Effects of Human Mesenchymal Stem Cell Transplantation Combined with Polymer on Functional Recovery Following Spinal Cord Hemisection

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Effects of Human Mesenchymal Stem Cell Transplantation Combined with Polymer on Functional Recovery Following Spinal Cord Hemisection

Directed by Professor Bae Hwan Lee

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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This certifies that the Master's Thesis of Ji Soo Choi is approved.

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석사생활을 시작할 때부터 석사논문을 마칠 때까지 아낌없는 충고와 도움을 주셨던 이경희 선생님, 부족한 후배를 항상 이뿌게 봐준 김은정 선생님과 세정이 누나에게도 고마움을 전합니다. 덤벙대는 저를 사소한 일에서부터 항상 일일이 챙겨준 우리 전기생리학연구실의 명훈, 은진, 나영, 경하와 2층의 모든 식구들에게도 고맙다는 말을 하고 싶습니다. 어려울 때 함께 하고 서로에게 든든한 버팀목이 되어준 오랜 친구 상욱이, 준형이, 호섭이에게도 고맙다는 말을 전하고 싶습니다. 곁에 없지만 마음으로 응원해준 명호, 명섭, 승재, 용우에게도 고마움을 전합니다.

늘 사랑으로 지켜봐 주시고 격려해 주신 부모님과 형에게 감사의 말씀을 전해드리고 싶습니다. 아울러 항상 건강하시길 기원합니다.

많은 분들의 도움이 없었다면 지금의 저는 없었을 것입니다. 저의 부족함을 깨닫는 시간들이었지만 그렇기에 또한 더 겸손해질 수 있었고 성장할 수 있었음에 감사드립니다. 훌륭한 연구자가 되기 위해 더 매진할 것을 다짐하며 부족한 논문을 마치려 합니다.

저 자 씀

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Effects of human mesenchymal stem cell transplantation combined with polymer on functional recovery following spinal cord hemisection

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(Directed by Professor Bae Hwan Lee)

Spinal cord injury (SCI) results in loss of motor and sensory function. Following injury to central nervous tissues, spontaneous axon regeneration of damaged neurons is restrictive. Cell transplantation is considered to be the most effective way to repair SCI. Over the past few years, many attempts have been made in animals to produce cellular regeneration in the spinal cord using transplantation of different cell types. Recently, transplantation of mesenchymal stem cells (MSCs) has been considered as a potential approach for enhancing nerve regeneration, avoiding ethical problems. As SCI is a complex pathological entity, the treatment of SCI requires a multipronged approach. Application of biosynthetic polymer for spinal cord repair has recently been reported. The purpose of the present study was to investigate the functional recovery and the therapeutic potential of human MSCs (hMSCs) and polymer

when they transplanted into the spinal cord hemisection injury.

Adult male Sprague-Dawley rats were subjected to a hemisection at thoracic spinal cord level (T11) and then divided into three groups. Two groups of rats underwent partial thoracic hemisection injury followed by implantation of either polymer only or polymer with hMSCs. The hemisection group received no treatment. Behavioral (motor and pain test), electrophysiological (MEP: motor evoked potential, SSEP: somatosensory evoked potential), and immunohistochemical studies were performed to observe the improvement of functional recovery.

The BBB locomotion scores were showed significant improvement in polymer with hMSC-transplanted group compared with hemisection only group since 5 weeks after the transplantation. From the electrophysiologic study, SSEPs have shown significant difference in P1-peaks latency when polymer with hMSC-transplanted group was compared with hemisection only group. In the MEPs recording, latency had no significant difference among the groups, but P1-peak amplitudes in polymer with hMSCs transplanted group were significantly higher than hemisection only group. In the immunohistochemical study, the ß-gal positive cells were located in the injured and the adjacent sites after hMSCs transplantation. hMSCs survived in injured and the adjacent spinal cord were differentiatied into different types of cells, such as neuron, astrocyte and oligodendrocyte. These data suggest that MSC-transplantation may play an important role in functional recovery and axonal regeneration, and be potential therapeutic strategies for SCI.

Key Words: Spinal cord injury, mesenchymal stem cells, behavior, transplantation, electrophysiology, polymer

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I. INTRODUCTION

Spinal cord injury (SCI) usually results in long-lasting deficits, involving loss of motor and sensory function. Following injury to central nervous tissues, spontaneous axonal regeneration of damaged neurons is restrictive. The failure of regeneration is attributed to the nonpermissive environment of the damaged adult mammalian spinal cord, the milieu of which is formed of astrocyte-derived inhibitory molecules in the scar tissue, myelin components of oligodendrocytes interfering with the regeneration of axons and lack of trophic support for axotomized neurons, and the intrinsic neuronal changes, including cell atrophy and death after axotomy.[1,2] Therefore, effective repair strategies for SCI require the creation of a permissive environment within the injured spinal cord that protects damaged neurons from the effects of secondary injury and also facilitates axonal regeneration. A variety of experimental strategies including stem cell transplantation are emerging to promote regeneration of the

injured spinal cord.

Cell transplantation is one of the most promising therapeutic approaches for treating SCI. Bone marrow is composed of two types of cells, hematopoietic cells and stroma cells.[3] The marrow stroma is a complex tissue. Although they were initially thought to be primarily hematopoietic support cells, the marrow stromal cells have been shown to have the potential to differentiate into a variety of mesenchymal cell types, including bone,[4,5] cartilage,[5,6] muscle,[7] glia and neurons.[8-11] Also, recent studies proposed a more extensive differentiation potential of MSCs showing phenotypic plasticity that appears to cross the boundaries of the traditional germ layers including cardiac cells,[23] skeletal muscle,[24] and neural cells.[25] Whether this apparent plasticity represents transdifferentiation, a pool of persistent pluripotent stem cells, cell fusion, or artifacts of culturing remains controversial.[26-29] Bone marrow-derived mesenchymal stem cells (MSCs) constitute an alternative source of pluripotent stem cells. Transplantation of MSCs into cerebral ischemia models has demonstrated that it reduced lesion size and improved functional outcome.[15-18] MSCs were reported to have an ability to migrate throughout the forebrain and cerebellum and to integrate into the parenchyma after injection into the corpus striatum in rodents.[12] Moreover, MSCs transplantation into demyelinated[19,20,21] and x-irradiation SCI models[22] had demonstrated remyelination and improved functional recovery, respectively.

The treatment of SCI is complex with many barriers to the patient's recovery, including limited ability of the adult CNS to regenerate, excessive scarring, and cavitation after injury.[30] Therefore, repair of the injured human spinal cord in many cases will require not only neuronal survival and axonal growth and remyelination[31] but also reconnection across the trauma cavity by means of bridging grafts. From a clinical point of view, the limited access to autologous donor material and the immunological problems associated with allograft rejection have prompted a search for artificial biomaterials that may be implanted as bridges in the injured spinal cord.

Investigators have attempted to overcome some of these barriers by using implantable scaffolds made of different materials, including biodegradable polymers[13,14] and other nondegradable materials such as polycarbonate and hydrogel.[13]

In the present study, we investigated the efficacy of polymer and hMSC transplantation into the injured spinal cord. To support this study, the BBB open field locomotion[37] and von Frey tests were used to evaluate the degree of functional recovery following different treatments after SCI. Electrophysiological studies of somatosensory and motor evoked potentials (SSEPs and MEPs) activities was used to determine the axonal conduction. And immunohistochemical study was used to observe the trends of cells that have undergone migration and differentiation.

