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Title 論文題目	Intravenous preload of mesenchymal stem cells rescues erectile function in a rat model of cavernous nerve injury （骨髄間葉系幹細胞の経静脈的事前移植による治療効果－実験的海綿体神経損傷に起因する勃起機能の温存－）
Author(s) 著　者	髙柳，明夫
Degree number 学位記番号	甲第 2852 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2015-09-30
Original Article 原著論文	Journal of Sexual Medicine. 2015 Aug;12(8):1713-21. doi: 10.1111/jsm.12957.
Doc URL	
DOI	
Resource Version	Author Edition. This is the peer reviewed version of the following article: Takayanagi, A., Sasaki, M., Kataoka-Sasaki, Y., Kobayashi, K., Matsuda, Y., Oka, S., Masumori, N., Kocsis, J. D. and Honmou, O. (2015), Intravenous Preload of Mesenchymal Stem Cells Rescues Erectile Function in a Rat Model of Cavernous Nerve Injury. Journal of Sexual Medicine, 12: 1713–1721, which has been published in final form at http://dx.doi:10.1111/jsm.12957. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

THE JOURNAL OF
Sexual
Medicine

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Journal:	*Journal of Sexual Medicine*
Manuscript ID:	JSM-03-2015-154.R2
Wiley - Manuscript type:	Original Research
Subject Area:	Animal models < BASIC SCIENCE < BOTH GENDERS, Molecular biologic studies of sexual dysfunction < BASIC SCIENCE < BOTH GENDERS, Neurophysiologic studies of sexual function < BASIC SCIENCE < BOTH GENDERS
Keywords:	mesenchymal stem cells, erectile dysfunction, transplantation

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Intravenous preload of mesenchymal stem cells rescues erectile function in a rat model of cavernous nerve injury

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Running head: *Preload of MSC infusion rescues erectile function in cavernous nerve injury*

Take Home Message:

Intravenous delivery of mesenchymal stem cells (MSCs) prior to a cavernous nerve (CN) injury model for experimental erectile dysfunction (ED), resulted in increased intracavernous pressure (ICP) / arterial pressure (AP), neuronal innervation and higher levels of GDNF and Neurturin mRNA in the major pelvic ganglia (MPG).

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Abstract

Introduction:

We evaluated the potential preventive effects and mechanisms of intravenously preloaded mesenchymal stem cells (MSCs) for erectile dysfunction (ED) in a cavernous nerve (CN) injury model.

Methods:

Male Sprague Dawley rats were used for this study. Rats were randomized into two groups. One group was intravenously preloaded with MSCs (1.0×10^6 cells in 1 ml total fluid volume) and the other was infused with medium alone (1ml DMEM) for sham control, respectively. Crushed CN injury was induced immediately after infusion. The surgeon was blind to the experimental conditions (MSC or medium).

Main Outcome Measures:

To assess erectile function, we measured the intracavernous pressure (ICP) and arterial pressure (AP) at 1 hour and 2 weeks after CN injury. After measuring the initial ICP/AP of pre-injury (normal) male SD rats, they were randomized into the two groups and infused with MSCs or medium. PKH26-labelled MSCs were used for tracking. To investigate the mRNA expression levels of neurotrophins in the major pelvic ganglia (MPG), we performed real-time quantitative RT-PCR.

Results:

The reduction of ICP/AP and area under the curve of ICP (ICP-AUC) in the MSC group was significantly lower than in the DMEM group ($P<0.05$; $P<0.05$) at one hour. The ICP/AP and ICP-AUC at 2 weeks post-injury in the MSC group was significantly higher than in the DMEM group ($P<0.01$; $p<0.05$). The preloaded PKH26-labelled MSCs were detected in the MPG and CN using confocal microscopy indicating homing of the cells to the injured nerve and ganglia. GDNF and neurturin, which are

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important neurotrophic factors for erection, had expression levels in MPG significantly higher in the MSC group than in the DMEM group (P<0.01, 0.05).

