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A DISSERTATION  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Effect of brain derived neurotrophic factor-  
expressing mesenchymal stromal cells and  
chondroitinaseABC on chronic canine  
spinal cord injury**

개의 만성 척수 손상에 대한 뇌유래신경인자발현  
중간엽줄기세포와 콘드로이치네이즈ABC의  
신경 재생 효과

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February 2017

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the Graduate School of Seoul National University  
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for the degree of Doctor of Philosophy in Veterinary Surgery

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# **Effect of brain derived neurotrophic factor-expressing mesenchymal stromal cells and chondroitinaseABC on chronic canine spinal cord injury**

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## **ABSTRACT**

Successful repair of spinal cord injury (SCI) is a major issue in veterinary neurosurgery. Multipotent mesenchymal stromal cells (MSCs) have effective potentials of neuronal regeneration. Besides, chondroitinaseABC (chABC) and neuronal factors such as brain-derived neurotrophic factor (BDNF) are widely investigated to repair chronic SCI. Single treatments with those factors, for neurotropic effects or lysis of chondroitin sulfate proteoglycans as barrier to neuronal regeneration have been evaluated. It was hypothesized that combination therapy with factors having different traits could be more effective

than single treatment.

The studies were composed of two parts. First, combinational therapy of canine adipose tissue derived MSCs (cADMSCs) and chABC was evaluated in relation to functional recovery and neuronal regeneration. Second, local injections of chABC and BDNF-expressed cADMSCs with intravenous injection of cADMSCs were evaluated.

In the first chapter, it was clarified that 5 U/mL chABC did not have a harmful effect on the viability of cADMSCs. The dogs treated with cADMSCs + chABC and cADMSCs showed significantly better functional recovery 8 weeks after transplantation compared with the negative control and chABC groups ( $p < 0.05$ ). In addition, the combination of cADMSCs and chABC increased the expression of digested chondroitin sulfate proteoglycans (CSPGs),  $\beta$ 3-tubulin, and neurofilament microtubule (NF-M). However, the levels of cyclooxygenase2 (COX2) ( $p < 0.05$ ) and tissue necrosis factor  $\alpha$  (TNF $\alpha$ ) were higher in the treatment groups than in the control. Transplantation of cADMSCs + chABC was more effective in improving clinical signs and neural regeneration, but a strategy for anti-inflammation after the treatment for chronic SCI would be needed for further improvement. Higher expressions of inflammation markers could have negative effect on the microenvironment of the injured spinal cord. Neurotrophic factors were not detected even after transplantation of cADMSCs. To improve the clinical outcome after the SCI, the suitable methods that decrease inflammation and increase neurogenic

factors are needed.

In the second chapter, combinatorial transplantation of chABC and cADMSCs genetically modified to secrete BDNF with intravenous administration of cADMSCs were investigated. BDNF-expressing MSCs (MSC-BDNF) were generated using a lentivirus packaging protocol. The dogs in the chABC/MSC-BDNF included groups had significantly improved functional recovery 8 weeks after transplantation compared to those in the chABC/MSC-GFP group. The animals in the chABC/MSC-BDNF/IV group showed significant improvements in functional recovery at 6, 7, and 8 weeks compared to those in the chABC/MSC-BDNF group ( $p < 0.05$ ). Fibrotic changes were significantly decreased in the chABC/MSC-BDNF/IV group. Significant decreases in the expression levels of  $\text{TNF}\alpha$ , interleukin-6 (IL-6), COX2, glial fibrillary acidic protein (GFAP), and galactosylceramidase (GalC) and increased expression levels of BDNF,  $\beta$ 3-tubulin, NF-M, and nestin in the chABC/MSC-BDNF/IV group were observed. These findings suggested that degradation of the glial scar by chABC combined with the presence of neurotrophic factors released by of the transplanted MSCs secreting BDNF can enhance functional recovery after chronic SCI.

It was demonstrated that lentiviral-mediated BDNF gene modification of cADMSCs allowed for stable BDNF production. In addition, chABC combined with cADMSCs transplantation, along with the IV administration of cADMSCs promoted clinical recovery in the injured spinal cord via microenvironment

modification, anti-inflammation, and neuronal regeneration. Thus the combinatorial treatment of direct and intraveonus injections of BDNF expressing cADMSCs and chABC has a good therapeutic potential in the treatment of chronic SCI and this can be used as an alternative treatment modality in neuronal regeneration.