II. MATERIALS AND METHODS

1. Preparation of human mesenchymal stem cells

In this study, we used LacZ-expressing hMSCs. LacZ-expressing hMSCs were cultured in 100-mm tissue culture Petri dish (Falcon, Flanklin Lakes, NJ, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated standard fetal bovine serum (FBS) (HyClone, Logan, Utah, USA) and penicillin-streptomycin (GIBCO, Grand Island, NY, USA), 0.01% bFGF (Sigma, Saint Louis, Missouri, USA). And hMSCs were incubated at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed every 2 days. After reaching confluence, they were harvested.

2. Spinal cord hemisection injury and transplantation

Male Sprague-Dawley rats (300~350g) were used for this study. Animals were housed in group of four in plastic cages with soft bedding under a 12/12 h reversed light and dark cycle. Food and water were available *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Under an operating microscope, a laminectomy was conducted at vertebral levels of T10-11. After opening the dura mater, followed by a lateral hemisection at the T11 spinal cord level by creating a 3mm-long longitudinal cut along the midline of the cord with micro-scissor (Fig. 1). After hemostasis was achieved, polymer (BD™ PuraMatrix™ Peptide Hydrogel, Qume Drive San Jose, CA USA) or polymer with hMSCs (4×10^5) were implanted into the cavity. Following spinal

cord hemisection, animals were divided into three groups (hemisection only, polymer only transplanted, and polymer with hMSCs transplanted groups). Muscle and fascia were sutured and the skin was closed with silk suture. During recovery, rectal temperature was maintained at 37℃ by a feedback regulated heating pad. Postoperative nursing care included bladder expression two times a day. Prophylactic gentamycin sulfate (1mg/kg, i.m) was regularly administered for a week. And Cyclosporine A (1mg/100gm, i.p) was injected daily since 2 days before the transplantation.

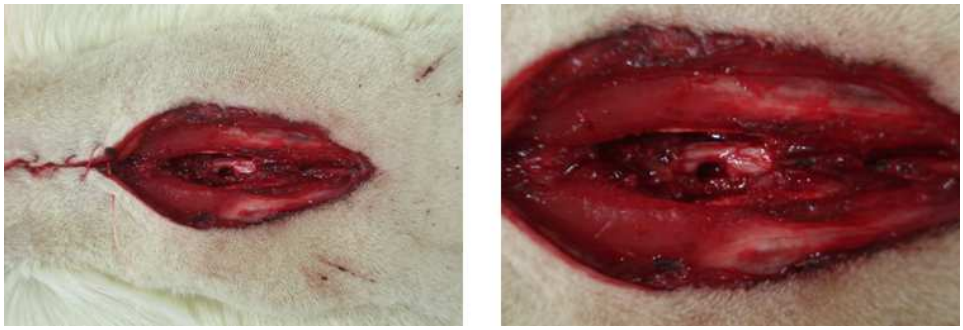

Fig. 1. Photos of spinal cord hemisection injury. Hemisection injury was created a 3mm-long longitudinal cut along the midline of the cord at the T11 level by micro-scissor.

3. Behavioral study

Behavioral testing was done blindly, thus the person doing the test was not aware of manupulations done to animals. Testing was done in the following conditions: normal rats before hemisection surgery, 1d, 4d, and once a week from 1 to 8 weeks after hemisection surgery.

1) BBB locomotor test scale

BBB(Basso, Beattie, Bresnahan) test was performed to measure functional

recovery of hindlimb. Open field testing procedure have been described by Basso et al.[37] This scale measures hindlimb movement, with increasing score for use of individual joints, coordinated joint movement, coordinated limb movement, weight-bearing and so on to a maximum score of 21. Rats were gently adapted to the open field. Once a rat walked continuously in the open field, two examiners conducted 5 min testing sessions in each leg, postoperative (p.o.) open field testing was performed at least once a week from 1 day to 8 weeks for all animals after operation.

2) Somatosensory pain test

The 50% paw withdrawal threshold (PWT) to mechanical stimuli applied to the paw was measured and used as an indicator of mechanical sensitivity of the affected paw. The mechanical thresholds were measured using the up-and-down method[32] following the procedures described in previous studies.[33-35] In brief, rats were housed in clear plastic boxes (8 x 8 x 18 cm) above a metal mesh (0.5 x 0.5 cm) and acclimated for 30 min to avoid the stress associated with environmental change. Mechanical PWT to the application of von Frey (VF) filament was measured by using up-down method.[36] A series of eleven von Frey (VF) filaments with approximately equal logarithmic incremental (0.19) von Frey values (3.38, 3.56, 3.75, 3.93, 4.12, 4.31, 4.49, 4.68, 4.86, 5.05 and 5.24) were used to determine the threshold stiffness required for 50% paw withdrawal. Since VF values are logarithmically related to gram (g) values (VF = log (10,000×g)), these chosen von Frey numbers are equivalent to 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10 and 12 in gram value, respectively. Starting with filament 4.31, von Frey filaments were applied perpendicularly to the ventral surface of the paw. Details of the testing procedure and the calculation of the mechanical thresholds were as described by Chaplan et al.[32]

4. Electrophysiological study

1) Animal preparation

Eight weeks after the hemisection and polymer only, polymer with hMSC transplantation, the animals were anesthetized with urethane (1.25 g/kg). Each animal was also given atropine sulfate (0.8 mg/kg) to reduce tracheal secretions. Pancuronium bromide (0.4 ml) was then perfused trough the tail vein to induce muscle relaxation. The rat was then artificially respired using a small animal respirator (Model 683, Rodent Ventilator, Harvard, Holliston, MA, USA) and CO_2 in expired air was maintained within the physiological range using a capnometer (Model 2200, Traverse Medical Monitors, Saline, Michigan, USA). The animal was placed on the stereotaxic device (Narishige Scientific instrument laboratory, Setagaya-ku, Tokyo, Japan) and the rectal temperature was maintained between 36.5~37.5℃.

2) Somatosensory evoked potentials (SSEPs)

SSEPs were recorded to measure the conduction recovery of the sensory system. A special electrode (NE-120, Rhodes Medical Instruments, Inc., Woodland Hills, CA, USA) was used for SSEPs recording. For the recording, the electrode was placed in the sensorimotor cortex (bregma: -2 mm, lateral: 2 mm). Bipolar platinum wire electrode placed in contralateral sciatic nerve was used as a stimulating electrode. A single square pulse (0.1 ms duration) of electrical stimulus was delivered by a stimulus isolator (A365D or A365, World Precision Instruments, Inc., New Heaven, Connecticut, USA), which was driven by a pulse generator (Pulsemaster A300, World Precision Instruments, Inc., New Heaven, Connecticut, USA). The analog signals of the evoked potentials were amplified (x10000), filtered (bandpass 300-1000 Hz), and fed to IBM-compatible PC through AD/DA converter (CED, Cambridge, UK) to be averaged using Spike 2 software. Each SSEP consisted of an average of 100-300 single sweep epochs. The threshold of electrical stimulation was first determined in each experiment. The effect of the stimulation intensity on SSEPs

was analyzed in the wave forms by latencies and amplitudes.

3) Motor evoked potentials (MEPs)

MEPs were recorded to measure the conduction recovery of the motor system. It was necessary to increase the area of stimulation in the motor cortex in order to properly monitor the MEPs with low stimulus intensity. The special electrodes that were identical to the recording electrodes to record SSEPs were used as stimulating and recording electrodes. In order for cortical stimulation, a single square pulse (0.1 ms duration) of electrical stimulus was delivered by a stimulus isolator, which was driven by a pulse generator. Laminectomy was performed at L1 of the spinal cord for the placement of the epidural recording electrodes. Following the laminectomy, the electrode was inserted into the contralateral gray matter of the L1 spinal cord. The analog signals of the evoked potentials were amplified (x10000), filtered (bandpass 300-1000 Hz), and fed to IBM-compatible PC through AD/DA converter to be averaged using Spike 2 software. Each MEP consisted of an average of 100-300 single sweep epochs. The threshold of electrical stimulation was determined in each experiment. The effect of the stimulation intensity on MEPs was analyzed in the wave forms by latencies and amplitudes.

