A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury

Dong-In Jung a, Jeongim Ha d, Byeong-Teck Kang b, Ju-Won Kim b, Fu-Shi Quan f, Jong-Hwan Lee c, Eung-Je Woo e, Hee-Myung Park b,

⁎ Corresponding author. Department of Veterinary Internal Medicine, College of Veterinary Medicine, Konkuk University, # 1 Hwayang-dong, Kwangjin-gu, Seoul 143-701, South Korea. Tel.: +82 2 450 4140; fax: +82 2 450 3037.  
E-mail address: parkhee@konkuk.ac.kr (H.-M. Park).

Department of Anatomy, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea.

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A B S T R A C T

The purpose of this study is to compare the therapeutic effects between autologous and allogenic bone-marrow-derived mesenchymal stem cell (MSC) transplantation in experimentally-induced spinal cord injury (SCI) of dogs. Thirty adult Beagle dogs (control group = 10, autologous group = 10, and allogenic group = 10) were used in this study. Prelabeled MSCs were intrathecally transplanted through the lumbar spinal cord into the injured lesion at a density of 1 × 10⁷ cells 7 days after SCI. Neurological signs of dogs in both autologous and allogenic groups were improved in their pelvic limbs after SCI compared with those in control group. Both autologous and allogenic groups showed significantly higher the Olby scores than control group (p < 0.05). This finding was consistent with results of MRI and histopathological examination in both groups. Immunofluorescence analysis revealed that prelabeled autologous and allogenic MSCs were detected in the injured lesions both at 1 and 4 weeks after transplantation. However, the distribution ratio of MSCs on the injured lesion in allogenic group was significantly decreased at 4 weeks after transplantation relatively to at 1 week after transplantation. The mRNA expression for neurotrophic factors in both allogenic and autologous groups was significantly higher than that in control groups (p < 0.05). Even though autologous MSC transplantation showed more beneficial effect than that of allogenic MSC transplantation, transplantation of allogenic MSCs also improved functional recovery following SCI. This study demonstrates that both autologous and allogenic MSC transplantation could be clinically useful therapeutic approaches for treating SCI.

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1. Introduction

Severe spinal cord injury (SCI), which leads to complete loss of sensory and motor functions, is one of the most serious neurological problems [11,22,23,25,32]. Although it has been long believed that the damaged central nervous system (CNS) does not regenerate upon injury, there is an emerging hope for regeneration-based therapy of the damaged CNS due to the progress of developmental biology and regenerative medicine including stem cell biology [18,22,25]. Previously, several cell therapy studies in experimental rodent SCI models have been reported [22,23,29,32]. In these models, transplantation of different types of cells, including Schwann cells, microglia cells, oligodendrocyte precursors, macrophages, and stem cells, partially improved the functional abilities of the animals by promoting the survival, regeneration, and remyelination of spinal axons [22,23,31,32]. Several studies have suggested that bone marrow cells are a potential source of neural progenitor cells and are clinically important in applications for neuronal tissue repairing [4,16,19,33,37]. MSCs are nonhematopoietic progenitor cells that are initially present in bone marrow [7,19,29,37]. Bone marrow-derived MSCs are also known as bone marrow stromal cells and are capable of in vitro differentiation into marrow and non-marrow cell types, such as adipocytes, chondrocytes, osteocytes, myocytes, and neurons [13,17,19,33,37]. A recent study has shown that canine bone marrow-derived MSC can form nerve-fascicle-like clumps and differentiate into neuron-like cells expressing neuronal markers [19,37]. These cells have attracted interest concerned with their capacity for self renewal in a number of nonhematopoietic tissues, their multipotentiality for differentiation, and their possible use for both cell and gene therapy. Transplantation of adult MSCs into damaged brain and spinal cord reduces functional deficits [1,4,5,12,15,28].
Bone marrow-derived MSCs are attractive for transplantation and spinal cord repair as they can be easily isolated, expanded in culture and delivered [7,19,33,37]. Several studies have evaluated the potential of bone marrow-derived MSC for treatment of SCI [1,5,30].

In stem cell research, both autologous and allogenic stem cell transplantation has exhibited considerable therapeutic potential in SCI [15,22,23,31,32]. However, all stem cell experiments in SCI have recently been separately performed by autologous or allogenic. Autologous stem cell transplantation is difficult to attempt on SCI patients in clinical medicine, because of a cell preparatory period and cell transplantation timing. Therefore, allogenic stem cell transplantation has more practical therapeutic value in clinical medicine. Unfortunately, no studies have ever tried to compare the therapeutic efficacy of autologous and allogenic stem cell transplantation in SCI.

