Human adipose-derived mesenchymal stem cells improve neuropathic pain and enhance nerve regeneration in a neuropathic pain model

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This certifies that the Master's Thesis

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Abstract

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Adipose derived mesnchymal stem cell(ADMSC) has been a promising source of cell therapy due to its capability of autologous transplantation, availabily of easy acquisition ,and potency to cross differentiation. While researchers has vigorously investigated on the efficacy of ADMSCs on nervous diseases, it is still not clear whether positive results are coming from its neural differentiation or anti-inflammatory effects. Currently, direct transplantation has been accepted as the standard method of stem cell delivery. However, a fragile lesion may be further damaged by a small volume of transplanting cell. Therefore, we evaluated the neuronal differentiation of ADMSCs and compared indirect transplantation with direct injection of h-ADMSCs in a rat model of sciatic nerve crush injury. In our result, h-ADMSCs significantly improved mechanical allodynia and neuronal regeneration compared with control group (p=8). Epineural injection was superior to intraneural injection in terms of less mechanical allodynia and better neuronal regeneration (p=8). It is assumed that secondary injury by needling and

volume expansion was avoided by less-invasive epineural soaking. In conclusion, h-ADMSCs transplantation with epineural soaking may be a good alternative for the treatment of peripheral nerve injury and neuropathic pain.

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Ⅰ. Introduction

Neuropathic pain is a result of neural injury or dysfunction. The nervous system has inherent regenerative potential after damage. Schwann cells and macrophages rapidly remove myelin debris and produce inflammatory cytokines, chemokines, extracellular matrix molecules, and proteolytic enzymes as well as a number of growth factors that play critical roles in neuronal survival and axonal regeneration. However, nerve regeneration is often limited by incomplete and non-specific regeneration, axonal loss, variable clinical results, or the development of chronic neuropathic pain disorders[1]. Recently, cell transplantation has become a challenging field of interest in neuro-degeneration; in experimental nerve gap models, the local implantation of neural stem cells or bone marrow mesenchymal stem cells has been shown to improve peripheral nerve regeneration through multiple mechanisms: trophic factor secretion, synthesis of extracellular matrix molecule, microenvironment stabilization, and immune modulation[2].

Human adipose-derived mesenchymal stem cells (h-ADMSCs) influenced relevant immune modulatory activity both in vivo and in vitro in a broad range of immune cells. Moreover, several research groups have found evidence for neural and glial trans differentiation of h-ADMSCs in vitro and in vivo. Finally, h-ADMSCs secrete a variety of neurotrophins and cytokines, which might positively impact neural cell survival and neural degeneration[3,4].

The intrinsic limitation of the general method is the additional trauma necessary in reaching the injection site as well as the difficulty in treating multiple or diffused sites of injury. In such circumstances, a systemic administration of cells capable of reaching the damaged PNS would be desirable.

The effects of h-ADMSCs treatment were evaluated in terms of functional recovery and nerve regeneration, assessing the ability of injected cells to integrate into the injured peripheral nerve, to modulate the immune response, and to influence the local micro environment through the release of neurotrophic molecules[5].

Ⅱ. Methods

1. Human adipose-derived mesenchymal stem cells culture and characterization

The cell were cultured at 1×10^4 cells in a 25 cm^2 flask in alpha MEM (Gibco, Grand Island, NY), Fetal bovine serum (10%; Gibco, Grand Island, NY), penicillin streptomycin (1%; Gibco, Grand Island, NY), at 37℃ in a 5% CO_2 atmosphere. When 70~80% confluent adhesion cells were trypsinized with 0.5% trypsin/EDTA (Gibco, Grand Island, NY) they were expanded in 75 cm^2 flask. The medium was replaced every 3~4 days with fresh subculture[6].

To confirm the presence isolation of h-ADMSCs, immunocytochemical analysis was conducted. h-ADMSCs were specified by the expression of CD44 and CD90 and absence of CD14 and CD19 (All primary antibodies purchased for Millipore, Billerica, MA).

For all experiments in this study, cells at passage 5~6 were used.

2. Neuronal differentiation

To induce neuronal differentiation for h-ADMSCs, to methods were used: serum-free cytokine induction, and butylated hydroxyanisole chemical induction.

Serum-free induction. h-ADMSCs were cultured in suspension using a bacterial dish containing sphere medium composed of neurobasal medium (Gibco, Grand Island, NY) with B27 (2%; Gibco, Grand Island, NY), L-glutamine (2 mM; Gibco, Grand Island, NY), EGF (20 ng/ml; R&D system, Minneapolis, MN), and bFGF (20 ng/ml; R&D system, Minneapolis, MN). After 4~7 days, h-ADMSCs formed spheroid aggregate (i.e., neurospheres). Neurospheres were plated on Matrigel (BD bioscience, San Jose, CA)-coated coverslip in neurobasal medium containing the 2% B27, 2 mM L-glutamine, 10

ng/ml recombinant human-BDNF (Peprotech, Rocky Hill, NJ), and 1% penicillin streptomycin. The medium was replaced every 3 days up to 10~14 days.[7,8]

Chemical induction. There are also other ways to induce neuronal differentiation in h-ADMSCs. Cells were plated on Matrigel-coated coverslips in DMEM (Gibco, Grand Island, NY) containing 20% FBS for 1 day. Then, the medium was changed to DMEM with butylated hydroxyanisole (200 μM; Sigma, St. Louis, MO), KCl (5 mM; Sigma, St. Louis, MO), valproic acid (2 μM; Sigma, St. Louis, MO), forskolin (10 μM; Sigma, St. Louis, MO), hydrocortisone (1 μM; Sigma, St. Louis, MO), and insulin (5 μg/ml; Sigma, St. Louis, MO) for 3 days.[7]

Immunocytochemistry. To detect changes in cell morphology following neuronal induction, we analyzed of h-ADMSCs culture from 4~14 days following neuronal induction. Images were viewed using bright field microscopy. For immunocytochemistry, we grew cells on cover slips, fixed h-ADMSCs with 4% paraformaldehyde, and washed two times in wash buffer with BSA (0.1%; Sigma, St. Louis, MO) in 1xPBS (Gibco, Grand Island, NY). These were then incubated with normal donkey serum (10%; Jackson, Bar harbor, ME) and Triton® X-100 (0.3%; Sigma, St. Louis, MO) in 1xPBS blocking buffer to prevent non-specific antibody binding. Primary was antibodies were allowed to react for 16 hours at 4℃. Subsequently, the cover slips were rinsed three times with PBS containing 0.1% BSA. Next, the samples were treated with a secondary antibody at room temperature for 1 hour. In addition to the nuclear staining, the cells were mounted with DAPI (4', 6-diamidino-2-phenylindole)-conjugated mounting medium.[9]

3. Establish of a neuropathic pain animal model

Adult male Sprague Dawley rats (200±20 g) were housed in a facility accredited by the Association for Assessment and Accreditation of laboratory Animal Care (AAALAC). Animals were randomized into four groups of eight animals each: a PBS epineural

soaking group, a phosphate-buffered saline (PBS) intraneural injection group, a h-ADMSCs epineural soaking group, and a h-ADMSCs intraneural injection group. All animals were anaesthetized with Rompun (10 mg/kg; Bayer Korea, Seoul, South Korea) and Zoletil 50 (25 mg/kg; Virbac Laboratories, Carros, France). The left hind leg was shaved and the biceps femoris incised to expose the sciatic nerve. The crush injury was performed 10 mm proximal to its trifurcation using a 60-g force vascular clip (Micro Serrefines; Fine Science tools, North Vancouver, BC, Canada) for 2 minutes. The nerve were kept moist with 37℃ sterile saline solution throughout the surgical procedure. After the injury, the muscles were approximated, and the wound was closed by suturing the skin with 4/0 interrupted nylon sutures.[3]

5×10^5 h-ADMSCs were administered directly or indirectly 7 days after the surgery in 3ul PBS. The animals were sacrificed by CO_2 chamber euthanasia at 35days after the crush, and the sciatic nerves were dissected. Sciatic nerves stored at -80℃ for histological and immunohistochemical analyses.[10,11]

After surgery, cefazolin (25 mg/kg; Chong Kun Dang, Seoul, south Korea) injection twice daily for 5 days and cyclosporine (20 mg/kg; Chong Kun Dang, Seoul, South Korea) injection until the end of examination after transplantation of cells.