5. X- gal positive cell count

Eight weeks after the induction of hemisection injury and transplantation, the rats were perfused, by normal saline and 4% paraformaldehyde in PB solution. The spinal cords were removed and post-fixed for 24 h in 4% paraformaldehyde followed by 30% sucrose in PBS overnight. Serial longitudinal sections of the spinal cord were made by cryostat (10μm thickness: Microm/HM500V, CE, Germany) and the specimens were stored at -20°C. For the detection of β -galactosidase activity in the spinal cord tissue, fresh frozen sections stained with X-gal reagent (5-bromo-4-chloro-3- indolyl-β-d-galacto pyranoside in dimethylformamide) were stored in a 37 °C thermostat

container overnight to produce a blue color in β-galactosidase-expressing cells. Five slides from each 4 animals were used for counting X-gal positive cells. The number of X-gal positive cells was counted in the tissue of whole 9 mm long and within 3 mm around the SCI region respectively (Fig. 2). The ratio of cell numbers in the central 3 mm region/whole 9 mm section was calculated.[61]

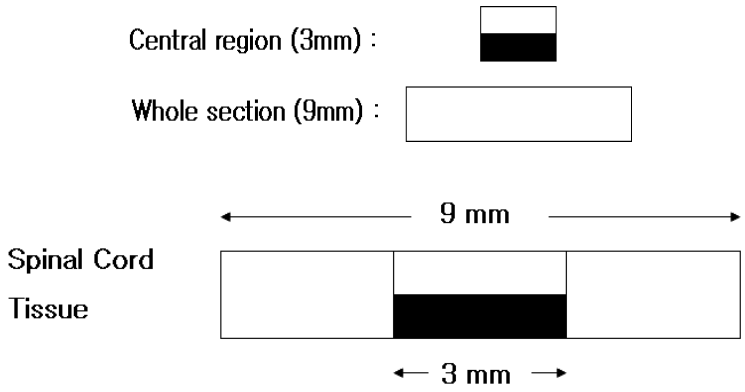

Fig. 2. Cell count methods. X-gal positive cell count in the central lesion (3 mm) and in the whole section (9 mm) of each longitudinal frozen section.

6. Immunohistochemical study

Immunohistochemistry was performed to evaluate the morphological features of transplanted cells in vivo. Eight weeks after the induction of hemisection injury and transplantation, the rats were perfused, by normal saline and 4% paraformaldehyde in PB solution. The spinal cords were removed and post-fixed for 24 h in 4% paraformaldehyde followed by 30% sucrose in PBS overnight. Serial longitudinal sections of the spinal cord were made by cryostat (10μm thickness: Microm/HM500V, CE, Germany) and the specimens were stored at -20°C. The sections were fixed in 4% paraformaldehyde for

10~15min. Then the sections were rinsed in PBS for 13min. Blocking solution was used to treat the sections. The sections were incubated for one day with primary antibodies at 4°C. Following primary antibodies were used: anti β -galactosidase (1:100, Biogenesis, Kingston, NH, USA) and anti-GFAP (glial fibrillary acidic protein, 1:100, BD Biosciences, San JOse, CA, USA), anti-Tau (neuron, 1:50, abcam, Cambridge, UK), or anti-APC (mature oligodendtocyte, 1:50, Chemicon, Temecula, CA, USA). Secondary antibodies, Cy3 (β -galactosidase, 1:500, Jackson, West Grove, PA, USA) and FITC (GFAP, Tau, APC, 1:250, Jackson, West Grove, PA, USA), were applied to the sections for three hours. The sections were mounted on slide glasses with fluorescent mounting medium (Vector, Burligame, CA, USA) and observed by confocal microscope (LSM 510, Olympus, Tokyo, Japan).

7. Statistical analysis

The one-way ANOVA followed by Dunnett's post-hoc multiple comparisons was used to determine statistical differences among the hemisection only, polymer only-transaplanted, and polymer with hMSC-transplanted groups for BBB scores and paw withdrawal threshold (PWT) value at each time point. One-way ANOVA followed by Dunnett's post-hoc multiple comparisons was conducted in electrophysiological studies in order to compare the among the hemisection only, polymer only-transaplanted, and polymer with hMSC-transplanted groups.

III. RESULTS

1. Behavioral study

1) BBB scale

Hindlimb locomotor performance was tested in all rats using the BBB open field scaling.[37] Hemisection group (n=19) has scored 0 in injured (left) and intact (right) leg at 1 day post injury then gradually increased to a final score of 7.8 ± 0.7 in injured leg and 8.9 ± 0.6 in intact leg at 8 weeks after the injury (Fig. 3). In injured(left) leg, the polymer with hMSC-transplanted group showed significantly improved hindlimb performance since 5 weeks after the transplantation compared to hemisection only group. In intact(right) leg, the polymer with hMSC-transplanted group showed significantly improved hindlimb performance since 4 weeks after the transplantation compared to hemisection only group. The final BBB scores of the polymer with hMSC-transplanted group (n= 22) were 9.7 ± 0.5 (injured) and 10.6 ± 0.3 (intact) in both legs.

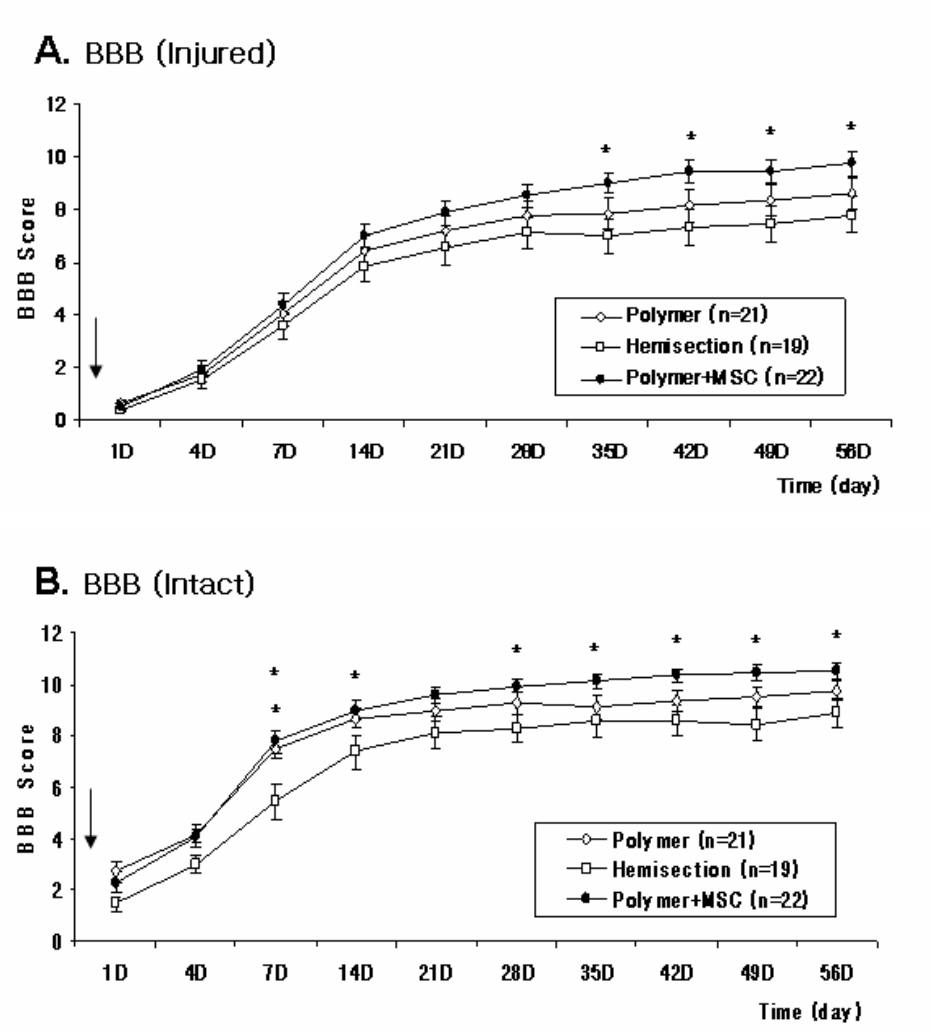

Fig. 3. BBB scores of SCI rats after the hemisection injury and transplantation. A: Polymer and hMSC-transplanted group significantly improved hindlimb performance in injured (left) legs since 5weeks, B: In intact (right) legs, polymer and hMSC-transplanted group significantly improved hindlimb performancesince 4 weeks after transplantation (↓: hemisection and transplantation time, * $p<0.05$).

