효과 연구

안 재 열

서울대학교 대학원

의학과 해부학전공

척수손상(Spinal cord injury, SCI)은 척추 내에 존재하는 중추신경계인 척수에 외상 또는 질병으로 인해 손상이 생김으로 손상 부위 이하의 운동과 감각 기능이 마비되고 자율신경계 기능에 이상이 생기는 질환이다.

척수손상은 인간 중추신경계의 자연적 신경재생능이 제한됨에 따라 조직 손상 후 만성 기능 장애가 수반되는 등 예후가 좋지 않으며 효과적인 치료법이 없는 것으로 알려져 있다.

본 연구에 사용된 성체신경줄기세포(adult human neural stem cells, ahNSC)는 성인 뇌조직에서 유래한 세포로써 신경줄기세포의 주요 특성을

나타내고 있으며, 퇴행성신경질환 등 다양한 중추신경계질환에서 치료 효능이 보고되었다.

본 연구의 목표는 국소피질이형성증 3a형(focal cortical dysplasia type IIIa) 성인 환자의 측두엽(temporal lobe) 조직에서 분리, 배양한 ahNSC의 SCI 치료 기전을 분석하고 효능을 평가하는 것이다.

실험 결과 SCI 대조군 대비 ahNSC를 이식한 실험군에서 척수손상 동물의 운동신경 회복이 유의미하게 증가하였으며 특히 손상된 척수 조직 주변에서 신경섬유(nerve fiber)의 재생(regeneration) 및 확산(spreading)이 관찰되었다. 아울러 손상된 조직내 재생된 신경세포들의 신경핵(neuronal nuclei)간 거리는 대조군대비 실험군에서 유의미하게 가깝게 분포 되어있음을 확인하였다.

면역조직화학염색(immunohistochemistry, IHC) 분석을 통해 SCI 손상 조직내 ahNSC의 신경보호기능(neuroprotection)은 척수신경세포 사멸을 억제하므로 인해 유도되는 것으로 관찰되었다.

이에 더해, ahNSC 배양액(conditioned medium, CM) 성분 분석을 통해 ahNSC의 신경보호기능은 측분비효과(paracrine effect)로 인해 세포 외부로 분비되는 다양한 종류의 사이토카인(cytokine) 중 monocyte

chemoattractant protein-1(MCP-1 또는 CCL2)에 의해 이뤄지는 것으로 확인하였다.

아울러, 본 연구를 통해 SCI에 신경손상 보호기능 등의 치료 효능을 보이는 적합한 수준의 ahNSC 투여 농도와 투여 방법을 확인할 수 있었으며, 향후 임상시험 진입을 위한 안전성, 기능적 회복, 임상시험용 투여농도, 투여방법, 투여기간 등에 대한 추가 비임상 시험이 필요할 것으로 사료된다.

본 연구 결과는 향후 SCI 비임상 시험 및 임상시험에 활용되어 ahNSC가 안전하고 효과적인 세포치료제로 개발될 가능성을 제시한다.

주요어 : 척수손상, 신경줄기세포, 측두엽, 사이토카인, monocyte chemoattractant protein-1

학 번 : 2020-37944