4. Dynamic plantar aesthesiometer

To estimate rat hind paw mechanical threshold, rats were adapt to the plastic box with a metal wire mesh table for 15 minutes. The mechanical stimulus was delivered to the plantar surface of the hind paw from below the floor of the test chamber by an automated testing device (Dynamic Plantar Aesthesiometer; Ugo Basile, Comerio, Varese, Italy).[12-14] A 0.5 mm-diameter steel rod of was pushed against the hind paw with ascending force of 0 to 50 g over a period of 20 seconds at a rate of 2.5 g/s. When the animal withdrew its hind paw, the mechanical stimulus automatically stopped, and the force at which the

animal withdrew its paw was recorded to the nearest 0.1 g. Animals were subjected to three consecutive trials with at least 5–6 minutes between the trials and averaged to select animals.

5. Sciatic functional index using foot print analysis

Walking track analysis was performed the day before the surgery and then weekly on the 7th, 14th, 21st, 28th, and 35th days post-operatively. Briefly, the plantar surface of the hind paws was dipped with red ink, and the rats were allowed to ambulate down a standard walking trace on which a strip of graph paper (60x15 cm) was displayed. Several measurements were taken from the footprints: (I) distance from the heel to the third toe, the print length (PL); (II) distance from the first to the fifth toe, the toe spread (TS); and (III) distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides.[15-17]

$$\text{SFI} = -38.3 \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \left(\frac{EIT - NIT}{NIT} \right) - 8.8$$

Measurements were made according to the Sciatic Functional Index (SFI), where 0 corresponds to normal function, and -100 corresponds to a total functional impairment of one side.

6. Evoked potential

Somatosensory evoked potentials (SSEPs) (n = 6 in each group) and motor evoked potentials (MEPs) (n = 6 in each group) were measured 5 weeks after transplantation. The rats were randomly assigned to the recording of SSEPs or MEPs without any indication of the extent of the lesion.[10,18,19] The animals were anesthetized with Rompun and Zoletil 50 Rompun (10 mg/kg, 25 mg/kg) before electrophysiological examinations.

A pulse generator (DG2A, Digitimer, Hertfordshire, England) was used to generate trigger pulses required for repetitive stimulation. It makes a constant output pulse, and a current stimulator (DS3, Digitimer, Hertfordshire, England) added amplitude to the signal.[20] A signal with desired pulsed current was given to the animal. For SSEP recording, left sciatic nerve was exposed and bipolar stimulating electrodes (730338, Harvard apparatus, Holliston, MA) was placed using a stereotaxic frame (WPI, Sarasota, FL) on the sensorimotor cortex at 2mm posterior to bregma and 2mm lateral to sagittal suture after craniectomy.[21] For MEP recording, the stimulating electrode was fixed at the temporalis   muscle and the recording electrode was inserted in the gastrocnemius muscle (with the injured sciatic nerve). The signal was amplified by a bio amplifier (axoclamp 900A, Molecular Devices, Union city, CA) with the specific parameters. The amplified wave was analyzed by software (qCLAMP 10, Molecular devices, Union city, CA)

A. Latency

B. Amplitude

Initial

N1

P1

Negative peak

Positive peak

Peak to peak
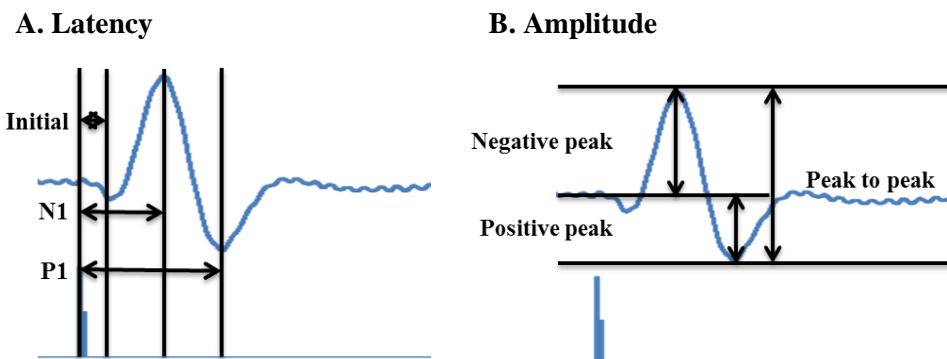
Figure 1. Point of measurement for electrophysiological assessment

A) The latency of the evoked potentials was measured from the onset of the first negative deflection (initial latency), from the peak of the first negative deflection (N1 latency), and from the peak of the positive deflection (P1 latency). B) The amplitudes was also measured from the peak of the first negative deflection to the baseline (negative peak amplitude), from the peak of the first positive deflection to the baseline (positive peak amplitude), and from the peak of the first negative deflection to the peak of the positive deflection (peak-to-peak amplitude)

7. Immunohistochemistry

The animals were sacrificed at 2 weeks and 5 weeks. For immunohistochemistry, L4-L6 DRGs and sciatic nerves were removed, fixed in 4% paraformaldehyde in PBS overnight, cryoprotected in 10, 20, and 30% sucrose, and cryostat-cut into 10um-thick section.

Sections were incubated overnight at 4℃ with a primary antibody with incubation buffer (1% bovine serum albumin, 1% normal donkey serum, 0.3% Trion® X-100, and 0.01% sodium azide in PBS) and followed by secondary species-specific fluorescent antibodies for 1 hour at room temperature. Then, sections were mounted with mounting solution (UltraCruz™ Mounting Medium, Santa Cruz Biotechnology, Dallas, Texas)[22,23]

A.

B.
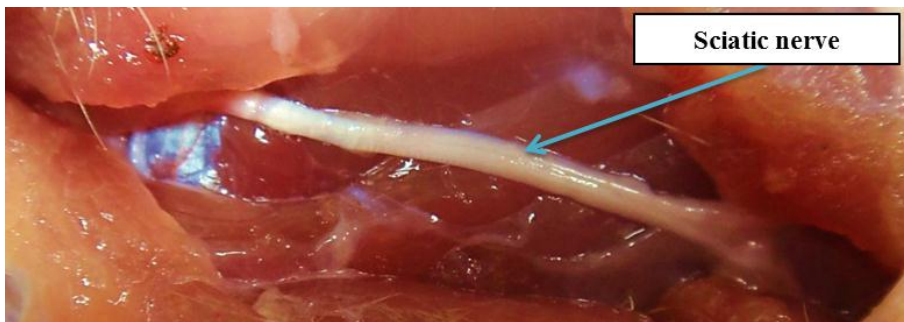
C.

Figure 2. Operative views.

A) Sciatic nerve crush injury model B) Sciatic nerve of rat treated with h-ADMSCs 4 weeks after transplantation. C) L4, L5, L6 dorsal root ganglion of rat treats treated with h-ADMSCs 4 weeks after transplantation.