2) Pain test

The time course of changes in PWT of hemisected rats without transplantation (n=19), with polymer transplantation (n=21), and polymer with hMSC transplantation (n=22) are shown in Fig. 4. After hemisection of the left spinal cord at T11, the PWT value to VF filament stimuli showed a remarkable decrease in both hind paws as compared with the pre-hemisection value in all groups. The PWT of polymer with hMSC-transplanted group was increased compared with that of hemisection only group since 3 weeks after transplantation. And in injured(left) leg, the PWT had significant difference at 5, 8 weeks after transplantation. In intact(right) leg, the PWT was increased significantly since 7 weeks after transplantation.

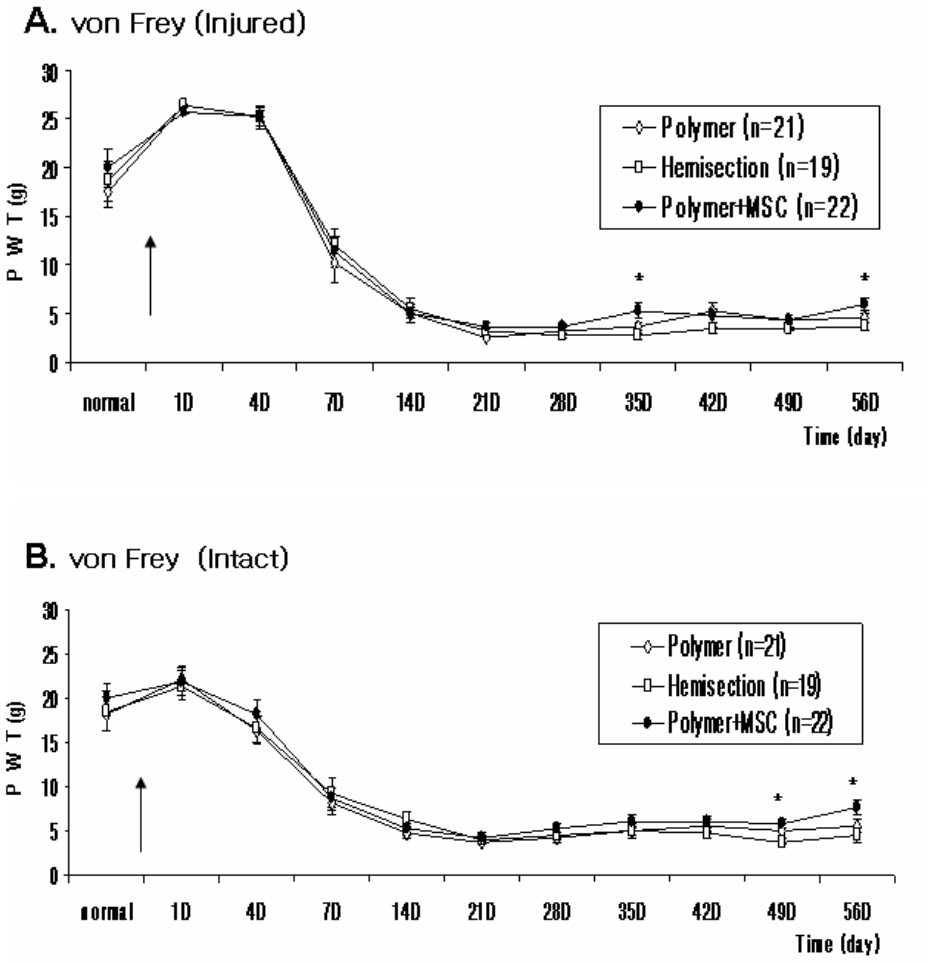

Fig. 4. Changes in paw withdrawal threshold (PWT) before and after the transplantation. A: In injured(left) hindlimb, the PWT had significant difference at 5, 8 weeks after transplantation. B: In intact(right) hindlimb, the PWT had significant difference since 7 weeks after transplantation. (↑: hemisection and transplantation time, * $p<0.05$)

2. Electrophysiology study

1) Somatosensory evoked potentials (SSEPs)

The SSEPs were recorded in the sensorimotor cortex following the stimulation of sciatic nerve. Fig. 5 shows representative wave forms of SSEPs by different intensity stimulations. When the sciatic nerve was stimulated, a negative-positive -negative deflection with a short latency was observed at the sensorimotor cortex. The latencies of SSEPs were classified as initial, N1- and P1-peak latencies. Table 1 and 2 shows the numerical data of the SSEP recording. In injured side, P1-peak latencies of polymer with hMSC-transplanted group were showed significant difference compared to hemisection only group. However, in latencies of intact side, no significance was observed. Amplitudes of experimental groups (polymer and polymer with hMSC group) were tend to increase compared to hemisection only group in both sides, and in amplitudes of intact side, P1-peak amplitudes of polymer with hMSC -transplanted group were showed significant difference compared to hemisection only group (Fig. 6).

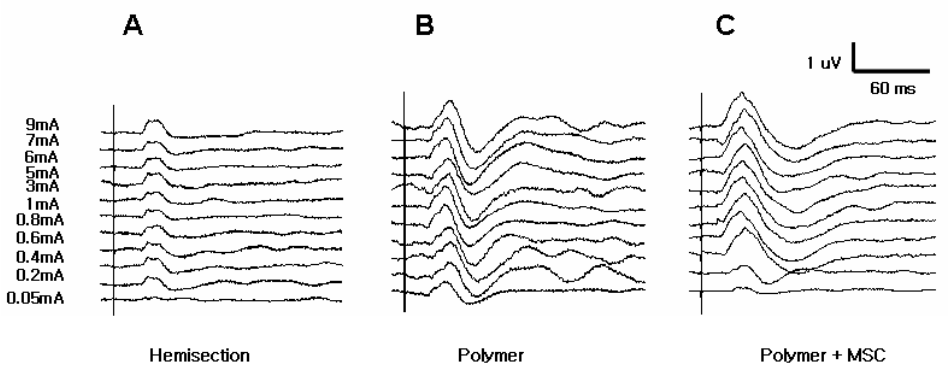

Fig. 5. Representative wave forms of somatosensoty evoked potentials by different intensity stimulations. Reduced latency and increased amplitude were seen in polymer with hMSC and polymer groups compared to hemisection group. A: Hemisection group. B: Polymer transplanted group. C: Polymer with hMSC transplanted group.