Conclusion: Intravenous preload of MSCs before a CN injury may prevent or reduce experimental ED.

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1. Introduction

Radical prostatectomy (RP) is a recommended curative procedure for patients with low and intermediate risk in localized prostate cancer and is associated with a life expectancy of more than 10 years [1]. Erectile dysfunction (ED) following RP, however, is the major problem and it can reduce patients' quality of life (QOL) after surgery [2, 3]. The etiology of ED after RP is considered to be multifactorial [4-6], but a neurogenic etiology resulting from cavernous nerve (CN) injuries during a nerve-sparing procedure is important[7].

Despite the progress of nerve sparing techniques that include spreading the robot-assisted procedures, recent post-operative ED rates at one year ranged from 10 to 46% [8]. Common drug therapy is oral intake of phosphodiesterase type 5 inhibitors, but the success rate is significantly lower in this population [9]. Penile rehabilitation is also beneficial treatment for these populations, but the effectiveness is limited and it takes several months to years to recover [4]. Therefore, it is an important research issue to develop approaches to better reduce post-operative ED in patients.

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Mesenchymal stem cells (MSCs) derived from adult bone marrow are considered to secrete a number of neurotrophic factors, cytokines and anti-inflammatory molecules that provide neuroprotection, induction of axonal regeneration/sprouting, remyelination and immunomodulation [10]. Much work in animal models and clinical trials have shown that intravenous infusion of MSCs following peripheral nerve injury including sciatic [11] and recurrent laryngeal [12] nerve injuries and central nervous diseases such as stroke [13-15] and spinal cord injury [16] is safe and improves functional recovery. In this study we evaluated the effects of systemically delivered MSCs in an experimental ED model in the rat.

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2. Aim

The aim of this study is to find a cell therapy approach to prevent or reduce post-operative ED using intravenous delivery of MSCs prior to induction of CN injury in the rat.

3. Materials and Methods

3.1. *Preparation of mesenchymal stem cells from rat bone marrow*

The use of animals in this study was approved by the animal care and use committee at Sapporo Medical University (#12-030), and all procedures were carried out in accordance with institutional guidelines. Methodology of MSC culture was based upon our previous studies [17]. Briefly, bone marrow was obtained from femoral bones in adult Sprague–Dawley rats (male, 200-250 g), diluted to 25 ml with Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM l-glutamine (SIGMA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Thermo Fisher Scientific Inc.) and incubated for 3 days (5% CO_2, 37 °C). When cultures almost reached confluence, the adherent cells were detached with trypsin-EDTA solution (SIGMA) and subcultured at 1×10^4 cells/ml. In the present study, we have used MSCs after three passages.

3.2. *Cavernous nerve injury model and assessment of erectile function*

Male Sprague-Dawley rats weighing 250–350 g were anesthetized with pentobarbital (45 mg/kg). Through a lower abdominal midline incision, bilateral major pelvic ganglia (MPG) and CNs were identified and exposed. The CNs

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(approximately 5 mm distal to the MPG) were first crushed using a micro needle holder (FD231R, B. Braun Aesculap Japan, Tokyo, Japan) for 15 sec as previously described with minor modification[18]. The procedure was carried out carefully so as not to cut the CNs to avoid major injury causing deterioration of erectile function. Abdominal wounds were then closed with sutures, and animals were allowed to recover from anesthesia.

The intracavernous pressure (ICP) and arterial pressure (AP) were measured using the methods similar to those previously reported [19-22]. Animals were anesthetized with pentobarbital (45 mg/kg). The skin of the left neck was incised and a PE50 (#427400, Becton Dickinson, MD, USA) tube was inserted into the left carotid artery to monitor AP. Bilateral MPG and CNs were exposed as described above and the incision was extended to the skin around the penis. A 23G needle connected to a PE50 tube filled with heparinized saline (250 IU/ml) was inserted into the left penile crus. Electrostimulation (20 Hz and 1.5mA for 60 seconds) of the CNs was applied with a bipolar hook electrode (TF-206-011, Unique Medical Co., Tokyo, Japan), and changes of ICP were measured. Maximal ICP, mean AP and area under the curve of ICP (ICP-AUC) during the period of electrostimulation were analyzed using LabChart (ADinstruments Inc., Colorado Springs, CO, USA). The maximal ICP value divided by simultaneous mean AP (ICP/AP) and ICP-AUC were used to evaluate erectile function.