Table 1. Antibodies used in the experiments

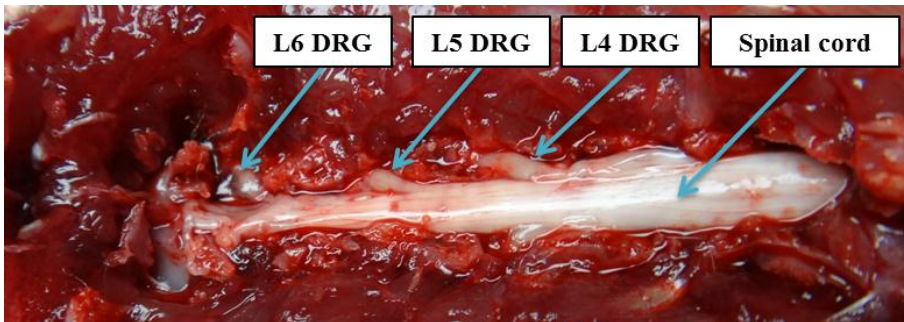| Antibody	Target	Dilution Ratio	Product Information
Mouse anti-CD44 (H-CAM)	Mesenchymal stem cells	1:500	Millipore CBL154-20UL
Mouse anti-CD90 (THY-1)	Mesenchymal stem cells	1:500	Millipore CBL415-20UL
Mouse anti-CD14	Leukocyte	1:500	Millipore MAB1219-20UL
Mouse anti-CD19	B lymphocyte	1:500	Millipore MAB1794-20UL
Rabbit anti-NCAM	Neuroectodermal cell line	1:250	Abcam Ab75813
Chicken anti-NSE	Early neuronal	1:5000	Abcam Ab134015
Rabbit anti-Nestin	Developing nervous system	1:500	Millipore ABD69
Rabbit anti-βⅢtubulin	Central and peripheral nervous system	1ug/ml	Abcam Ab18207
Mouse anti-NeuN	Terminal differentiation of neuron	1:100	Millipore MAB377
Rabbit anti-Neurofilament-66	Mature neuronal lineage	1:500	Millipore 04-1032
Mouse anti-GFAP	Mature glial cell (astrocyte)	1:100	Abcam Ab10062

Rabbit anti-S100	Astrocyte, Shwann cell	1:40	Abcam Ab52642
Mouse anti-CNPase	Oligodendrocyte	1:200	Abcam Ab6319
Chicken anti-vimentin	Peripheral nerve (Schwann)	1:5000	Millipore AB5733
Mouse anti-human nuclei	Nuclei of all human cell type	1:200	Millipore MAB1281
Mouse anti-CGRP	Inflammation marker	1:100	Abcam Ab81887
FITC-Donkey anti-mouse IgG(H+L)	Mouse primary antibody	1:100	Jackson 751-095-151
Cy^{TM}3-Donkey anti-mouse IgG(H+L)	Mouse primary antibody	1:500	Jackson 715-164-151
FITC-Donkey anti-rabbit IgG(H+L)	Rabbit primary antibody	1:100	Jackson 711-095-152
Cy^{TM}3-Donkey anti-rabbit IgG(H+L)	Rabbit primary antibody	1:500	Jackson 711-165-152
CyT^{M}3-Donkey anti-chicken IgG(H+L)	Chicken primary antibody	1:500	Jackson 703-165-155

8. Luxol fast blue staining, toluidine blue staining and electron microscopy

Luxol Fast Blue Staining. The sectioned tissue was stained by Luxol Fast Blue (LFB) to examine general morphology and the extent of demyelination. At first, the sections hydrated and then were stained with LFB solution (Luxol® fast blue solution, L0294) at 60℃ overnight. Using lithium carbonate solution and 70% alcohol, excess staining and permount were removed.[24]

Electron microscopy and toluidine blue staining. The regenerated nerves were harvested and immersion-fixed in Karnovsky's solution (2% Glutaraldehyde, 2% Paraformaldehyde, 0.1M Phosphate buffer) overnight at 4℃. The specimens had post fixation in 1% OsO_4 solution, dehydrated with an ascending series of ethanol followed by infiltration with propylene oxide. Specimens were then embedded in mixture solution including EPON mixture (EPON 812, MNA, DDSA, DMP30) and propylene oxide. Semithin sections of 250~300nm were cut then stained with 0.5% toluidine blue. Thin section for transmission electron microscopy were placed on glider thin bar mesh grids and double stained with uranyl acetate and lead citrate.[25]

Ⅲ. Results

1. Human adipose-derived mesenchymal stem cells cultures

The isolated human adipose tissue cells, which were cultured in alpha-MEM medium, were positive for CD44 and CD 90, but negative for CD14 and CD19. It tested using immunofluorescence staining (Fig. 3).

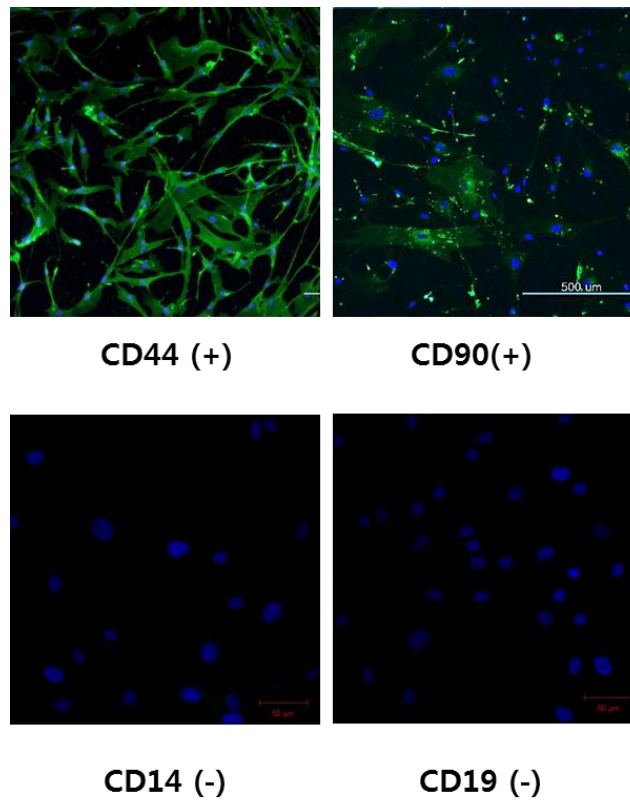**Figure 3**. Characterization of h-ADMSCs

Isolated h-ADMSCs approached with confluency, showing spindle shape-like fibroblastic morphology. In addition, they have mesenchymal stem cell markers CD44 and CD99 and were not reactive at CD14 and CD19.

2. Differentiation to a neuronal cell phenotype using two different methods

In our study, we used two protocols for confirming neuronal differentiation of h-ADMSCs. The early neural marker Tuj1 was positively expressed by cells by serum-free induction method. However, astrocyte markers, GFAP and oligoendrocyte markers, and CNPase were positively expressed (Fig. 4A,B). On the other hand, early neural markers Tuj1 and Nestin were positively expressed by chemical induction method. In addition, astrocyte markers, S100 oligoendrocyte markers, CNPase, and peripheral nerve markers vimentin were positively expressed (Fig. 4C). These two method show that h-ADMSCs have a neuronal differentiation ability.