The present study investigated the hypothesis that allogenic MSC transplantation is a clinically useful method for treating SCI compared with autologous MSC transplantation. Numerous attempts have been made by researchers to clarify the side effects of allogenic stem cell transplantation [8,14,27,32]. However, here the present study limits the discussion to therapeutic effect of autologous and allogenic MSC transplantation after SCI.

The purpose of this study is to compare the therapeutic efficacy of autologous and allogenic bone-marrow-derived mesenchymal stem cell transplantation in canine SCI.

2. Materials and methods

2.1. Animals

Thirty adult Beagle dogs (1 to 4 years old, weighing 5.0 to 12.2 kg, female; 19/male; 11) were divided into 3 groups of 10 dogs each, as follows: 1) Control group; no MSC transplantation after SCI, 2) Autologous group; autologous MSC transplantation after SCI, 3) Allogenic group; allogenic MSC transplantation after SCI. Five dogs in each group were euthanized at 2 weeks after SCI, and the other 5 dogs in each group were euthanized at 5 weeks after SCI. Additional not injured 5 normal dogs, exclusive of 30 dogs described above, were euthanized for comparative analysis of neuroprotective factor expression with 3 study groups. All dogs were treated in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

2.2. MSC isolation and characterization from canine bone marrow

MSC isolation and cultivation from bone marrow was performed according to reports described previously [16,19,33,37]. Cultured mononucleated cells were characterized by fluorescence-assisted cell sorting (FACS) analysis based on previous reports [13,29,37]. For immunophenotypic analysis, third-passage canine MSCs were stained under ice conditions, according to the manufacturer’s recommendations regarding the monoclonal antibodies (anti-CD9, anti-CD34, anti-CD44, and anti-CD45; Serotec, USA). All the antibodies used for FACS were shown by the manufacturer to cross-react with canine cells. Positive cells were detected on a Coulter Epics Elite fluorescence-activated cell sorter by using a 1:100 dilution of the secondary antibody (goat anti-mouse fluorescein isothiocyanate (FITC), Goat anti-rat FITC, Jackson Immunoresearch Laboratory, USA). Subsequently, the specimens were analyzed by flow cytometry (FACS calibur flow cytometer, BD, USA) using the CellQuest software (CellQuest, BD, USA).

2.3. SCI model

SCI was experimentally-induced using silicone balloon catheter compression methods, as described previously [11]. The dogs were anesthetized by intravenous administration of propofol (Anefol, Hana Pharm, South Korea) at 6 mg/kg with subcutaneous administration of atropine sulfate (Atropine, Jeil Pharm, South Korea) at 0.05 mg/kg. Anesthesia was maintained by inhalation of 3% isoflurane (Terrell, Minrad Inc., USA). Dogs were placed in ventral recumbency on the operating bed, and the dorsal approach was selected to spare the ligaments and the muscles between the spinous processes (between L2 and L3). When the dorsal intervertebral space between L2 and L3 was identified, the distance between the T13 and L2 spinous processes of the vertebrae was measured, and a 6-French silicone balloon catheter (Yushin Medical, South Korea) was inserted into the vertebral canal through the dorsal intervertebral space between L2 and L3. The silicone balloon catheter was inserted in the cranial direction to a distance corresponding to that measured previously between the T13 and L2 spinous processes. Positioning of the catheter was confirmed using fluoroscopy, and the balloon was then inflated to a volume of 1.5 ml in the spinal extradural space by injection of saline. The soft tissues and skin were closed as per standard methods, and the balloon was removed after 20 min.

2.4. Transplantation of MSCs after SCI

Seven days after SCI, autologous and allogenic MSCs were prelabeled with a carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) cell tracer kit (Molecular Probes, USA) and resuspended in 3 ml PBS at a density of 1 x 10^7 cells, and then transplanted into the injured lesion by an intrathecal injection between the L4 and L5 regions using 22-gauge spinal needle under fluoroscopic monitoring. MSCs were transplanted single time for each animal in autologous and allogenic groups under general anesthesia with xylazine (Rompun, Bayer Korea, South Korea: 1.1 mg/kg, IV) and ketamine (Ketamin, Yuhan Corporation, South Korea: 10 mg/kg, IV). In control group, only 3 ml PBS solution was transplanted at 7 days after SCI using the same methods as those used for autologous and allogenic group.