3.3. Experimental protocol (Fig.1)

First, we measured the initial ICP/AP from pre-injury rats. The rats were randomized into experimental and sham control groups (MSC and DMEM). Rats were intravenously infused with MSCs (1.0×10^6 cells in 1ml total fluid volume) and

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medium alone (fresh 1 ml DMEM only) respectively via right external jugular vein. CN injury was immediately induced after preload of MSCs or DMEM. The order of delivering MSC or DMEM was randomized. The surgeon (A.T.) did not know whether MSC or DMEM was administered before and during infusion. We measured ICP/AP at 1 hour and 2 weeks after CN injury. Each ICP/AP measurement at 1 hour (n=12/group) and 2 weeks (n=10/group) was a terminal experiment in a separate group.

3.4. Retrograde tracing study

Fluorogold (FG: Hydroxystilbamidine, methanesulfonate, H-22845, Thermo Fisher Scientific Inc.) injection was performed one week before histological examination as previously reported [19, 22, 23]. Briefly, animals were anesthetized with pentobarbital (45 mg/kg). The skin beside the penis was narrowly opened and 4μl of FG (4.0%) was injected into the right penile crus by using a 30G Hamilton syringe. After the ICP measurement the right MPG was collected and immediately fixed in 4% paraformaldehyde (PFA) for about 48 hours, and then immersed in 0.1 mol/L phosphate buffer (PB) containing 25% sucrose.

Quantification of retrograde labeled FG positive cells was performed as previously described. [20, 24, 25] Serial frozen transverse sections (14 μm thick) of the entire MPG were cut with a cryostat. Every sixth section was mounted on 3 aminopropyltriethoxysilane-coated slides (12–14 sections per animal). Images of each MPG were taken under fluorescent microscope through a wideband UV filter using 40x objective. We evaluated all FG positive neuronal profiles that had nuclei in each MPG section to avoid double-counting of neurons (10 rats each in the MSC and the DMEM groups). The standardized selection of sections through the entire MPG and

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counting all FG positive cells on each section allowed an unbiased assessment of retrogradely labelled cells between the MSC and DMEM groups spanning the entire MPG.

3.5. *Detection of PKH26 labeled MSCs*

Cells were prelabeled with the membrane dye PKH26 as previously reported [10]. Intracardiac perfusion (4 % PFA) (n=10) were performed immediately after CN injury to collect MPGs and CNs and store at -80 ℃ until use. Transverse cryostat sections (20 μm) were obtained through the lesion area. Immunohistochemistry was performed using anti-neurofilament (#N4142, Sigma; 1:500). The sections were examined using a confocal microscopy with Ex/Em (405; 488; 561: LSM780 ELYRA system; Carl Zeiss, Oberkochen, Germany).

3.6. Real-Time Polymerase Chain Reaction

One hour after nerve injury, animals (n=5/group) were anesthetized, and the MPGs were collected in each group using a microscope. Total RNA was extracted using a RNeasy Plus Mini kit (#74134, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions [21, 25]. The concentration of the RNA was quantified by determination of optical density at 260 nm. The RNA (1μg) was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Qiagen) and nucleotide oligo-dT. Real-time polymerase chain reaction (PCR) for each sample was performed in triplicates with TaqMan Universal Master Mix II with UNG (Thermo Fisher Scientific Inc.). Specific sets of primers and TaqMan probes were purchased from Thermo Fisher Scientific Inc.: glyceraldehyde-3-phospate dehydrogenase (GAPDH) (TaqMan rodent GAPDH control reagents, Rn01775763-g1) as an endogenous control,

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GDNF (Rn00569510_m1) and Neurturin (Rn01527513-g1) as target genes. The reactions were run in an ABI-StepOne real-time PCR system (Thermo Fisher Scientific Inc.) using the 48-well plate format. The cycling conditions included an initial phase at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, and 55°C for 60 s. Relative quantification of target gene expression was calculated using the comparative threshold cycle method according to the guidelines of the manufacturer.