Table 1. Latencies of SSEPs. (unit: msec)

	Group	Initial	N1	P1
Injured	Hemisection (n=11)	27.6 ± 3.05	50.3 ± 3.86	82.0 ± 5.61
	Polymer (n=15)	22.9 ± 3.07	44.1 ± 3.51	72.8 ± 4.32
	Polymer+hMSC (n=14)	23.2 ± 2.78	39.9 ± 3.59	64.3 ± 4.62*
Intact	Hemisection (n=12)	18.5 ± 2.45	33.8 ± 2.81	56.8 ± 4.50
	Polymer (n=18)	20.1 ± 1.32	37.5 ± 1.98	70.9 ± 3.78
	Polymer+hMSC (n=19)	18.7 ± 1.13	34.1 ± 1.41	60.6 ± 2.89

Numerical data of SSEPs showed significant difference in P1-peak indicating the recovery of conduction velocity in sensory system. Asterisk (*) indicates statistically significant difference compared to hemisection group.

Table 2. Amplitudes of SSEPs. (unit: μV)

	Group	N1	P1
Injured	Hemisection (n=11)	0.56 ± 0.11	0.78 ± 0.19
	Polymer (n=13)	0.65 ± 0.12	0.98 ± 0.17
	Polymer+hMSC (n=13)	1.86 ± 0.69	2.96 ± 1.15
Intact	Hemisection (n=12)	2.38 ± 0.78	4.19 ± 1.53
	Polymer (n=18)	3.32 ± 0.75	5.68 ± 1.32
	Polymer+hMSC (n=19)	7.08 ± 1.77*	10.77 ± 2.44

Numerical data of SSEPs showed no significant difference among the three groups in injured side. Asterisk (*) indicates statistically significant difference compared to hemisection group.

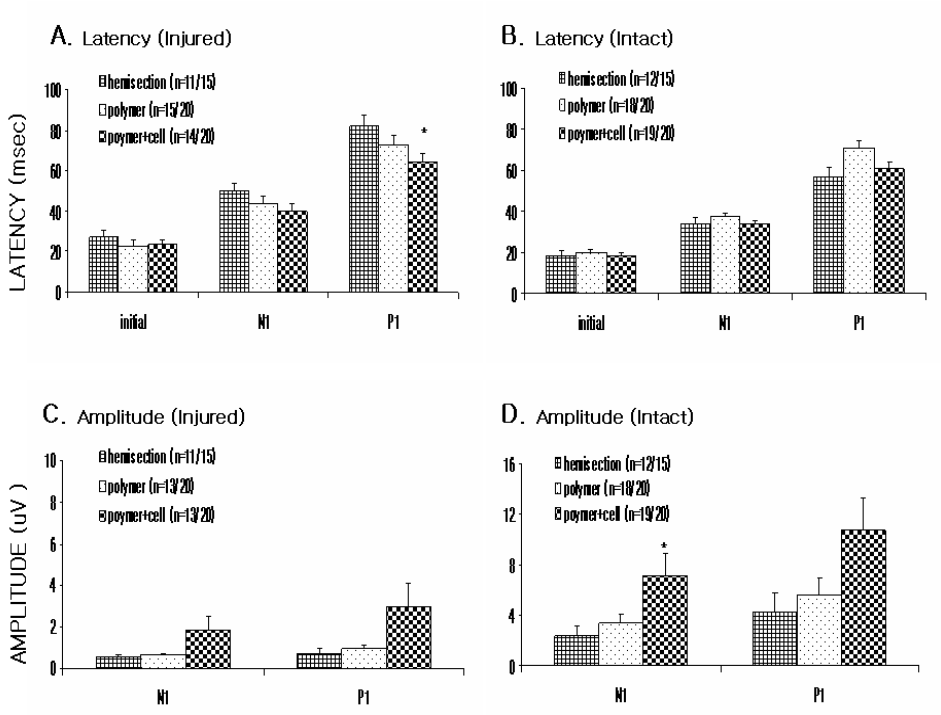

Fig. 6. Latencies and amplitudes of SSEPs. A, B: Comparison of initial, N1- and P1-peaks latencies in different groups. P1-peaks latencies in polymer with hMSC group were shorter than hemisection group on injured(A) side. C, D: Comparison of N1- and P1-peak amplitudes in different groups. No differences were observed in latencies among the three groups on injured(C) side. Asterisks (*) indicate statistically differences between polymer with hMSC group and hemisection group by Dunnett's post-hoc multiple comparisons.

2) Motor evoked potentials (MEPs)

The MEPs were recorded using a bipolar disk electrode in the L1 spinal cord after hindlimb area of the sensorimotor cortex was stimulated. Fig. 7 shows representative wave forms of MEPs recorded in hemisection, polymer-transplanted and polymer with hMSC-transplanted animals. The wave forms were very similar to SSEPs with negative-positive-negative deflection. Table 3 and 4 show the numerical data of the MEPs recording. After spinal hemisection injury, the animals showed lengthened MEP latencies and reduced amplitudes. In MEPs, the latency of polymer with hMSC-transplanted group was tend to shorten compared to the hemisection only group in both sides, and in N1-and P1-peak latencies of intact side were showed significant difference compared to the hemisection only group. In injured side, the P1-peak amplitudes of the polymer with hMSC-transplanted group were significantly increased ($p < 0.05$) compared to hemisection only group. However, the amplitude of intact side have shown no significant differences among the three groups (Fig. 8).

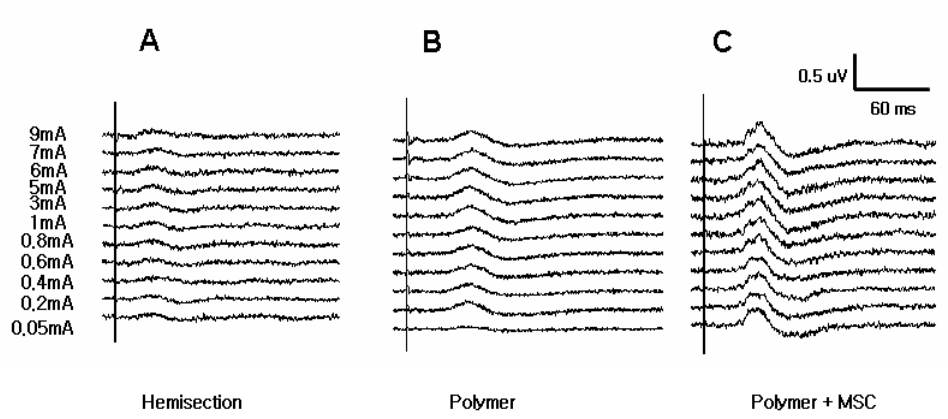

Fig. 7. Representative wave forms of motor evoked potentials by different intensity stimulations. Increased amplitude was seen in polymer with hMSC and polymer groups compared to hemisection group. A: Hemisection group. B: Polymer transplanted group. C: Polymer with hMSC transplanted group.

Table 3. Latencies of MEPs. (unit: msec)

	Group	Initial	N1	P1
Injured	Hemisection (n=8)	27.3 ± 5.12	44.9 ± 4.40	73.9 ± 4.24
	Polymer (n=18)	22.0 ± 2.62	40.5 ± 2.90	66.7 ± 2.93
	Polymer+hMSC (n=19)	20.5 ± 1.20	41.2 ± 1.84	70.6 ± 2.57
Intact	Hemisection (n=12)	22.5 ± 2.21	42.7 ± 2.93	70.9 ± 4.31
	Polymer (n=17)	18.7 ± 1.68	38.7 ± 2.35	68.8 ± 2.89
	Polymer+hMSC (n=16)	14.8 ± 1.06	33.0 ± 1.80*	61.1 ± 3.22*

Numerical data of MEPs showed no significant difference among the three groups in injured side. Asterisks (*) indicate statistically significant difference compared to hemisection group.