2.5. Behavioral analysis based on the Olby score

Behavioral analysis was performed before operation and at 1, 2, 3, 4, and 5 weeks after SCI in order to assess the functional recovery of the pelvic limbs after SCI. Behavioral recovery was scored according to the Olby scoring system [26], which is composed of 15 different criteria. The Olby scoring system is the modified scoring system for dogs based on pelvic limbs, and confirmed the reliability by several investigators [10,24,25]. The gait of dogs were recorded using video camera, and two different investigators scored the gait.

2.6. Magnetic resonance imaging (MRI)

A 3.0-Tesla MRI system (Magnum3, Medius, Korea) was used to examine the location, extent, and progress of each injury. All dogs in each group were examined at 1, 2, and 5 weeks after SCI (at 1 and 4 weeks after MSC transplantation in autologous and allogenic group). Both T1- (TR/TE = 550.0/12.4) and T2-weighted (TR/TE = 4400.0/ 96.0) transverse and sagittal images with a scan thickness of 4 mm were obtained under general anesthesia with xylazine (1.1 mg/kg, IV) and ketamine (10 mg/kg, IV). The size of injured spinal cord lesion which showed hypointense or hyperintense signals were calculated from midsagittal view MR images using an image analyzer program (Image, version 1.38; National Institutes of Health, USA). The size of lesion at 1 and 5 weeks after SCI was calculated and analyzed among three groups.

2.7. Postmortem examination, histopathological and immunohistochemical analysis

Postmortem examination, histopathological and immunohistochemical changes were evaluated in the following manner: 1) Five dogs in each group were euthanized at 2 weeks after SCI, and 2) Five
dogs in each group were euthanized at 5 weeks after SCI. In all dogs, the spinal cord from T5 to L5 was sampled and separated to 3 sections (T5–T6, T12–L1, and L4–L5). The half of each section was frozen with liquid nitrogen. Slices (4 μm thick) were cut from the each 3 sections and mounted on silane-coated slide glasses. The slides were stained with hematoxylin and eosin (H & E) to identify vacuolar and cavity formation. The volumes of cystic cavities in each slide were calculated using an image analyzer program (ImageJ, version 1.38; National Institutes of Health, USA) according to a report described previously [25]. First, the area of spinal cord was calculated from image of the transverse section at the epicenter of the injured spinal cord. And then, the area of cystic cavity was calculated from the same image. Subsequently, percentage of cavity formation area in transverse section of the epicenter of injured spinal cord was analyzed.

For immunohistochemical analysis, the sections on each slide were incubated in 10% bovine serum albumin with the anti-glial fibrillary acidic protein (GFAP) (rabbit anti-GFAP, Chemicon, USA; 1:1000) and anti-neuronal nuclei (NeuN) (Mouse anti-NeuN, Chemicon, USA; 1:100) at 4 °C overnight. After washing, secondary antibodies (goat anti-mouse Alexa 647 and goat anti-rabbit Alexa 647, Molecular Probes, USA; 1:100) were added and incubated for 1 h. To determine whether the transplanted CFDA-SE prelabeled MSCs had successfully migrated into the injured spinal cord lesion and could influence to glial cell and neuron regenerations in the injured spinal cord, immunofluorescence colocalization studies using a laser-scanning confocal microscope (FV-1000 spectral, Olympus, Japan) were performed.

2.8. Neurotrophic factor expression in the SCI lesion

Total RNA from the injured spinal cord (T13–L1; frozen tissue section) was isolated using TRIZOL (Invitrogen, USA), according to the manufacturer’s instructions. One microgram RNA was used for reverse transcription (RT). Using canine-specific primers, the polymerase chain reaction (PCR) was performed for the glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), stromal cell-derived factor (SDF), and nerve growth factor (NGF) from the cDNA of the injured spinal cords of all dogs. The initial denaturation step was at 94 °C for 5 min. This was followed by denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The sequences of the primers used in the PCR of the neurotrophic factors are summarized in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The band from RT-PCR was analyzed by densitometry using an image analyzer program (ImageJ, version 1.38; National Institutes of Health, USA). Ratio of each neurotrophic factors to GAPDH density values was analyzed.

2.9. Statistical analysis

All data in the present study are shown as mean ± SD. A probability value of p < 0.05 was considered to be statistical significance. Statistical analyses were performed by using SPSS (version 12. 0 KO, USA). Repeated measure Kruskal–Wallis test (nonparametric method) followed by Bonferroni corrected Mann–Whitney U test (nonparametric method) were used to compare three groups.