3.7. Statistical analysis

All statistical analyses were performed using SPSS 18 (SPSS, Inc., IL, USA). For multiple comparisons, we used Kruskal-Wallis one-way analysis of variance followed by Bonferroni *post hoc* test. Comparison between two groups was performed using the Mann-Whitney *U*-test.

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4. Results

4.1. *Erectile function after MSC transplantation*

To assess the erectile function, a physiological study with ICP/AP and ICP-AUC analyses was carried out. Representative waveforms of the ICP and AP at pre-injury in the MSC group (Fig. 2A), pre-injury in the DMEM group (Fig. 2B), 1 hour after injury in the MSC group (Fig. 2C) and 1 hour after injury in the DMEM group (Fig. 2D) are shown in Fig. 2. The ICP/AP and ICP-AUC were significantly decreased one hour after injury in both groups (Fig. 2E, 2F: $P<0.01$). The reduction of ICP/AP in the MSC group (54.7%) was 12.4% lower than in the DMEM group (67.1%) at 1 hour after transplantation. (Fig. 2E, $P<0.05$). The reduction of ICP-AUC in the MSC group (60.2%) was 12.3% lower than in the DMEM group (72.5%) at 1 hour after transplantation (Fig. 2F, $P<0.05$).

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Representative waveforms of the ICP and AP at 2 weeks after transplantation in the MSC group (Fig. 3A) and the DMEM group (Fig. 3B) are shown in Fig. 3. It should be noted that waveform of the MSC group (Fig. 3A) shows similar trends as the one of pre-injury state (Fig. 2A). No change of the waveform in the DMEM groups at both 1 hour (Fig. 2D) and 2 weeks (Fig. 3B) was observed. The ICP/AP in the MSC group (0.65±0.04) was 41.3% higher than in the DMEM group (0.46±0.04) at 2 weeks after transplantation (Fig. 3C, $P<0.01$). The ICP-AUC in the MSC group (5695±553 cmH_2O) was 53.7% higher than in the DMEM group (3705±527cmH_2O) at 2 weeks after transplantation. (Fig. 3D, $P<0.05$). The MSC-preloaded group displayed less ED shortly after CN injury (Fig.2) and erectile function evaluated with physiological analyses reached near normal values over the time course of two weeks (Fig.3). Collectively, these results indicate that the intravenous preload of MSCs prevented or reduced ED after RP.

4.2. *FG-positive cells in the MPGs*

To assess the degree of neuronal survival, a retrograde tracing study using FG was carried out. Representative sections from four DMEM (Fig. 4A-D) and four MSC (Fig.4 E-H) animals are shown in Fig. 4A-H. However, we counted the number of FG-positive cells in the entire MPG in both groups. The mean number of FG-positive cells was 58.3±4.7 (cells/MPG) in the MSC group as compared to the DMEM group (36.9±3.8 cells/animal) ($P<0.01$) at 2 weeks after CN injury (Fig.4I). This indicated that there was 58.0 % higher number of FG detected neurons in the MSC treated rats.

4.3. Distribution of transplanted PKH26-labelled MSCs (PKH26-MSCs) in the injured lesion site

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The PKH26-MSCs (red) survived and distributed to the lesion sites. The distribution of the transplanted PKH26-MSCs demonstrates a homing effect into the MPGs (5-10 cells/slide) and lesioned CNs (10-20 cells/slide) in relation to the neurofilament stained axons after systemic delivery (Fig. 5A, B). To determine if autofluorescence of the MSCs was present at the wavelengths used to study PKH26 fluorescence, we examined MPGs and CNs of animals infused MSCs without labeling PKH26. No red cells were observed in these lesions (Fig 5C, D).