A

ADMSC   STEP1   Neurosphere   STEP2   Neuronal cell
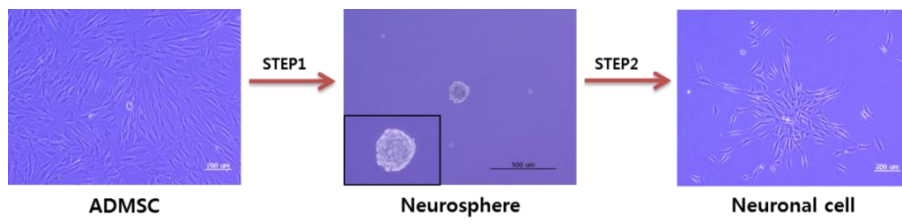
B
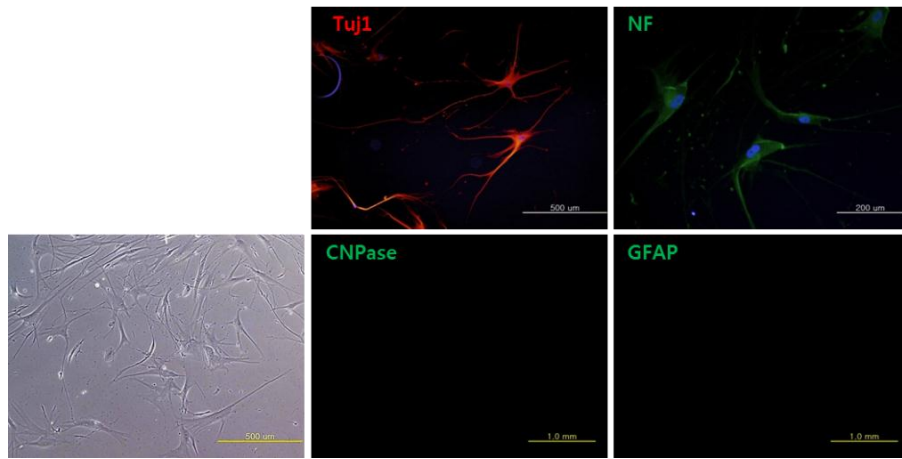
C
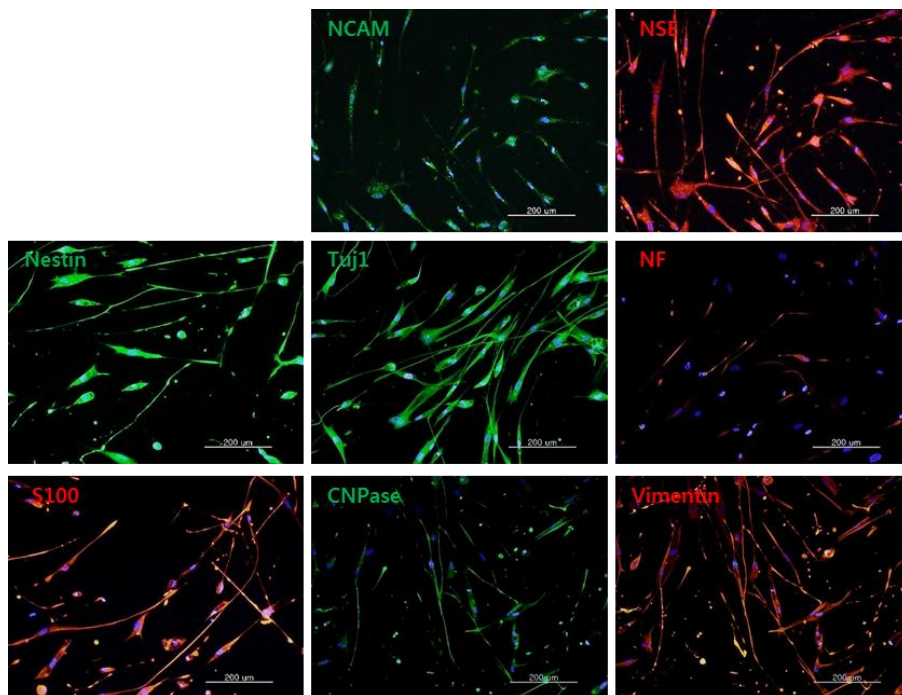
Figure 4. h-ADMSCs differentiate to neuronal cell phenotype.

A) Scheme of neuronal differentiation in serum free induction. B) Inflected h-ADMSCs differentiated into Tuj1-positive neuron (red) and NF-positive mature neuron (green), but were negative for CNPase and GFAP. C) Inducted h-ADMSCs differentiated into NCAM positive neuroectodermal cell line (green), NSE positive early neuronal cell line (red), Nestine, Tuj1 positive neuron (green), S100 positive astrocyte (red), CNPse positive oligodendrocyte (green), and vimentine positive Schwann cell (red)

3. h-ADMSCs relieve mechanical allodynia

When tested with dynamic plantar aesthesiometer, mean paw withdrawal threshold of the PBS epineural soaking group was 13.12 ± 0.72, 16.13 ± 1.81, 16.94 ± 1.24, 19.77 ± 1.34, and 21.18 ± 1.90g; PBS intraneural injection group was 13.12 ± 0.72, 14.19 ± 1.31, 16.09 ± 1.16, 16.42 ± 1.45, and 18.94 ± 1.94; h-ADMSCs epineural soaking group was 13.12 ± 0.72, 17.76 ± 1.60, 17.98 ± 1.29, 22.97 ± 1.06, and 24.05 ± 1.69; and h-ADMSCs intraneural injection group was 13.12 ± 0.72, 14.92 ± 1.02, 17.02 ± 1.66, and 21.53 ± 1.76g. Paw withdrawal thresholds were recorded at various time intervals, with values ranging from 31.37 ± 0.37g to 34.73 ± 0.32 g in sham operated animals. h-ADMSCs epineural soaking transplantation showed the best convalescence, while there was no significant difference between the h-ADMSCs intraneural injection and PBS epineural soaking groups (Fig. 5A).

A.
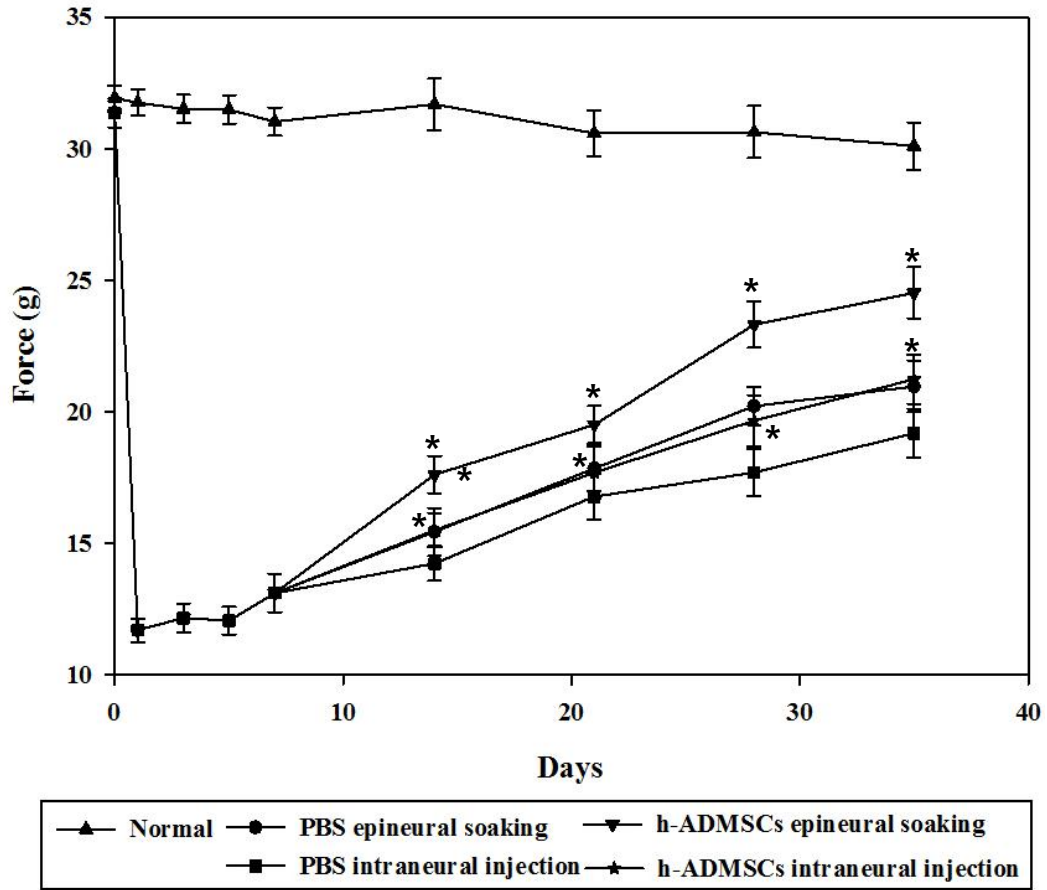
B.
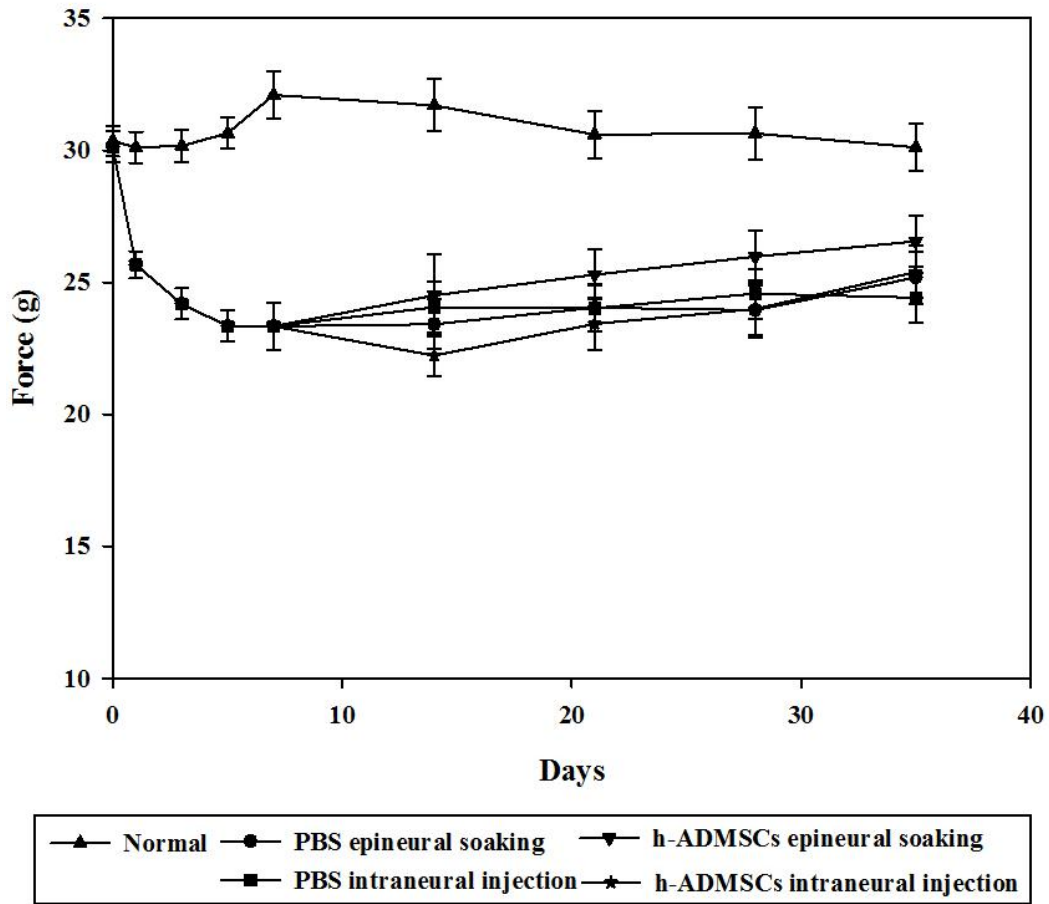
Figure 5. Pain reducing effect of h-ADMSCs on mechanical allodynia