3. Results

3.1. Characterization of canine MSCs

Approximately 4–5 days after the bone marrow-derived mononuclear cells were seeded, they began to attach to the culture dish and proliferate in colonies. Cells with differing morphological characteristics were observed in the culture; these included loosely attached round cells and firmly attached spindle-shaped cells. The nonadherent round cells were removed while changing the culture medium, and spindle-shaped cells were predominantly observed. These spindleshaped cells were long, flattened cells exhibiting a fibroblastic morphology. Morphologically, the canine MSCs appeared very similar to MSCs of other species.

Fig. 1 shows the cell-surface antigen profile of the spindle-shaped cells, as determined by flow cytometry. The cells were positive for CD9 and CD44, and negative for CD34 and CD45. The negative results obtained for CD34 and CD45 demonstrated that cells of hematopoietic origin were excluded during the MSC culture. The positive results obtained for CD9 and CD44 were consistent with previous reports on MSCs isolated from other species [22,33,37].

3.2. Behavioral analysis based on the Olby score

During the study period, the functional behavior of each group of animals was evaluated using the Olby score (Fig. 2). All injured dogs manifested complete pelvic limb paralysis after SCI. The Olby score of all groups was 14 before SCI. The Olby score of control, autologous and allogenic was zero at 1 week after SCI. Following transplantation of the MSCs in autologous and allogenic group, functional recovery gradually increased. At 1 week after transplantation, dogs in autologous and allogenic group had an Olby score of 1.8 ± 0.44 and 1.6 ± 0.54, respectively. At 4 weeks after transplantation, dogs in autologous and allogenic group had an Olby score of 5.4 ± 0.54 and 3.2 ± 0.83.
respectively. The scores were in the range of 0–1 (0.2 ± 0.44) in control group at 5 weeks after SCI.

### 3.3. MRI analysis

MRI studies were performed at baseline to determine the size and location of the injury (Fig. 3). Most injured dogs belonging to control, autologous and allogenic group revealed hyperintense signals on T2-weighted images at 1 week after SCI at the T13–L1 lesion (19/30 dogs). Several injured dogs showed hypointense signals (6/30 dogs) or mixed-intense signals (5/30 dogs) on T2-weighted images that suggested hemorrhages at 1 week after SCI at the same region.

At 5 weeks after SCI, the T2-weighted images showed that the lesion in control group had expanded cranially and caudally with hypointense signals. The areas of the lesions at 4 weeks after MSC transplantation in dogs from autologous and allogenic group also showed hypointense signals on T2-weighted images. These changes were not evident on T1-weighted images.

The size of injured spinal cord lesion at 1 week after SCI in control, autologous, and allogenic groups were 594.8 ± 254.4, 763.8 ± 314.7, and 710.2 ± 283.2, respectively. And the size of injured spinal cord lesion at 5 weeks after SCI in control, autologous, and allogenic groups were 1583.8 ± 608.1, 878 ± 578.2, and 884.8 ± 131.6, respectively (*p < 0.05, autologous versus control, and **p < 0.05, allogenic versus control) (Table 2). There was not significant difference among three groups at 1 week after SCI. However, the size of injured spinal cord lesion was significantly reduced in autologous and allogenic groups at 5 weeks after SCI when compared with those in control group.

### 3.4. Postmortem examination, histopathological and immunohistochemical analysis

On postmortem gross findings, there were no significant differences among the 3 groups. At 2 weeks after SCI, myelomalacic changes were observed in all groups (Fig. 4A). At 5 weeks after SCI, atrophic and fibrotic changes were detected in all groups. The yellowish connective tissues were infiltrated into the damaged site and replaced (Fig. 4B).

Histopathological analysis with H & E staining revealed severe parenchymal damages in both the white and grey matter. At 2 weeks after SCI, hemorrhage and diffused parenchymal necrosis with vacuolar formations were detected in all groups (Fig. 4C). However, epidural and subarachnoid hemorrhages were more severe in control group than in MSC-transplanted groups. All dogs in control group showed severe hemorrhages. At 5 weeks after SCI, diffused parenchymal necrosis with cystic cavity formation was observed in all groups (Fig. 4D). The percentage of vacuolar formation area in transverse section of the epicenter of injured spinal cord at 2 weeks after SCI in control, autologous, and allogenic groups were 89.3 ± 0.32, 84.2 ± 3.24, and 88.9 ± 0.21, respectively. And, the percentage of cavity formation area in transverse section of the epicenter of injured spinal cord at 5 weeks after SCI in control, autologous, and allogenic groups were 36.08 ± 4.51, 8.13 ± 1.89, and 12.96 ± 2.5, respectively (*p < 0.05, autologous versus control, **p < 0.05, allogenic versus control, and †p < 0.05, autologous versus allogenic) (Table 3). Although the percentage of cavity formation area was more reduced in autologous group than in allogenic group, these results indicated that the area of the cystic cavity was significantly more reduced and the residual white matter area was higher in autologous and allogenic group than in control group.