Table 4. Amplitudes of MEPs. (unit: μV)

	Group	N1	P1
Injured	Hemisection (n=12)	0.24 ± 0.05	0.23 ± 0.04
	Polymer (n=18)	0.41 ± 0.08	0.48 ± 0.08
	Polymer+hMSC (n=19)	0.50 ± 0.09	0.66 ± 0.13*
Intact	Hemisection (n=11)	0.59 ± 0.20	0.83 ± 0.26
	Polymer (n=13)	0.54 ± 0.09	0.75 ± 0.11
	Polymer+hMSC (n=13)	0.84 ± 0.22	1.19 ± 0.33

Numerical data of MEPs showed significant difference in P1- peak amplitudes, indicating the recovery of conduction velocity in motor system. Asterisk (*) indicates statistically significant difference compared to hemisection group.

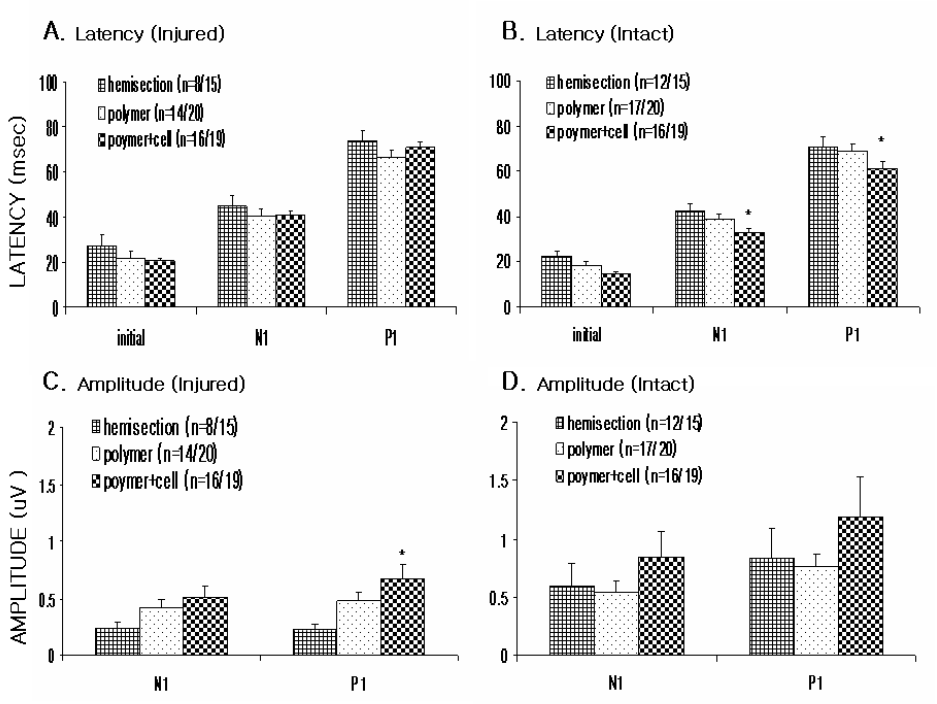

Fig. 8. Latencies and amplitudes of MEPs. A, B: Comparison of initial, N1- and P1-peak latencies in different groups. No differences were observed in latencies among the three groups on injured(A) side. C, D: Comparison of N1- and P1-peak amplitudes in different groups. P1-peaks amplitudes in polymer with hMSC were significantly higher than hemisection group on injured(C) side. Asterisks (*) indicate statistically significant differences between hydrogel with hMSC group and hemisection group by Dunnett's post-hoc multiple comparisons.

3. X-gal positive cell count

The amount of cells that have settled and survived after the transplantation of cells was determined by X-gal stain. The X-gal positive cells appeared in injury site and adjacent site of the spinal cord (Fig. 9). The results of cell count were summarized in Fig 10. In samples, rostral site, injured site and caudal site were averaged 25.5, 7.3 and 29.6 X-gal positive cells in the whole section, respectively. The results of the cell gathering ratio were summarized in Fig 10. Cell gathering ratios of rostral site, injured site, caudal site were 40.9%, 11.6%, 47.4% in the whole section, respectively. And these data were divided into left (injured) and right (intact) side. Cell gathering ratios of rostral site, injured site and caudal site were 42.3%, 13.4% and 44.4% in left side. And Cell gathering ratios of rostral site, injured site and caudal site were 40.2%, 10.2% and 49.5% in right side. Cell gathering ratios of left and right side were 42.8%, 57.2%.

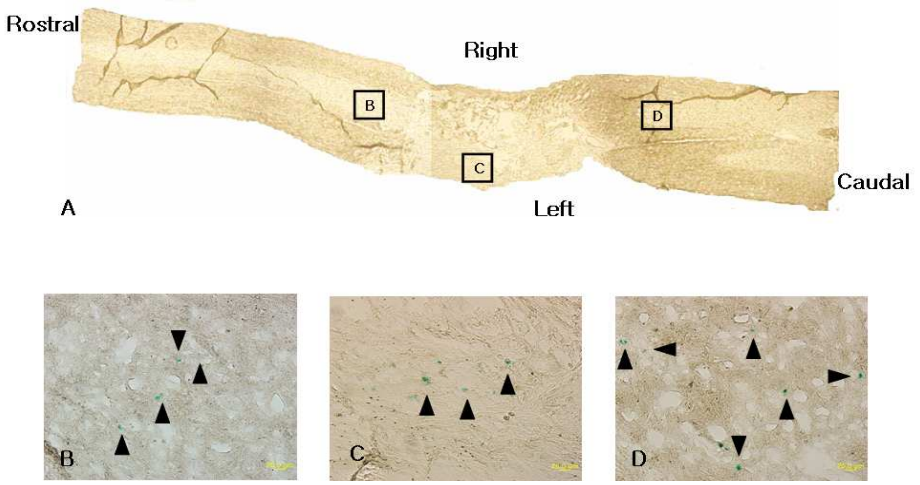

Fig. 9. Staining with X-gal. A: X-gal positive cells were revealed around the injury site and adjacent site, which show migrated cells. B, C, D: High magnification of box B, C, D in A (arrowhead: X-gal positive cell).

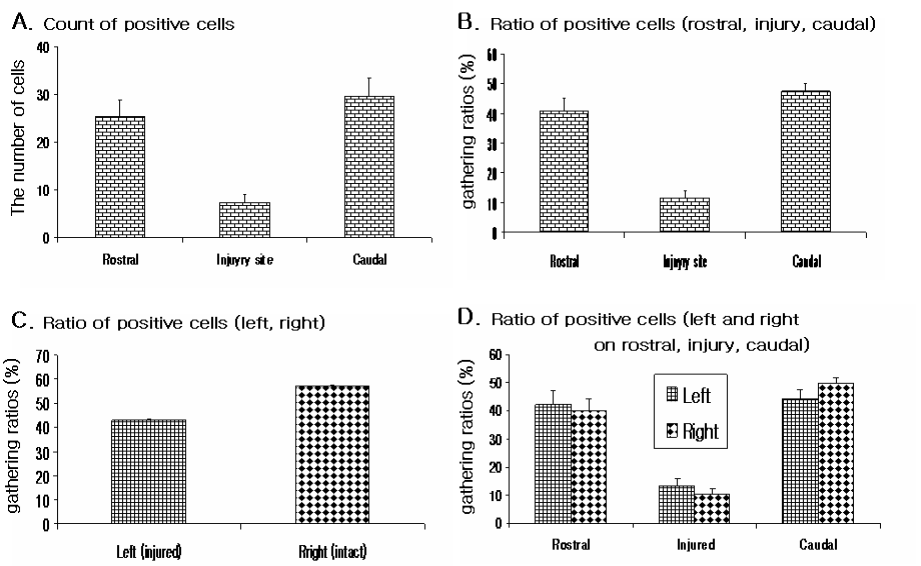

Fig. 10. X-gal positive cell count. A: The number of X-gal positive cells in whole section (rostral, injured, caudal site). B: The ratios of X-gal positive cells in whole section (9mm). C: The ratios of X-gal positive cells in left (injured) and right (intact) side of whole section. D: The ratios of X-gal positive cells in rostral, injured, caudal site in left and right side.