A) Result of dynamic plantar aesthesiometer test for left leg. B) Result of dynamic plantar aesthesiometer test for right leg. (*P<0.05 vs PBS intraneural injection)

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4. Assessment of allodynia using dynamic plantar aesthesiometer

The data show each group's SFI score at each time point. One week later, the SFI score was -51.98±2.51 in the injured group and -6.25±1.22 in the normal group. This showed a difference 1 week later after transplantation (PBS epineural soaking: -32.41±1.42; PBS intraneural injection: -33.05±1.27; h-ADMSCs epineural soaking: -27.14±1.26; and h-ADMSCs intraneural injection: -30.52±1.53), which was maintained until the end of experimentation (PBS epineural soaking: -13.29±2.05; PBS intraneural injection: -18.10±0.78; h-ADMSCs epineural soaking: -9.18±1.36; and h-ADMSCs intraneural injection: -14.16±2.11). Sciatic nerve damage led to a decrease of TS and IT length and increase of PI length by release of paw flexion. After 35 days following injury, the h-ADMSCs epineural soaking transplantation group showed recovery matching the normal group (Fig. 6).

A.

B.

| Normal | 1 week (crush) | PBS epidural soaking | PBS intraneural injection | h-ADMSCs epidural soaking | h-ADMSCs intraneural injection |
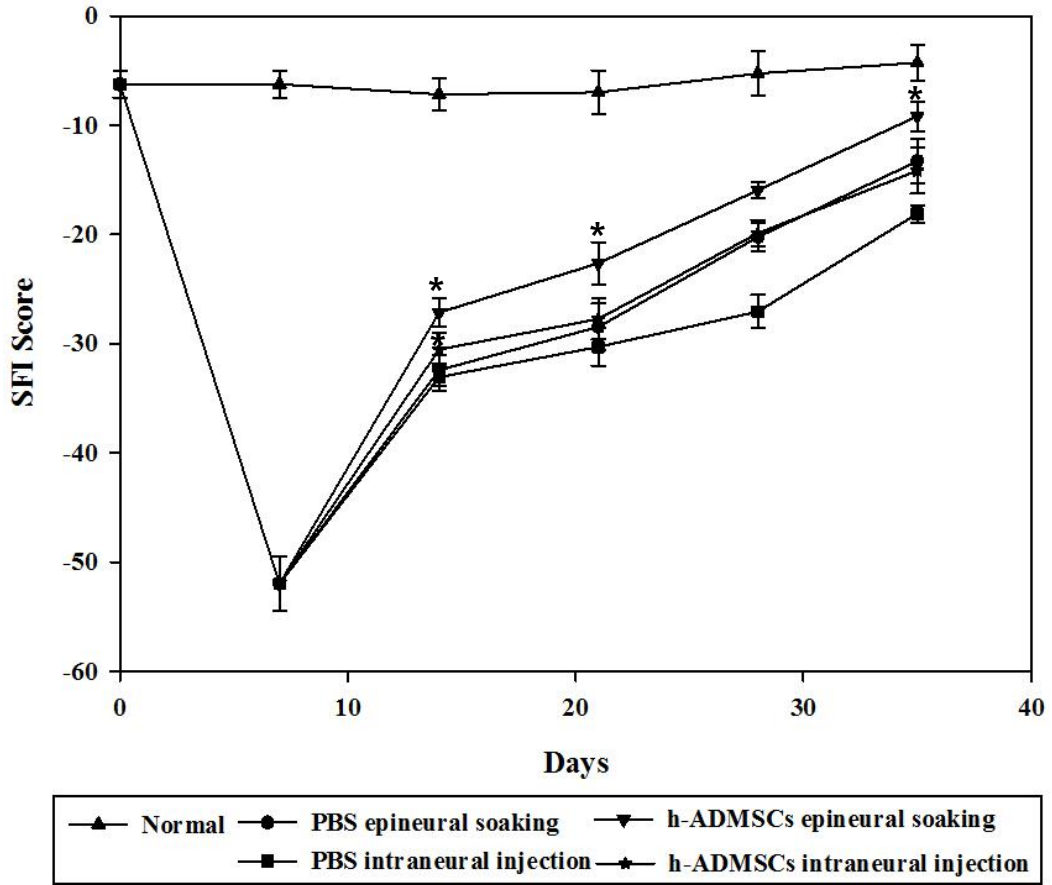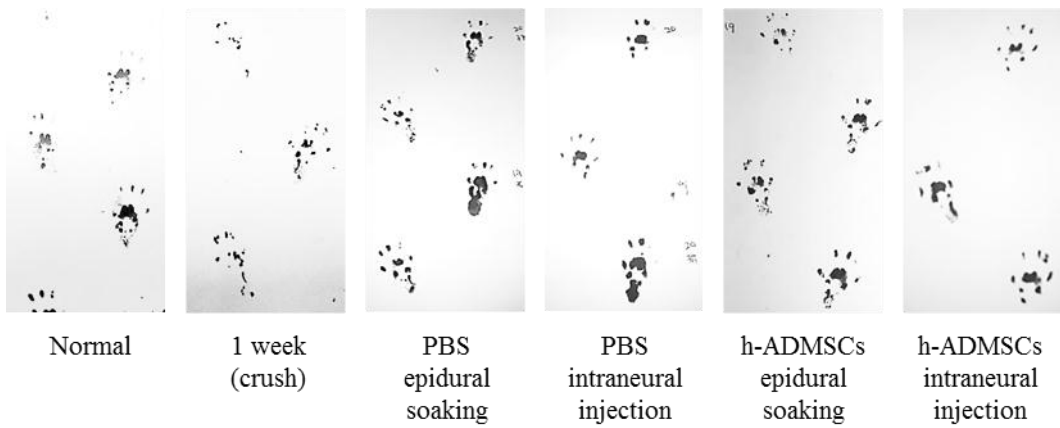
Figure 6. Motor functional recovery is accelerated after h-ADMSCs treatment.