In confocal microscopic analysis, CFDA-SE prelabeled autologous and allogenic MSCs were detected on the injured lesion both at 1 and 4 weeks after transplantation (autologous; Fig. 5, allogenic; Fig. 6). The prelabeled MSCs were centralized in the epicenter of injured spinal cord lesion and were not detected in the uninjured spinal cord regions. The numbers of CFDA-SE prelabeled MSCs on the epicenter of injured spinal cord were calculated using a FLUOVIEW program in confocal microscope system. The number of prelabeled MSCs was counted in ten randomly selected areas (×400) under confocal microscopy. Based on these data, the average number of prelabeled cells per area between at 1 week and at 4 weeks after transplantation was calculated and compared relatively (Table 4). In autologous group, the distribution numbers of transplanted MSCs that had migrated to the injured spinal cord lesion between at 1 week and 4 weeks after transplantation demonstrated no significant differences. In allogenic group, however, the distribution numbers of transplanted MSCs in the injured site was significantly decreased at 4 weeks after transplantation in comparison with the number at 1 week after transplantation.

The immunofluorescence colocalization study between prelabeled MSCs and NeuN/GFAP positive area in the epicenter of injured spinal cord was analyzed by FLUOVIEW program in confocal microscope.
The results were showed as merged reconstruction images (Figs. 5 and 6). At 1 week after transplantation, the majority of transplanted MSCs were partially colocalized with GFAP and NeuN in both autologous and allogenic group (Figs. 5A and 6A). At 4 weeks after autologous MSC transplantation, the ratio of non-colocalized GFAP and NeuN positive areas surrounding the nearby transplanted MSCs was signifi-

cantly increased (Fig. 5B). At 4 weeks after allogenic MSC transplantation, the few remaining transplanted MSCs showed partial colocalization with GFAP and NeuN. Non-colocalized GFAP positive areas are also observed, while non-colocalized NeuN positive areas are occasionally observed (Fig. 6B). The percentage of dual labeled cells was calculated in ten randomly selected colocalized areas (×400) under confocal microscopy. Based on these data, the average percentage of dual labeled cells per area between at 1 week and at 4 weeks after transplantation was calculated (Table 5). In autologous and allogenic groups, the average percentage of dual labeled cells

Fig. 3. T2-weighted sagittal MR images of the spinal cord of dogs from control, autologous and allogenic group. Panels A-1, B-1, C-1, D-1, E-1, F-1, G-1, and H-1 show MR images at 1 week after SCI (circles), and panels A-5, B-5, C-5, D-5, E-5, F-5, G-5, H-5, and I-5 show MR images of same dogs at 5 weeks after SCI (rectangles). Panels A-1, C-1, G-1 indicate hyperintense signals on T2-weighted images 1 week after SCI. Panels E-1, H-1, and I-1 show hypointense signals on T2-weighted images at 1 week after SCI, and panel B-1, D-1, and F-1 shows mixed-intense signals on T2-weighted images at 1 week after SCI. At 5 weeks after SCI, T2-weighted images show that the lesion had expanded cranially and caudally with hypointense signals (A-5, B-5, C-5, D-5, E-5, F-5, G-5, H-5, and I-5). Control group: A, B, C; Autologous group: D, E, F; Allogenic group: G, H, I.
expression levels in allogenic group were markedly decreased
than in control group (Fig. 7B). Whereas, TGF-β, VEGF, and SDF-1 expression levels in autologous group were higher among groups with respect to the expression of BDNF, bFGF, and NGF. There was no difference while GDNF was not expressed in any group. There was no difference of therapeutic efficacy after severe damage. These MRI findings in the present study are in agreement with several prior reports [20,25]. According to one previous report, most of dogs transplanted allogenic umbilical cord blood-derived MSC after SCI showed improvement on MRI, and one dog showed a normointense intramedullar lesion at 8 weeks after transplantation [25].

Postmortem gross findings showed no difference among three groups. And histopathological findings at 2 weeks after SCI also showed no significant differences. However, histopathological findings at 5 weeks after SCI indicated that autologous and allogenic MSC transplantation reduced cystic cavity formation after crushing damages in spinal cord compared to control group. These results did not mean that transplanted MSCs promoted remyelination to neuronal repair. Nevertheless, it is likely that autologous and allogenic MSC transplantation protected spinal cord tissue against damage.