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4.4. Expression of GDNF and Neurturin mRNA after transplantation

To investigate the expression levels of GDNF and Neurturin mRNA in the MPGs, we performed real-time quantitative RT-PCR. GDNF and Neurturin are neurotrophic factors in MPG that are important for erection [26, 27]. Relative expression levels of GDNF mRNA (Fig 6A) in MPG was significantly higher (3.6 fold) in the MSC group (7.22±2.59) compared to the DMEM group (2.02±1.27) ($P<0.01$) and the relative expression level of Neurturin mRNA (Fig 6B) in MPGs was also significantly higher (2.4 fold) in the MSC group (4.03±1.84) compared to the DMEM group (1.67±0.65) ($P<0.05$).

5. Discussion

The primary objective of this study was to develop a new approach to prevent or reduce post-operative ED with intravenous preload of MSCs in a CN injury rat model. Intravenous preload of the MSC-infused group exhibited a significant preservation of erectile function. Erectile functional in the MSC transplanted animals as assessed using ICP/AP and ICP-AUC analysis was significantly preserved compared to the control group. A retrograde tracing study using FG also demonstrated that the number

of FG-positive cells in the MPG was higher in the MSC group compared to the control group. MSCs labeled with PKH26 that is known to have a long half-life *in vivo*, were found in the both MPGs and injured CNs after the transplantation. We observed significantly higher expression of GDNF and neurturin mRNA in MPG in the MSC group by real-time quantitative RT-PCR. These results suggest that intravenous preload (delivered before the surgical procedure) of MSCs rescues erectile function by neuroprotection in a rat model of cavernous nerve injury. Given that prostatectomy surgery unlike traumatic disorders is timed, the preparation and pre-delivery of MSCs before surgery is practical. Thus, the therapeutic effects of MSCs could have a more immediate beneficial effect.

Although the post-operative ED occurred in both groups, the MSC-preloaded group displayed less ED shortly after CN injury (Fig. 2) and significant improvement at 2 weeks (Fig. 3) evaluated with physiological analyses suggesting that the ED in the MSC group gradually improved over time. Acute accumulation (less than 1 hour) of the intravenously preloaded PKH-labelled MSCs observed in the MPG neurons and injured CNs might provide neuroprotection. Fandel et al. reported that intracavernously injectioned adipose-derived stem cells were detected in the MPG and found the elevation of stromal cell-derived factor-1 (SDF-1) in the MPG [18]. SDF-1 is a chemokine that has been implicated in cell recruitment (homing) after stem cell infusion [18]. MSCs also have a tendency to home to damaged lesion sites and enhance their therapeutic effectiveness with similar molecular mechanisms [28].

Elevation of GDNF and neurturin mRNA in MPG was associated with the preservation of the erectile function in the MSC group. GDNF and neurturin are two of the neurotrophic factors acting in the MPG for erection[26]. Kato et al. demonstrated that the recovery from ED after CN crush was facilitated when GDNF and neurturin

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were administered into nerves with herpes simplex virus vector-mediated delivery[20, 23]. Another possible therapeutic mechanisms of intravenously transplanted MSCs in various models of nervous diseases are not only differentiation to neuronal and/or glial cells, but also secretion of neurotrophic factors which can provide for neuroprotection, regeneration, recruitment of progenitors, facilitation of endogenous neurotrophic secretion, induction of axonal sprouting, remyelination and modulation of inflammatory/immune responses [10, 29]. Recent studies also suggested the involvement of paracrine pathways. Paracrine action might be an important mechanism for erectile function by which transplantation of MSCs facilitate regeneration in corpus cavernosum after CN injury [30, 31].