A,B) The disease curve was measured weekly by SFI in each group. Improved plantar flexion and toe spread are visible in track obtained from the h-ADMSCs epineural soaking group as compared with the PBS intraneural injected group 2 weeks after injury. (*$P<0.05$ vs PBS intraneural injection)

5. Electrophysiological assessment

SSEPs and MEPs were recorded 4 weeks after transplantation. The curves of MEPs have patterns of positive-negative-positive-negative potentials, and SSEPs have positive-negative-positive potentials (Fig. 7A,B). Sciatic nerve crush injury rat transplanted with h-ADMSCs epineural soaking group showed shortened initial latency compared with the intraneural injection group. However, it did not show any difference with the PBS epineural soaking group, though amplitude of MEP in the h-ADMSCs epineural soaking group show increased amplitude compared with other groups (Table. 2). The h-ADMSCs epineural soaking group showed shortened initial latency and N1 and P1 latency of SSEPs. Overall, the amplitude showed a clear tendency to be increased in other groups (Table. 3).

A. Motor evoked potential (MEP)

| | Normal | h-ADMSCs epineural soaking | h-ADMSCs Intraneural injection | PBS epineural soaking | PBS Intraneural injection |

B. Somatosensory evoked potential (SSEP)

| | Normal | h-ADMSCs epineural soaking | h-ADMSCs Intraneural injection | PBS epineural soaking | PBS Intraneural injection |
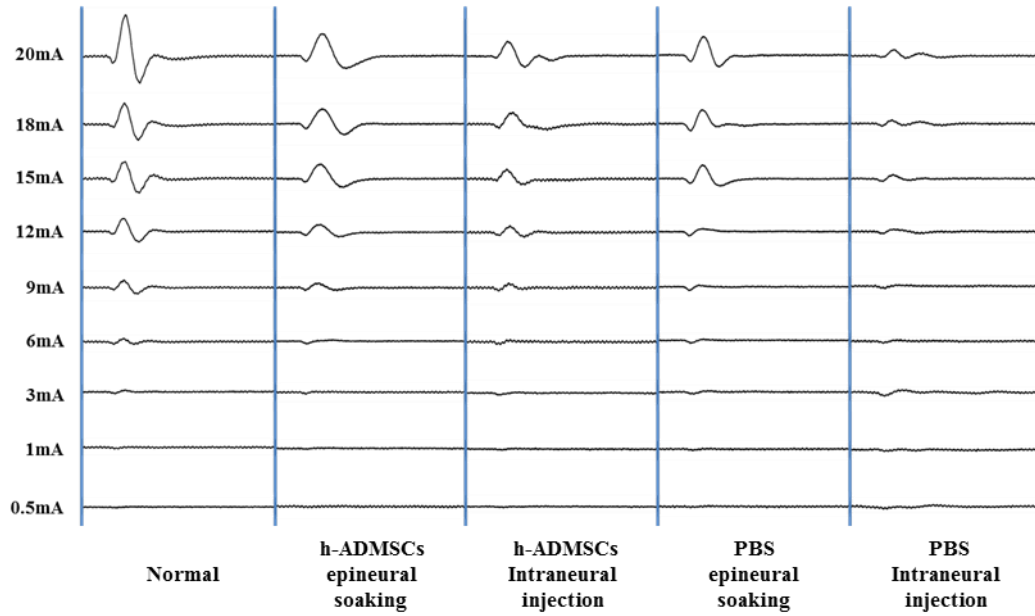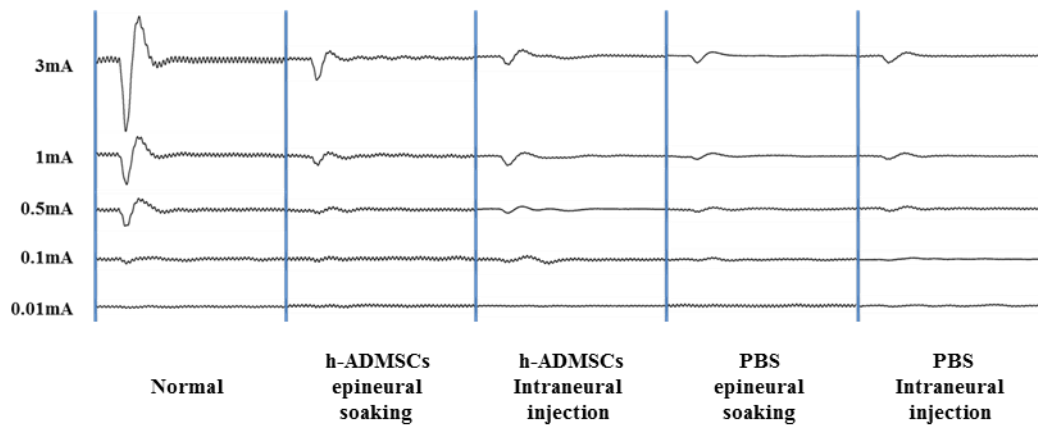
Figure 7. Electrophysiological studies after transplantation of h-ADMSCs.

A) Sciatic nerve crush injury rat transplanted with h-ADMSCs epineural soaking group showed shortened initial latency similar to that in the normal group, though N1 latency and P1 latency did not. In addition, amplitude of MEP significantly increased in the h-ADMSCs epineural soaking group. B) The h-ADMSCs epineural soaking group showed shortened initial latency and N1and P1 latency. Overall, the amplitude showed a clear tendency to increase in h-ADMSCs epineural soaking group the other groups.

Table 2. Motor evoked potential in rat treated with h-ADMSCs 4 weeks after transplantation.

MEP	Latency (msec)			Amplitude (mV)		
	Initial	N1	P1	Negative peak	Positive peak	Peak to peak
Normal	0.11±0.00	0.16±0.00	0.24±0.02	21.27±3.60	14.88±2.56	36.15±5.63
h-ADMSCs epineural soaking	9.56±5.12	55.86±12.78	148.44±28.39	12.77±3.12	12.12±2.86*	24.89±5.95
h-ADMSCs intraneural injection	13.65±7.77	70.85±11.89	183.85±20.10	4.68±1.18	7.32±1.81	12.00±1.96
PBS epineural soaking	12.54±3.49	64.83±10.66	145.16±15.91	9.03±3.29	5.67±2.06	14.70±5.34
PBS intraneural injection	21.35±7.68	83.88±8.31	180.28±19.18	4.15±0.92	3.29±0.80	7.45±1.70

Values are mean ±SE. Input power is 20mA and the number of experiments is 8 MEP, Motor evoked potential; h-ADMSCs, human adipose derived mesenchymal stem cells; PBS, phosphate-buffered saline. (*$P<0.05$ vs PBS intraneural injection)

Table 3. Somatosensory evoked potential in rat treated with h-ADMSCs 4 weeks after transplantation.

SSEP	Latency (msec)			Amplitude (mV)		
	Initial	**N1**	**P1**	**Positive peak**	**Negative peak**	**Peak to peak**
Normal	3.90±1.09	28.07±0.66	79.68±3.62	86.23±37.81	59.60±12.09	145.83±36.68
h-ADMSCs epineural soaking	4.50±1.05	39.74±7.58	103.20±9.85	19.82±8.21	27.27±12.00	47.08±20.21
h-ADMSCs intraneural injection	5.35±1.09	41.06±8.01	108.40±6.58	10.23±3.58	11.71±3.46	21.94±6.79
PBS epineural soaking	5.49±1.48	46.00±11.10	117.16±26.77	8.28±2.24	9.75±2.73	18.03±4.94
PBS intraneural injection	7.00±1.35	42.94±7.77	112.24±7.85	8.04±3.33	6.89±2.24	14.94±5.54

Values are mean ±SE. Input power is 3mA, and the number of experiment experiments number is 8 SSEP, somatosensory evoked potential; h-ADMSCs, human adipose derived mesenchymal stem cells; PBS, phosphate-buffered saline. (*$P<0.05$ vs PBS intraneural injection)