When MRI findings were compared to histopathological findings, hypointense signal areas on T2-weighted images at 1 week after SCI indicated parenchymal edema and myelomalacic changes after injury. Likewise, hypointense or mixed-intense signal areas on T2-weighted images at 1 week after SCI suggested hemorrhage or characteristics of blood ingredients in injured site. However, similar hypointense signal area on T2-weighted images at 5 weeks after SCI revealed fibrotic changes of injured lesion. Although MRI findings at 1 week after SCI indicated heterogeneity, all dogs showed paraplegia on pelvic limbs after SCI. With regard to behavioral status, heterogeneity of MR images might be not affecting the validity of this experiment.

To investigate the fate of the transplanted MSCs, MSCs in this study were prelabeled with fluorescent dye before transplantation. Many previous reports described that the fluorescent dye using in this study (CFDA-SE) was detected up to more than 4 weeks after labeling without leakage [22,34]. Furthermore, the label is inherited by daughter cells after cell division, or cell fusion and is not transferred to adjacent cells in a population [35,36]. In autologous and allogenic groups, exogenous transplanted MSCs were observed to migrate toward the injured spinal cord lesion successfully. One week after autologous and allogenic MSC transplantation, the transplanted cells were partially colocalized with GFAP and NeuN positive areas. Furthermore, at 4 weeks after MSCs transplantation, the ratio of endogenous GFAP and NeuN positive areas surrounding the nearby transplanted MSCs was increased. These results suggested the possibility that exogenous MSCs were provided a pleasant environment for neuronal repair through immunosuppressive, anti-inflammatory, and trophic effects of MSCs described as previously [30]. This result and hypothesis is in agreement with a prior report that showed new neuronal formation in the injured structures of the spinal cord after MSC transplantation [25]. In the allogenic MSC-transplanted groups, however, the distribution number of transplanted allogenic MSCs on injured area was significantly decreased at 4 weeks after transplantation. The author supposed that some difference of therapeutic efficacy between autologous and allogenic group might be due to the survival rate of MSCs at injured area. According to previous reports, remarkable immunological responses

were decreased at 4 weeks after transplantation compared to at 1 week after transplantation, because non-colocalized GFAP and NeuN positive areas surrounding the nearby transplanted MSCs was significantly increased at 4 weeks after transplantation.

### 3.5. Neurotrophic factor expression in the SCI lesion

To investigate the action of transplanted MSCs in injured spinal cord lesion, the expression of the mRNA species encoding for neurotrophic factors that are known to neuroprotect the injured area was examined.

Results from the RT-PCR analysis revealed that BDNF, bFGF, NGF, TGF-β, VEGF, and SDF-1 were expressed in all samples of each group, while GDNF was not expressed in any group. There was no difference among groups with respect to the expression of BDNF, bFGF, and NGF. Furthermore, there was no significant difference between normal and control group.

At 2 weeks after SCI (at 1 week after MSC transplantation), TGF-β, VEGF, and SDF-1 expression levels in autologous and allogenic groups were significantly higher than in normal and control groups (Fig. 7A).

At 5 weeks after SCI (at 4 weeks after MSC transplantation), TGF-β, VEGF, and SDF-1 expression levels in autologous group were higher than in control group (Fig. 7B). Whereas, TGF-β, VEGF, and SDF-1 expression levels in allogenic group were markedly decreased at 4 weeks after transplantation as compared to the other groups (Fig. 7B).

### 4. Discussion

The majority of previous studies have employed parenchymal injection of cells directly into the lesion site at a density of 1 to 4 × 10⁶ cells [5,25,32]. This method was designed to efficiently deliver cells into the injured site. However, this method can be applied after laminectomy in canine models and have risk of secondary damage to the already injured spinal cord. In addition, many spinal cord diseases in small animals are multifocal diseases and would require delivery of engrafted cells over extensive, non-contiguous areas. This study demonstrated that transplanted MSCs by an intrathecal injection were effectively delivered to the injured spinal cord without dispersion to undesired areas. Furthermore, large number of MSCs (1 × 10⁷ cells) was transplanted in this study, based on a previous study that the intrathecal cell delivery should allow larger volume than other transplantation methods [6].