4. Immunohistochemical study

The hMSCs that have settled and survived after the transplantation of cells were determined by β-galactosidase (β-gal) immunoreactivity in longitudinal sections including cavity of spinal cord in 8 weeks after the transplantation. The β-gal-positive cells appeared in injured site and upper and lower site of the spinal cord. The double staining of β-gal and Tau, GFAP or APC-positive cells were present in transplanted site and the adjacent sites, indicating that the transplanted cells have well been settled and differentiated as neuron, astrocyte or oligodendrocyte (Fig. 11).

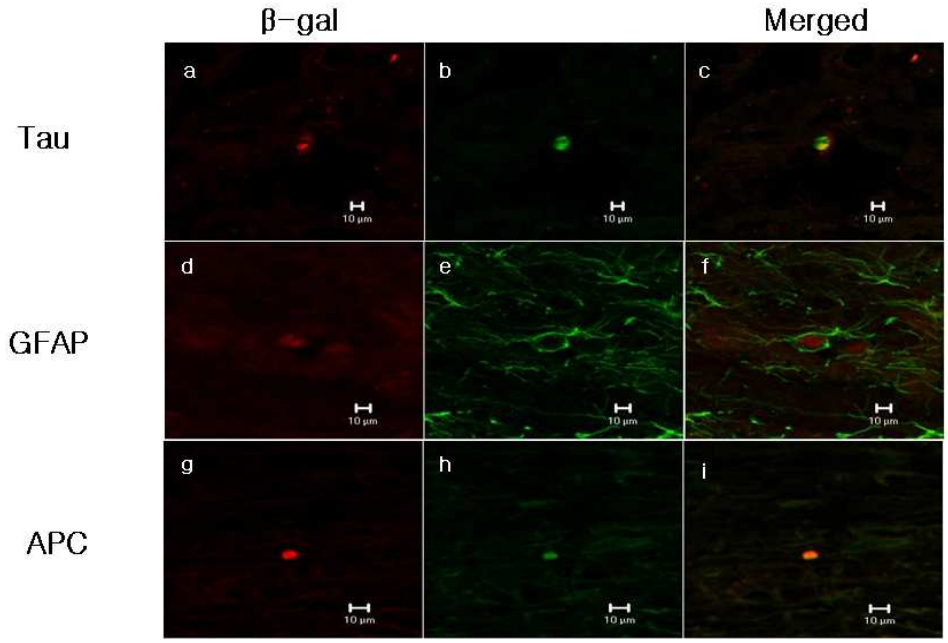

Fig. 11. Double staining of β-gal and Tau, GFAP or APC analyzed by confocal microscope. A,D,G: β-gal positive cell. B, E, H: Tau, GFAP, APC positive cell. C, F, I: merged.

IV. DISCUSSION

In the present study, after polymer with hMSC was transplanted into the injured site of the spinal cord, locomotion recovery by BBB scale was significantly improved compared with hemisection only group at 5 ~ 8w after transplantation. In pain test, PWT values have shown significant difference between polymer with hMSC transplanted and hemisection only group at 5 and 8w after transplantation. The electrophysiological study has shown no significant difference in SSEPs amplitude data among polymer with hMSC transplantation, polymer transplantation and hemisection group. However, from SSEP latency data, P1-peak latencies in polymer with hMSC-transplanted group were significantly shorter than hemisection only group. Also MEP amplitude data were shown significant difference between polymer with hMSC transplanted and hemisection only group in P1-peak amplitudes. The X-gal positive cell counting study showed that the number of X-gal positive cells in rostral and caudal site to the injured site was more than those of positive cells in the injured site. Double staining of β-gal and Tau, GFAP or APC showed positive cells after 8weeks of transplantation.

1. Behavior improvement by cell transplantation

1) Locomotion

Several reports have indicated the effectiveness of MSC transplantation for SCI. Transplantation of Bone marrow stromal cells (BMSCs) significantly improved hindlimb function in a rat spinal cord contusion injury.[38] BMSCs formed guiding strands in contused spinal cord and promoted hindlimb functional recovery.[56] BMSCs enhanced the differentiation of a cocultured neurosphere in vitro and promoted regeneration of injured spinal cord.[55] However, the precise mechanism by which MSCs promote the functional recovery after SCI is still unclear. In

the present study, hindlimb locomotor performances on both the injured and intact sides were significantly improved in the polymer with hMSC-transplanted group. We showed that animals transplanted polymer with hMSC into hemisection cavity significantly improve functional outcome as measured on the BBB test since 5 weeks after transplantation. Significant improvement of function appears to increase up to 8 weeks after transplantation. Although there was no significant difference, the injured hindlimb function of polymer transplanted group was tend to improve compared to hemisection only group. These results suggest that even the polymer only transplantation is capable of leading to improvement in functional recovery. Also, similar results were showed in the intact hindlimb performance. These data indicate that polymer and hMSC transplantation promoted regeneration of injured spinal cord, as a result hindlimb function may be improved.

In the present study, the group transplanted only hMSC was not included in experiment design. Because hMSC only transplantation in hemisection cavity was very difficult in the animal model used in the present study. But I think it is possible to transplant into rather than the hemisection cavity itself the adjacent spinal cord to the hemisection cavity.

2) Somatic sensation

SCI results in loss of sensory and motor function below the level of the lesion, as well as the development of chronic pain in the majority of patients. SCI induced pain is characterized as a spontaneous burning pain allodynia, and hyperalgesia, which occur at or below the level of the lesion.[44] Over the last decade, a few animal models have been developed to explore pain mechanism.[46,47,48] The underlying mechanism is not fully understood, and this pain is usually refractory to conventional analgesic treatments.[45] The finding in this study that hind PWT to VF stimulation was decreased after spinal hemisection injury indicates that mechanical allodynia developed below the level of the injury. And the PWT values of rats transplanted polymer with hMSCs in injured side tended to increase since 3

weeks after transplantation and had significant increment compared to hemisection only group at 5, 8 weeks after transplantation. The present study shows that spinal hemisection induces hyperalgesic responses in pain test data and that polymer with hMSC transplantation prevents the development of these responses following spinal hemisection.

In behavioral data, the PWT value of polymer only transplanted group tended to improve compared to hemisection only group, but there was no significant difference. And the PWT value of polymer with hMSC-transplanted group showed significant difference compared to hemisection only group. This results indicate that the transplantation of polymer with hMSC was more effective than the transplantation of polymer only, and may be due to reconnect across the trauma cavity by capability of polymer. But I doubt whether our animal model is suitable for pain test. Therefore, further study is there is required to explore the precise mechanism about pain of hemisection animal model.

Recently, there is growing evidence that MSCs produce a variety of neurotrophic factors as well as chemokines and cytokines in vitro and in vivo.[57] Neurotrophic and growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are produced by MSCs.[58,59] Also, Chen et al.[60] showed that the secretion profile of MSC is responsive to the environment with increased secretion of certain growth factors (e.g., BDNF, NGF) in injured brain. In the present study, we didn't investigate about the secretion produced by hMSC. However, the cells may create a permissive environment for axon outgrowth and axonal guidance mediated by their release of trophic factors, thereby improving self-repair in the damaged CNS.

2. Recovery of neural conduction

Many studies have reported for axonal regeneration and remyelination following transplantation of various cells. In the injured spinal cord, not only demyelination of neurons have occurred, but neuronal cell death have also occurred. When directly injected into the demyelinated rat spinal cord, marrow cells derived from the mononuclear layer remyelinate these axons.[21] In animal models of SCI, grafts of MSC have been shown to promote remyelination[51] as well as partial recovery of function.[38,39,52] However, the functional recovery of newly regenerated axons and reestablished myelins could hardly be observed.