6. Transplanted h-ADMSCs were differentiated to a neuronal lineage

Transplanted cells derived from h-ADMSCs were confirmed by human nuclei antibody. Double immunoreactivity for human nuclei and neuronal markers showed that transplanted cells differentiated into neurons and oligodendrocytes. However, definite astrocyte marker GFAP was not seen. In the epineural soaking group, cells were almost differentiated from Schwann cells; in the intraneural injected group, cells were differentiated from neurons. (Fig. 8)
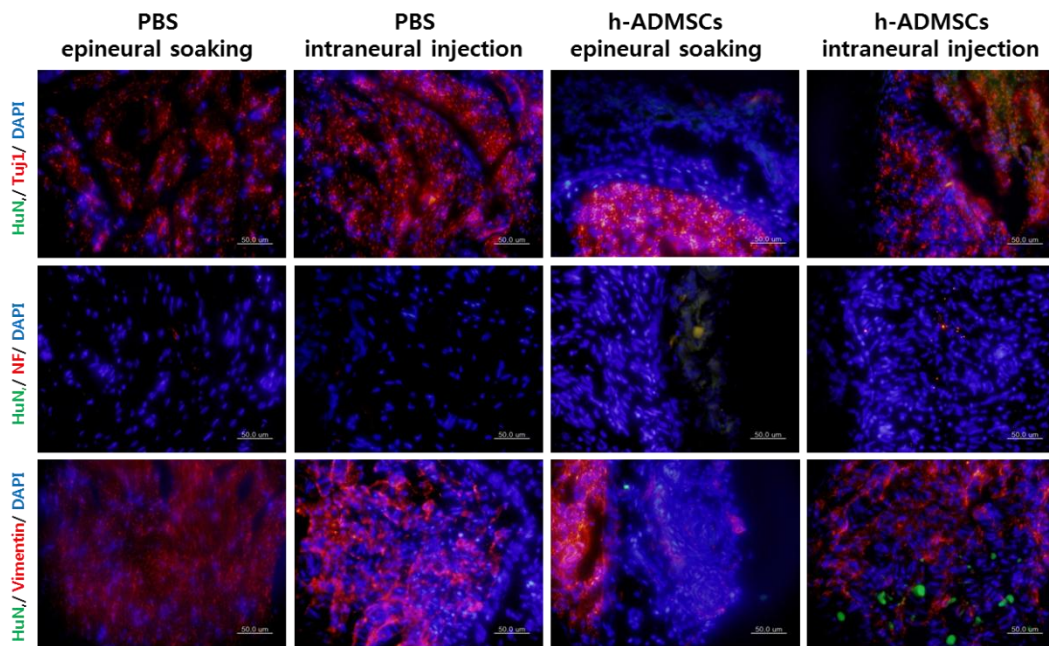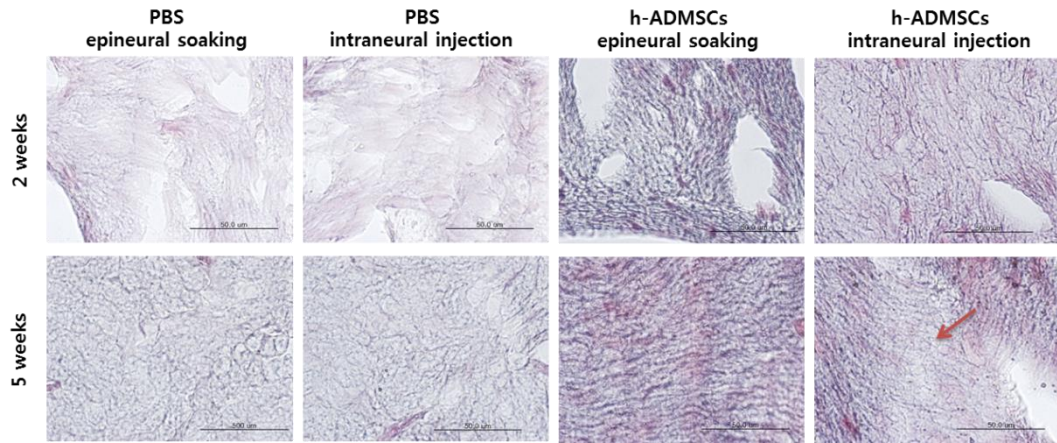
Figure 8. Immunohistochemistry showing recovery of neuronal regeneration

Immunostaining images 4 weeks after transplant of h-ADMSCs. Sciatic nerve sections were stained with anti-human nuclei (green), anti-Tuj1 of neuron (red), anti-NF of mature neuron (red), and anti-vimentin of Schwann cells (red).

7. Transplanted h-ADMSCs promote the remyelination of the injured nerve

In LFB staining, morphological changes were observed following the h-ADMSCs transplanted group. In the control group, the PBS was not repaired after 5 weeks. In the h-ADMSCs transplanted group, tissue recovery and remyelination occurred. However, in the h-ADMSCs intraneural injecting group, there remained a needle track that was not restored (Fig. 9A). In toluidine blue staining, the normal nerve showed numerous large well-myelinated axons. The h-ADMSCs transplanted group showed almost perfectly recovery similar to normal tissue, unlike with the PBS group, which showed abnormal shape of nerves as circular bundles (Fig. 9B). In transmission electron microscopic examination, transplantation of the h-ADMSCs enhanced structural maturation and organization of the injured sciatic nerve, characterized by better myelination and less fibrosis, which corroborates the findings of the toluidine blue staining. In the PBS groups with nerve injury, invasion of fibroblast or poorly-shape myelinated axon were observed (Fig. 9B).

A.

| | PBS epineural soaking | PBS intraneural injection | h-ADMSCs epineural soaking | h-ADMSCs intraneural injection |

B.
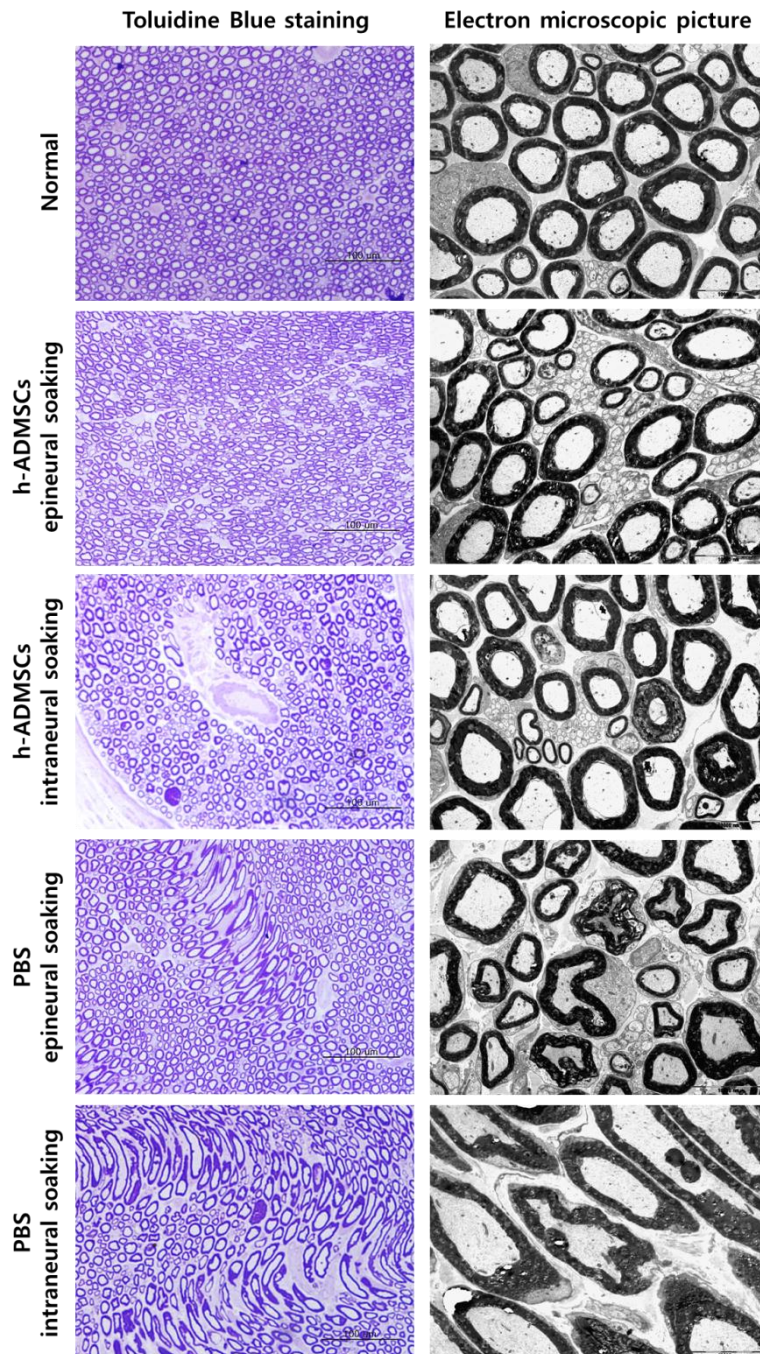
Figure 9. Microscopic findings showing the remyelination of the injured sciatic nerve following h-ADMSCs transplantation