The Olby scoring system enabled to accurately quantify the extent of recovery of dogs following SCI in this study. Dogs receiving autologous and allogenic MSC transplantation had improved neurofunctional recovery in their pelvic limbs after SCI in comparison with those that did not receive any treatment after SCI. In this study, autologous group showed more efficient result compared to allogenic group. However, allogenic group also showed significant improvement of spinal cord functional recovery after severe SCI. The important point to note is that both autologous and allogenic MSC transplantation had beneficial effect to rehabilitate locomotor and nociceptive function in SCI dogs from a therapeutical point of view. Although there was no evidence, the author supposed that transplanted MSCs enhanced spinal cord functional recovery by neuronal repair.

The valuable effects of autologous and allogenic MSC transplantation were also observed in results of MRI. To determine the efficacy of autologous and allogenic MSC transplantation, the size of injured spinal cord lesion among three groups at 1 week and 5 weeks after SCI was measured and analyzed. The size at 1 week after SCI showed no significant differences among three groups. However, the sizes in autologous and allogenic groups were significantly reduced compared to that of control group at 5 weeks after SCI. As a result, it is likely that both autologous and allogenic MSC has neuroprotective efficacy after severe damage. These MRI findings in the present study are in agreement with several prior reports [20,25]. According to one previous report, most of dogs transplanted allogenic umbilical cord blood-derived MSC after SCI showed improvement on MRI, and one dog showed a normointense intramedullar lesion at 8 weeks after transplantation [25].

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### Table 2

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<th>Groups</th>
<th>Post injury</th>
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<td></td>
<td>1 week[^a]</td>
<td>5 weeks[^a]</td>
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<td>Control</td>
<td>594.8 ± 254.4</td>
<td>1583.8 ± 608.1</td>
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<tr>
<td>Autologous</td>
<td>763.8 ± 314.7</td>
<td>878 ± 578.2⁹</td>
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<td>Allogenic</td>
<td>710.2 ± 283.2</td>
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[^a]: p < 0.05, autologous versus control, and **p < 0.05, allogenic versus control. ^: Size was calculated by image analyzer program. All values are mean ± SD.
to transplanted allogenic stem cells were observed, and transplanted cells disappeared several weeks after transplantation [8,32]. In the present study, dogs in allogenic group were not treated with immunosuppressant drugs; thus, transplanted allogenic MSCs may have been destroyed by immunological responses. Therefore, treatment with immunosuppressant drugs could be necessary to suppress the immunological rejection for the allogenic cells in allogenic MSCs transplantation. In this dual labeling experiment, it seems that GFAP positive cells and NeuN positive cells exceed considerably almost 100%, indicating that many of the MSCs express...
both GFAP and NeuN. This suggests that the cells did not actually transdifferentiate into specific neural lineages, but express these markers in a promiscuous manner. Moreover, the significant decrease in MSC numbers in the transplanted spinal cord between weeks 2 and 5 suggested that the recovery was not due to transdifferentiation and regeneration by graft cells, but rather by their trophic and protective properties.

To investigate the action of transplanted MSCs in injured spinal cord lesion, the expression of the mRNA species encoding for neurotrophic factors that are known to neuroprotect the injured area was examined. According to many previous reports, these factors play a significant role in damaged neuronal tissue after stem cell transplantation [2,3,4,9,18,21]. Thus, specific cytokines such as BDNF, bFGF, NGF, TGF-β, VEGF, GDNF, and SDF-1 were measured in the present study.

In the present study, SDF-1, VEGF, and TGF-β were significantly upregulated in MSC-transplanted dogs in comparison with normal and control dogs. According to prior reports, SDF-1 can promote the survival of neurons and help support neurons that have been damaged by injury or inflammation [3,9,18]. Furthermore, one study strongly suggested that SDF-1 is itself a migratory chemoattractant for neural crest cells [3]. It is well known that VEGF plays an important regulatory role in vascular growth and development [21]. Several studies have shown that VEGF is neurotrophic and neuroprotective and is independent of any vascular component [18,21]. Further, it plays a potentially pleiotropic role in CNS development and repair. TGF-β is also known to protect neurons against damage [2]. And TGF-β is known to have immunoregulatory effects in damaged areas [30]. Therefore, increases in the expression levels of SDF-1, VEGF, and TGF-β after MSC transplantation in this study demonstrated that MSCs promote the secretion of neurotrophic factors in order to enhance the generation of new neural tissue for the repair of injured areas of the spinal cord. The expression levels of these factors significantly increased at 1 week and 4 weeks after transplantation in autologous group. In allogenic group, the expression levels of these factors also

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Cavity formation (%)</th>
<th>2 weeks after SCI</th>
<th>5 weeks after SCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>89.3 ± 0.32</td>
<td>36.08 ± 4.51</td>
<td></td>
</tr>
<tr>
<td>Autologous</td>
<td>84.2 ± 3.24</td>
<td>8.13 ± 1.89†</td>
<td></td>
</tr>
<tr>
<td>Allogenic</td>
<td>88.9 ± 0.21</td>
<td>12.96 ± 2.51†</td>
<td></td>
</tr>
</tbody>
</table>

All values are mean ± SD.