In this study, electrophysiological measurements of SSEPs and MEPs activity were used to determine if axons carrying sensory and/or motor information crossed the damage site during the recovery period. These results indicate that the SSEP latency of injured side, which has been recorded from the sensorimotor cortex following sciatic nerve stimulation, tended to be shortened compared to the hemisection only group and the significant difference was observed in P1- peak latency. And the SSEPs amplitude tended to be increased compared to the hemisection only group. However, when MEPs were recorded from L1 spinal cord following the motor cortex stimulation, the MEP amplitudes of polymer with hMSC-transplanted group in injured side were statistically increased compared to hemisection only group. The improvement of SSEPs and MEPs was been shown because damaged neurons and axons may be recovered enough to meet the required amount of myelin sheaths to deliver the action potentials by the polymer with hMSC transplantation. In the present study, the secretion produced by hMSC was not studied. However, recovery of damaged neurons and axons, and remyelination may be due to secretion from hMSC as well as the direct effect of polymer and hMSC. Consequently, reconnection and remyelination of axons in the injured spinal cord might be easier to take place after the transplantation of the polymer with hMSC.

3. Immunohistochemical evidence to support survival and differentiation patterns of transplanted cells

The present study has used β-gal to observe survival and differentiation patterns of transplanted cells. The extent of cells that have settled and survived after the transplantation was determined by observing X-gal positive in longitudinal sections of the spinal cord including the hemisected injury site in 8 weeks after the transplantation. X-gal positive cells were observed in rostral and caudal sites. This result means that the hMSCs transplanted into hemisection cavity were migrated from injured site to rostral and caudal sites. And there was no difference between cell gathering ratios of injured and intact side. This result indicate that the transplanted polymer with hMSC was well implanted and polymer may play an important connection in cavity site.

In the previous study, MSCs can differentiate into cells with neural characteristics in vitro[40,41,42] and in vivo.[38,43] Neurofilament (NF-200), microassociate protein-2 (MAP-2), neuron-specific nuclear protein (NeuN), nestin, gial fibrillary acidic protein (GFAP), GAD and ChAT were detected by immunohisitochemistry.[50] Bone marrow stromal cells can differentiate into astrocytes when transplanted into rodent brain[49] and neurons in vitro under appropriate cell culture conditions.[53] The double staining of β-gal and Tau, GFAP or APC was observed from the transplanted and the adjacent sites, indicating that the transplanted cells have well settled and differentiated into neurons, astrocytes and oligodendrocytes. These data support the evidence that has been shown in the previous study. Also, those data indicate that the transplanted hMSCs are able to differentiate into a variety of cell-types and these cells may play an important role of functional recovery, including improvement of behavior performance and neural conduction.

In conclusion, our data showed significant functional outcome in the group transplanted with hMSCs and polymer compared with hemisection

only group, which means hMSCs and polymer might take an important role to improve functional outcome. Electrophysiological study showed that the transplantation of hMSCs and polymer may plat an important role in axonal regeneration. Immunohistostaining for β-gal (β-galactosidase) and GFAP (glial fibrillary acidic protein), Tau (neuron), or APC (mature oligodendtocyte) were positive in scattered cells derived from hMSCs, which exhibited transplanted hMSCs survived and had potential to neural differentiation in spinal cord injured rat. For clinical application, it is vital to solve the problems of stem cells survival and control of its differentiation. In this study, we have not demonstrated intrinsic mechanism of neurotrophic factor affecting neural repair. However, our experiment is consistent with a growing literature that MSCs and neurotrophic factor promote tissue repair and functional recovery after spinal cord injury and suggest that MSCs and polymer transplantation warrants investigation as a therapeutic intervention after spinal cord injury.

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ABSTRACT (IN KOREAN)

중합체를 이용한 인간 중간엽 줄기세포의 이식이 척수 반절단 손상 후 기능 회복에 미치는 효과

(지도교수 이 배 환)

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척수손상(spinal cord injury)이란 척수에 가해진 외상으로 인해 정상적인 운동, 감각 및 자율신경 기능에 이상이 생긴 상태를 말한다. 중추신경에 손상이 생기면, 손상된 신경의 자발적인 재생은 제한적이다. 세포이식은 척수손상을 치료하기 위한 매우 효과적인 방법으로 여겨지고 있다. 지난 몇 년간 척수손상 동물모델에서 다양한 세포의 이식을 이용하여 신경의 재생에 관한 많은 연구가 수행되었다. 최근, 중간엽 줄기세포(mesenchymal stem cell)의 이식이 신경재생을 증가시키고, 윤리적 문제를 피할 수 있는 잠재적인 접근법으로 여겨지고 있다. 척수손상은 병리학적 관점에서 매우 복잡하다. 따라서 척수손상의 치료는 다양한 방향에서의 접근이 필요하다. 최근에 척수손상으로 부터의 회복을 위해 생합성 중합체(polymer)의 응용이 보고되고 있다. 따라서 본 연구에서는 중합체를 이용한 인간 중간엽 줄기세포의 이식이 척수 반절단 손상 후 기능 회복에 미치는 영향을 알아보기 위해 중합체와 인간 중간엽 줄기세포를 척수 반절단 동물모델에 이식한 후 행동검사(BBB test, pain test), 전기생리학적 검사, 면역조직학적 검사를 실시하여 기능 회복에 미치는 영향을 알아보고자 하였다.

실험동물은 Sprague-Dawley종 수컷 흰쥐를 사용하였다. Pentobarbital로 마취한 후 제 10-11 흉추에서 척추후궁절제술을 실

시하고, 척수를 반절단하여 척수 반절단 모델을 만들었다. 동물모델은 반절단만을 시행한 그룹과 반절단 후 중합체를 이식한 그룹 그리고 중합체와 중간엽 줄기세포를 이식한 그룹으로 나누었다. 이식 후 8주 동안 운동과 감각기능의 회복정도를 행동검사를 통해 확인하고, 손상된 신경의 전도성 회복은 전기생리학적 방법을 통해 확인하였다. 또한 이식된 세포의 생존과 분화를 조직검사를 통하여 관찰하였다.

BBB검사 결과 반절단 후 중합체와 세포를 이식한 실험군이 이식 후 5주후부터 반절단만을 시행한 대조군과 비교하여 BBB점수가 유의미하게 향상되었다. 또한 통증검사 결과 반절단만 시행한 대조군과 중합체와 세포를 이식한 실험군을 비교하여 이식 후 5, 8주에서 paw withdrawal threshold수치가 유의미하게 증가되었다. 전기생리학적 검사에서 체성감각유발전위 (SSEP)는 세포를 이식한 그룹에서 전도성이 빨라지는 경향성을 보였지만 차이는 없었다. 그러나 운동유발전위(MEP)에서는 전도성의 차이를 확인할 수 없었다. 또한 조직검사에서는 이식한 세포의 생존과 분화를 알아보기 위해 β-gal 항체를 이용하여 면역염색을 실시하였고, β-gal 양성인 세포가 이식된 부위와 이식부위의 주변부에서 관찰되었다. Double 면역염색에서는 이식한 세포가 다양한 신경세포로 분화됨을 관찰할 수 있었다. 이러한 결과들은 중합체를 이용한 중간엽 줄기세포 이식이 기능 회복과 축삭의 재생에 중요한 역할을 할 것으로 보이고, 척수손상의 치료에 있어서 효과적인 치료전략이 될 수 있을 것으로 생각된다.

핵심되는 말: 척수 손상, 중간엽 줄기세포, 중합체, 이식, 행동검사, 전기생리