Preloaded MSCs could circulate in the blood stream and home to the injured tissues such as MPGs and injured CNs when the insults occurred and the distributed these cells could have therapeutic efficacy for post-operative ED. In experimental erectile function, several studies using intracavernous injection of MSCs have been reported therapeutic effects[32]. Yet, there are few studies using intravenous infusion of MSCs for ED. Intravenous preloading of MSCs, which may home to MPG and injured CNs after the CN injury, would be less invasive than intracavernous injection. The other potential merit of intravenous infusion of the MSCs is that infused MSCs accumulated not only in the injured CNs but also in the MPG providing significant recovery of erectile function after CN injury. Given that intravenous infusion of MSCs derived from bone marrow has been used in a number of clinical studies with demonstrated safety the prospect of using MSCs as an adjunct therapy for damaged nerve repairs should be considered. Functional improvement after intravenously delivered MSCs has been reported in several clinical studies dealing with other neurological diseases [13, 15]. Currently, clinical trials with direct injection of adipose-

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derived MSCs for ED (ClinicalTrials.gov Identifier: NCT02087397; NCT02240823) are listed and the intravenous infusion of bone marrow-derived MSCs for other neurological diseases such as cerebral stroke, spinal cord injury are also ongoing [13, 15].

Whether intravenous infusion of MSCs for prostate cancer promotes progression or not is still controversy. There are several papers evaluating in progression of experimental prostate cancer, most of them concluded MSC had no adverse effects on progression of prostate cancer[33, 34]. We can also select the patients with low-risk by combined information of MRI, biopsy Gleason score, and preoperative PSA level before RP. Although we agree that the intravenous infusion of MSCs would be contraindicated in case of aggressive cancers [35] , twelve-year cancer specific survival was reported more than 95% for patients with low risk prostate cancer who received RP [36]. Therefore, intravenous infusion of MSCs before RP for these selected patients might be safe in terms of recurrence, progression and overall survival.

Our study has limitations. In this study, we focused on the primary change by assessment of the degree of neural survival to examine FG-positive cells in the MPG and found that the number of FG-positive cells in the MPG was higher in the MSC group compared to the control group indicating greater neuronal connectivity. We also performed the ICP/AP and ICP-AUC analysis to examine erectile function and found that post-operative ED was prohibited in the MSC group. Since we did not focus on the secondary change, we did not assess the expression of nNOS and eNOS in the cavernous nerves and in the penis, which play a major role in erection and are impaired after CN injury. Future studies will be performed to elucidate the molecular mechanism remains to be determined.

In summary our results indicate that intravenous preload of MSCs prevents or reduces post-operative ED in a CN injury model. Potential mechanisms include

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distribution of the transplanted cells to the lesion area where they may provide neuroprotection and prevent neuropraxia. It should be noted that we infused MSCs before CN injury, suggesting that preload of MSCs can rescue erectile function.. Finally it may be possible to combine MSC therapy with other current penile rehabilitation therapies to achieve maximal function.

Acknowledgement

Source of funding:

This work was supported in part by the Japanese Ministry of Education, Culture, Sports, Science and Technology, Grant-in Aid for Scientific Grant-in Aid for Scientific Research (C) to A.T (25462516) and the Rehabilitation Research and Development (RR&D) Service of the Department of Veterans Affairs (B7335R, B9260L), the National Multiple Sclerosis Society and the CT Stem Cell Research Program (12-SCB-Yale-05).

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Figure legends

Figure 1

A timeline for experiments is shown. Animals were first assessed by ICP/AP. They were randomized to two groups and preloaded (MSC and DMEM groups), then induced CN injury. The ICP/AP was measured at 1 hour and two weeks. Tissue sampling for histological analyses were performed at 1 hour and 2 weeks.

Figure 2

ICP response to electrostimulation of CNs at 1 hour. ICP along with AP (ICP/AP): (A) in the pre-injury, MSC group, (B) pre-injury, DMEM group, (C) post 1 hour, MSC group, (D) post 1 hour, DMEM group. Quantitative analyses of erectile function as evidence by ICP/AP (E) and ICP-AUC (F). ICP/AP and ICP-AUC at 1 hour after injury in the MSC group was significantly higher compared to the ICP/AP at 1 hour after injury in the DMEM group (* $P<0.05$). The ICP/AP and ICP-AUC were significantly decreased one hour after injury in the both groups (** $P<0.01$). Black bars under the x axis indicate 1min period of stimulation.