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**Keywords: mesenchymal stromal cells, canine spinal cord injury, chondroitinase ABC, brain-derived neurotrophic factor, intravenous injection**

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## LIST OF ABBREVIATIONS

<b>BBB</b>	Basso, Beattie, and Bresnahan
<b>BDNF</b>	Brain-derived neurotrophic factor
<b>cADMSCs</b>	Canine adipose tissue derived mesenchymal stromal cells
<b>chABC</b>	ChondroitinaseABC
<b>CNS</b>	Central nervous system
<b>CSPGs</b>	Chondroitin sulfate proteoglycans
<b>COX2</b>	Cyclooxygenase2
<b>FITC</b>	Fluorescein isothiocyanate
<b>GalC</b>	Galactosylceramidase
<b>GFP</b>	Green fluorescent protein
<b>GFAP</b>	Gial fibrillary acidic protein
<b>H&amp;E</b>	Hematoxylin and eosin
<b>HO-1</b>	Heme oxygenase-1
<b>IHC</b>	Immunohistochemistry
<b>IL-6</b>	Interleukin-6
<b>MMP-2</b>	Matrix metalloproteinases-2
<b>MSCs</b>	Mesenchymal stromal cells
<b>NF-M</b>	Neurofilament microtubule
<b>NGF</b>	Nerve growth factor
<b>NT-3</b>	Neurotrophin-3
<b>PBS</b>	Phosphate-buffered saline

<b>pSTAT3</b>	Phosphorylated signal transducer and activator of transcription 3
<b>ROS</b>	Reactive oxygen species
<b>SCI</b>	Spinal cord injury
<b>TNF<math>\alpha</math></b>	Tissue necrosis factor $\alpha$

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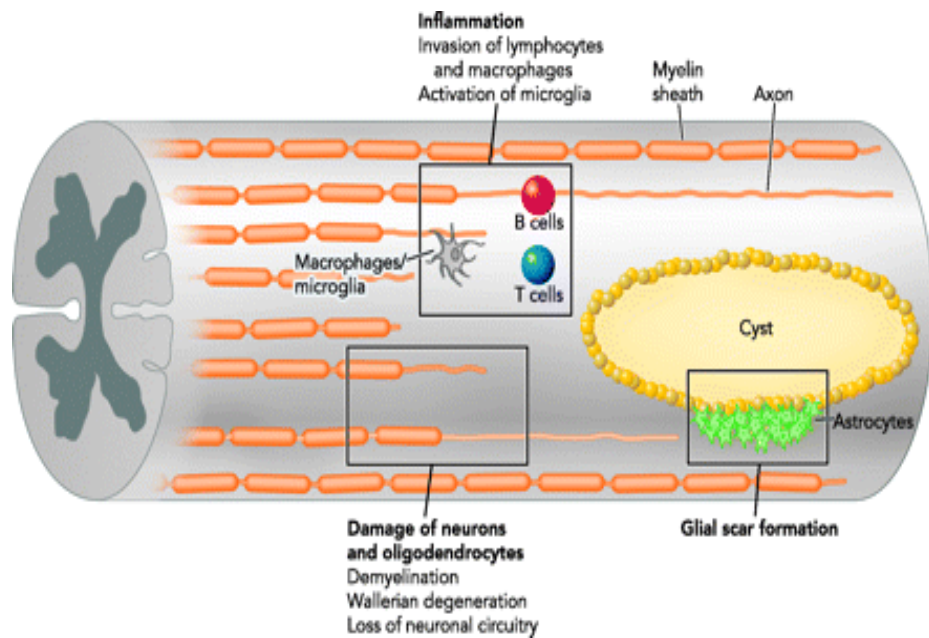
# **BACKGROUNDS**

## **1. Pathophysiology of SCI**

The regeneration of injured spinal cord occurs at very low levels spontaneously due to multiple factors. Inflammation, extensive cell loss, glial scarring, axonal disruption, and growth inhibitory molecules in glial scar prevent the regeneration of axons at the injured site (Fig. 1)(Somers 2001). To ameliorate these circumstances, it is necessary to know the injury mechanisms, which are primary mechanical damage due to spinal cord injury (SCI) results in local hemorrhage, edema, and tissue necrosis. Subsequently, the secondary events, including ischemia, anoxia, inflammation, apoptosis, and free radical production, occur over a period of hours to days post-SCI (Yick et al. 2000, Lu et al. 2005, Lee et al. 2010, Sharma et al. 2012). Of these, oxidative stress caused by reactive oxygen species (ROS) that yield free radical formation is the major contributor to secondary damage. Extravasated fluid from hemorrhages causes hematoma formation. The resulting exposure of the injured spinal cord to hemoglobin and other products leads to the generation of free radicals. Infiltrating neutrophils and macrophages are additional sources of ROS and production of free radicals thus exaggerates the SCI (Snyder and Teng 2012,

Mothe and Tator 2013). The level of free radicals constantly increases at the injured site and leads to massive cell death in SCI. Minimizing secondary injury is generally achieved by ensuring adequate perfusion and oxygenation and by ancillary administration of neuroprotective agents for improving clinical signs.