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A) Luxol fast blue staining. h-ADMSCs epineural transplantation group tissue recovered faster than that of any other group. B) Toluidine blue staining. h-ADMSCs transplantation made perfectly recovery, as normal tissue. Transmission electron microscopic examination. h-ADMSCs transplantation recovered as normal tissue. It checks observation of myelin sheath's thickness and shape.

VI. Discussion

Stem cell therapy has been showing a promising method for the treatment of neurological disorders affecting central and peripheral nerve system. At these diseases, embryonic stem cells and adult stem cell is provided by useful tools. Using embryonic stem cells in clinical situations is restricted due to cell regulations and technical and ethical considerations involved in the genetic manipulation of human ESCs, even though these cells are, theoretically, highly beneficial.[26] Adult stem cells, On the other hand, overcome the safety and ethical controversy, it also have differentiation potency to a specific organ and placiticy of trans-differentiation. As a result, cell therapy method using an adult stem cells is available to variable diseases. Among others, bone marrow derived stem cell is most popular cell to treat various neuronal diseases. It could have successful result for many research, and now in clinical trial, but obtaining of enough cells is limited at once and the isolation process need invasive method.[27-29] There are several reasons for why adipose tissue is ideal source for stem cells. It can be obtained high amounts of adipose stem cells with minimum risk like surgery. Also, quantity of stem cells from adipose tissue is 5000 times than isolated bone marrow stem cells. Especially, human adipose stem cells express cell surface antigen that are hardly detected by T cells and therefore h-ADMSCc can be transplanted autograft, allograft, and even xenograft.[30,31] Thus, we choose h-ADMSCs in this research.

There are ways how to deliver stem cell to nerve system, like intrathecal delivery, intralesional delivery, intravenous delivery, intraarterial delivery. The intralesional injection group showed the best engraftment, so the present study attempted to carry out direct transplantation cell therapy at injury region, which is known as an efficacious nerve tissue treatment. However it could bring secondary damage on treatment site.[32] Therefore, we investigated the effect of indirect transplantation is effective and stable.

In our neuropathic pain model, h-ADMSCs epineural soaking group showed differences with PBS treatment group at dynamic plantar aesthesiometer assay. SFI

analysis shows the difference in the h-ADMSCs epineural soaking group and PBS intraneural injection and epineural soaking group at 1 weeks after cell transplantation up to the end of the experiment. (Fig. 6A) The electrophysiology result also agrees with other experiment results. Particularly, amplitude of MEPs were significantly improved in h-ADMSCs epineural soaking group, compared with PBS intraneural injection group (Table. 2,3) Furthermore, histological assessment showed that h-ADMSCs transplantation induced recovery of injured sciatic nerve and remyelination at damage tissue. However, there were no differences between h-ADMSCs direct transplantation and PBS indirect transplantation groups at all experiment. These result suggested that secondary damage due to injection injury or injection pressure by intralesional delivery may be a reducing h-ADMSCs therapeutic effect.

The efficacy and viability of the transplanted cells were observed according to different transplantation routes. In the present study, mesenchymal stem cells what we used have been demonstrated to have an ability to move to the injured site and trans-differentiate into neural lineage cells.[33] Bakshi et al. reported that, when MSCs were injected into the cerebrospinal fluid three times, they observed a 2.3-fold higher engraftment rate than after only one injection in lesion site. And our priminary study, after intralesional transplantation group, engrafted cells were concentrated around the injection point, which induced mass effect on adjacent normal tissues and restricted dispersion to the damaged regions.[32] Others have also reported that the needle used in intralesional transplantation group can cause secondary damage to the injured spinal cord and the volume of the injected cells can result in increased pressure inside the spinal cord.[32] Furthermore, when we consider the application of intralesional transplantation in clinical practice, a patient must undergo general anesthesia and a mandatory surgical procedure, which can be obstacles for debilitated patents who have weakened immune systems. However, Takahashi et al, ILT would be assumed to be the most effective approach any other method. Also, Nakamura et al revealed that NS/PCs injected into non-injury sites such as

by intravenous or intrathecal administration did not engraft to the injury site in sufficient numbers, but instead were often "trapped" in the lungs and kidneys. They concluded that intralesional application might be the most effective and reliable method for transplanting cells.

Interestingly in our experiment contrast leg response at low force compared with non-surgery. This can explain why mirror-image allodynia occurs from the contralateral to the injured region of trauma or inflammation. Some researchers assert glial and proinflammatory cytokine action caused mirror-image neuropathic pain. However, the accurate mechanism of this phenomenon remains unknown (Fig. 5B)

It had been reported the rat stem cell transplantation in defected sciatic nerve induce peripheral nerve regeneration by Murakami. Apart from that, many investigations reported that adipose tissue derived stem cells transplantation could improve the injured tissues by morphologically, histologically and electromyographically.[34] However this doesn't mean that transplanted cell differentiate to neuronal cell. Also, several studies suggest that stem cells may contribute to the repair of injured tissue by releasing a large number of trophic factors, which can influence injured region, promote and maintain axonal sprouting and myelination of fibers.

Therefore, further study need to clear about the paracrine effect of transplanted cells on injury site. Also, the next experiment will be a mechanisms at the spinal cord and dorsal root ganglia, which undergo a response after any nerve injury possibly induce by variable neuropathic pain therapy.

VII. Conclusion

In this research, h-ADMSCs epineural soaking showed significant pain reduction, confirmed by dynamic plantar aesthesiometer, SFI analysis, and electrophysiological assessment. It is assumed that direction injection might induce additional trauma. Thus, h-ADMSCs indirect transplantation may be the safest and most effective method for delivering stem cells and reduction neuropathic pain in peripheral nerve injury.

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국문요약

백서의 신경병증성 통증 모델에서 인간중간엽줄기세포의 통증감소 및 신경재생효과

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중간엽줄기세포는 외배엽으로의 분화를 이용한 신경조직치료제로 사용 될 수 있다. 오늘날 손상 신경조직에 대한 중간엽줄기세포치료는 지역 이식만이 이루어 지고 있다. 하지만 신경조직 손상시 신경 조직에 직접적인 특이적 이식은 신경의 또 다른 손상을 야기할 수 있다. 이러한 이유로 본 실험에서는 두 가지 방법을 이용한 인간 지방 조직에서 유래한 중간엽줄기세포의 분화능을 확인하고 백서의 급성 신경 손상을 야기하는 좌골신경의 손상 일주일 후 세포의 간접적 이식을 통해 두드러진 기능적 회복을 돕는 것을 확인했다. 또한 인간 지방 조직에서 유래한 중간엽줄기세포를 이용한 좌골신경의 치료는 신경 조직의 재생과 운동력 향상을 향상 시키며, 통증을 완화시킴을 알 수 있었다.

이와 같은 결과를 통해 본 연구에서는 인간 지방 조직 유래 중배엽줄기세포의 간접적인 이식은 신경 재생을 촉진하고 통증을 완화시키며 재수초화를 촉진하는 것을 통해 급성 신경 손상 모델에서 확실한 치료효과를 보여주는 것을 통해 신경병증성 통증 질환에 대한 치료 방법을 제시 할 수 있을 것으로 사료 된다.