Figure 3

ICP response to electrostimulation of CNs at 2 weeks. ICP along with AP (ICP/AP): (A) MSC and (B) DMEM groups. Rats preloaded MSCs exhibited significant recovery of ICP/AP and ICP-AUC compared to the DMEM group. Quantitative analyses of erectile function as evidence by ICP/AP (C) and ICP-AUC (D). The ICP/AP and ICP-AUC at 2 weeks after injury in the MSC group was significantly higher compared to the DMEM group (** $P<0.01$, * $P<0.05$). Black bars under the x axis indicate 1min period of stimulation.

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Figure 4

Representative images of FG-positive cells in the MPGs from four animals per group 2 weeks after transplantation; (A-D) DMEM, (E-H) MSC groups. (I) The MSC group had more FG-positive cells in MPGs than DMEM group. ** p<0.01. Scale bar=250μm.

Figure 5

Transverse sections and immunohistochemical analysis of injured MPG (A) and CN (B) and intravenous preload of PKH26-MSCs. Demonstration of distributed infused PKH26-MSCs (red: white arrows) and stained with neurofilament (green). Endogenous cells stained with DAPI (blue). Note that we infused MSCs without labeling PKH26 and no red cells were observed in the MPGs (C) and CNs (D). Scale bar=150μm.

Figure 6

mRNA expression of GDNF (A) and Neurturin (B) in MPGs after intravenous preload of MSCs at 1 hour. Relative expression level of GDNF and Neurturin mRNA in MPGs was significantly higher in the MSC group (P<0.01, 0.05).

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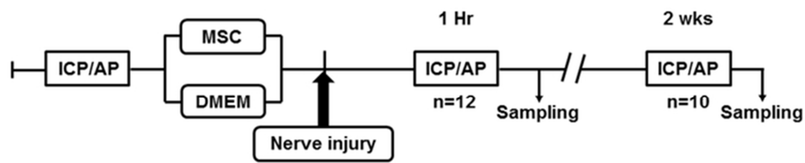

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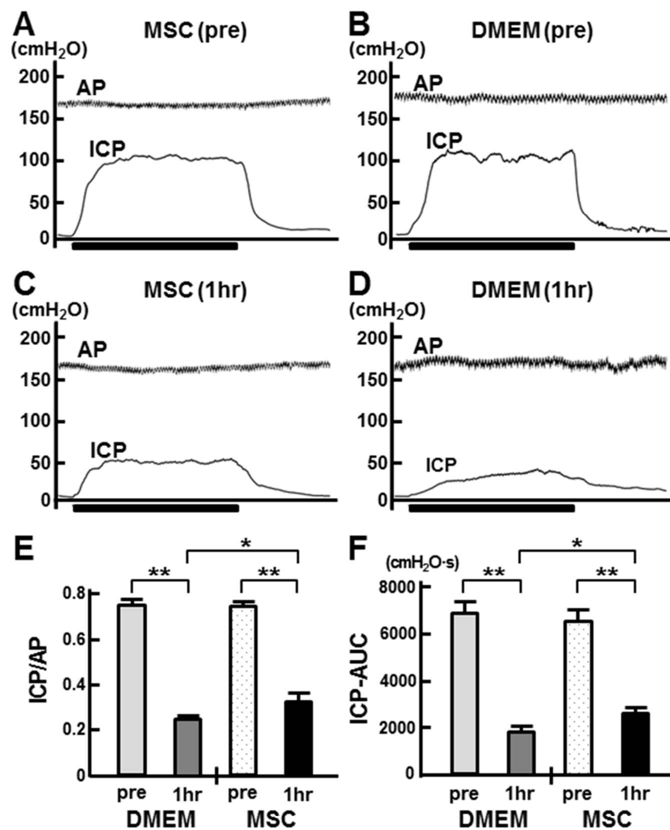