*p < 0.05, autologous versus control, **p < 0.05, allogenic versus control, and †p < 0.05, autologous versus allogenic.

* Area was calculated by image analyzer program.

Fig. 5. Immunofluorescence colocalization studies of autologous group at 1 week (A) and 4 weeks (B) after MSC transplantation. Double positive colocalized areas for MSCs and GFAP, MSCs and NeuN are indicated in confocal microscopic images. Non-colocalized GFAP and NeuN positive areas surrounding the nearby transplanted MSCs are also observed (B: big arrows) (scale bar = 20 μm).
increased at 1 week after MSC transplantation. However, the levels of these factors were markedly decreased at 4 weeks after transplantation. These results suggested that the immunological response to allogenic MSCs promoted the reduction of the nerve growth factor expression at the injury site. Nevertheless, it is clear that allogenic MSC transplantation promote the secretion of neurotrophic factors in early stage to protect the neuronal tissue against damage.

In the present study, there are several limitations should be noted. First, this study did not considered side effects of allogenic MSC transplantation. Second, the period of this study was relatively short. Third, comparatively small numbers of experimental dogs were used in this study. Therefore, more extensive, more controlled, and more long-term period investigations are needed to validate the conclusion of the present study. Moreover, further study about side effect is needed to better understand availability of allogenic MSC transplantation in clinical medicine.

It was obvious that autologous MSCs exhibited more beneficial therapeutic potential than allogenic MSCs. Furthermore, the majority of allogenic MSCs disappeared in the injured spinal cord area at 4 weeks after transplantation. However, allogenic MSCs had own advantages in view of the practical aspect. The present study focused

**Table 4**
The number of prelabeled MSCs on the epicenter of injured spinal cord in ten randomly selected areas (×400) at 1 and 4 weeks after transplantation.

<table>
<thead>
<tr>
<th>Group</th>
<th>The average numbers of prelabeled MSCs a (cells/area)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week after transplantation</td>
</tr>
<tr>
<td>Autologous</td>
<td>356 ± 55.4</td>
</tr>
<tr>
<td>Allogenic</td>
<td>312 ± 43.6</td>
</tr>
</tbody>
</table>

The average number of cells per area was calculated.

a The number was calculated by FLUOVIEW program in confocal microscope system. All values are mean ± SD.

b p<0.05, 1 week after transplantation versus 4 weeks after transplantation.

**Table 5**
The percentage of dual labeled cells in ten randomly selected colocalized areas (×400) at 1 and 4 weeks after transplantation.

<table>
<thead>
<tr>
<th>Group</th>
<th>The average percentage of dual labeled cells a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week after transplantation</td>
</tr>
</tbody>
</table>
| Autologous| (MSC and GFAP) 82.2 ± 11.4                      | 39.5 ± 12.6
|           | (MSC and NeuN) 86.5 ± 4.8                       | 34.8 ± 7.6
| Allogenic | (MSC and GFAP) 89.2 ± 5.9                       | 61.1 ± 5.1
|           | (MSC and NeuN) 90.6 ± 4.3                       | 69.2 ± 8.3

The average percentage of dual labeled cells per area was calculated.

a The percentage was calculated by FLUOVIEW program in confocal microscope system. All values are mean ± SD.

b p<0.05, 1 week after transplantation versus 4 weeks after transplantation.
on the comparison of therapeutic efficacy without consideration of the side effects of allogenic MSC transplantation.

Therefore, this study demonstrates that both autologous and allogenic MSC transplantation could be clinically useful therapeutic approaches for treating severe SCI.

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References


Fig. 7. Results of RT-PCR and densitometric analysis of neurotrophic factor expression at 2 weeks (A) and 5 weeks (B) after SCI. (A) TGF-β, VEGF, and SDF-1 expression levels in autologous and allogenic groups were significantly higher than in normal and control groups. (B) TGF-β, VEGF, and SDF-1 expression levels in autologous group were higher than in control group and allogenic group. In allogenic group, TGF-β, VEGF, and SDF-1 expression levels were markedly decreased as compared to the other groups (**p<0.05, autologous versus control, *p<0.05, autologous versus control and 1p<0.05, autologous versus allogenic).