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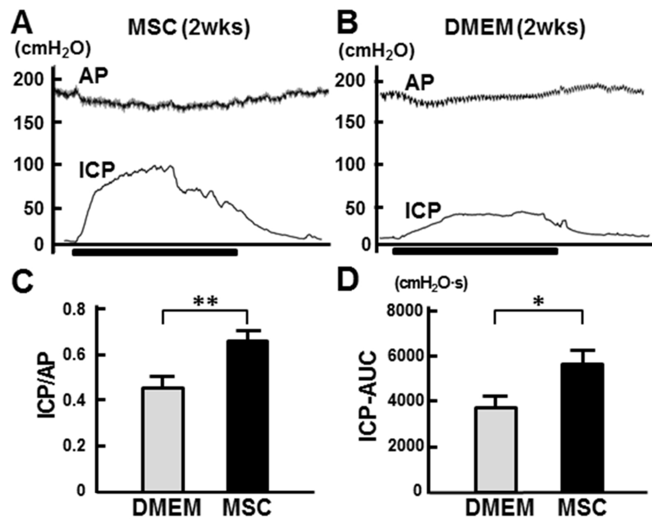

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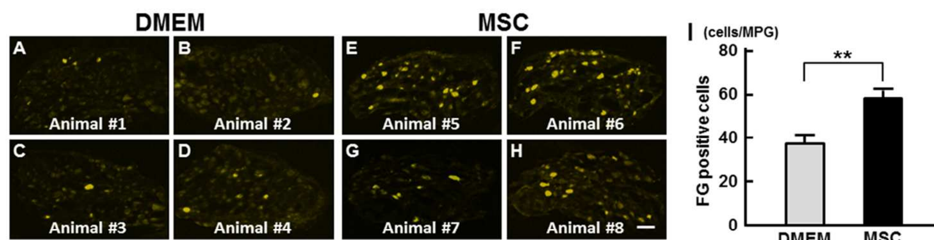

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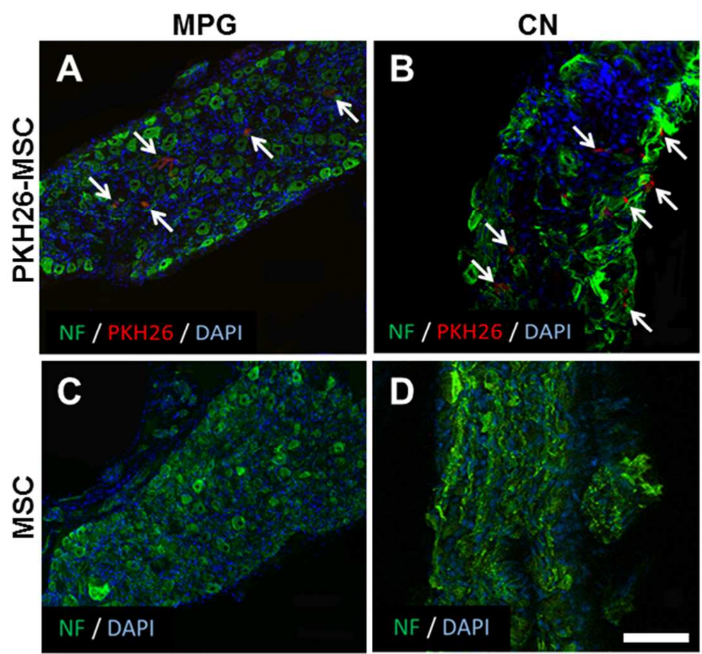

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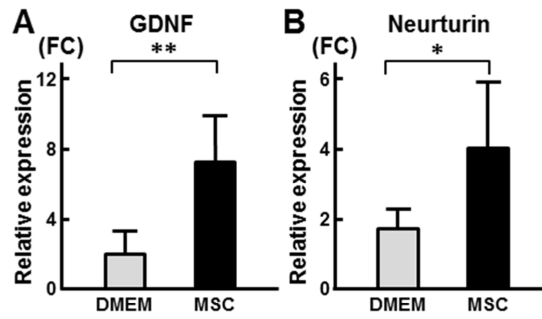

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