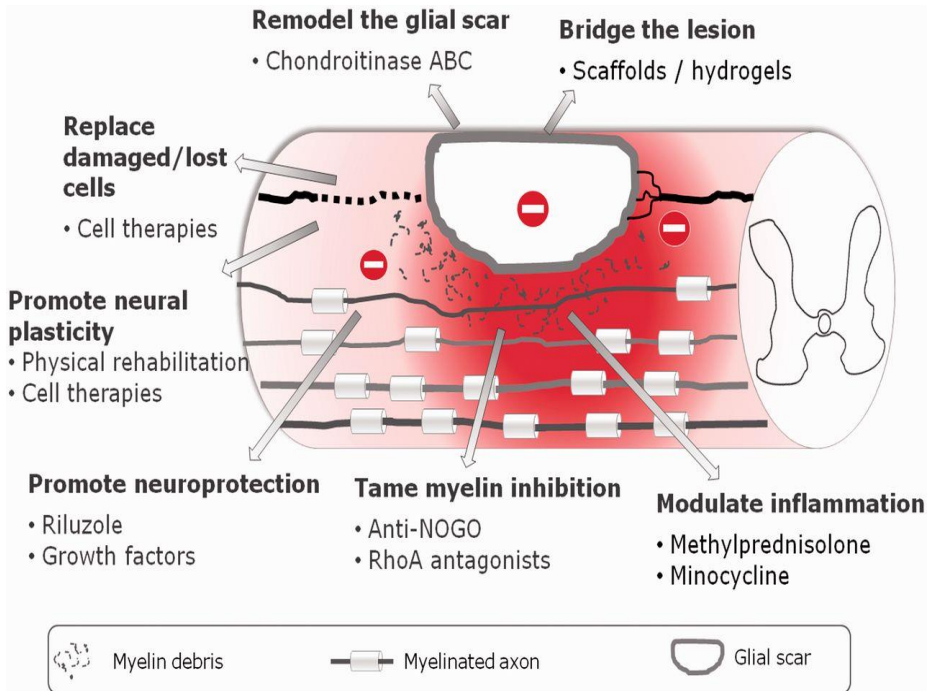
Furthermore, the damaged spinal cord undergoes an injury response, usually called reactive glial scarring. Macrophages are arrived from bloodstream and microglia migrates from the surrounding tissue. These are occurred within hours of injury. Chondroitin sulfate proteoglycans (CSPGs) are one of the inhibitory molecules deposited in the glial scar after SCI and contribute to regenerative failure. CSPGs form boundaries as barriers to axon growth during central nervous system (CNS) development and are expressed in gliotic regions surrounding injury sites in the CNS. Oligodendrocytes are recruited from the surrounding tissue 3 to 5 days after injury, and the final structure of the glial scar is formed with astrocyte. The glial scar consists of astrocytes by self-duplication of resident astrocytes that located at the lesional margins (Fawcett and Asher 1999, Sabelström et al. 2014). In particular, a major impediment of regeneration is the astroglial scarring at the lesion site.



**Figure 1.** Chain reaction after SCI. Inflammation, damages of neurons and oligodendrocytes and glial scar formation (Somers 2001).

## **2. Therapeutic strategies**

Three processes have been identified as main contributors to the inhibitory environment: the activation of an inflammatory response, extensive cell loss, and the formation of the glial scar. Based on the diverse sources of inhibitory signaling, multifarious strategies have been proposed to address it. Overall, these strategies focus on modulate inflammation, replace damaged and death cells, and remodeling the glial scar (Yick et al. 2000, Jin et al. 2002, Kim et al. 2015). Furthermore, designing a bridge to re-connect the injured spinal cord encloses several challenges, namely facing the hostile environment at the lesion site or providing adequate mechanical support. Nonetheless, to develop an implantable device provides a unique opportunity to combine different therapeutic signals in a single platform. Nerve regeneration research based on the use of biomaterials was primarily focused on the development of scaffolds that connect the lesion site, filling the cavity formed upon lesion, providing physical support and a path for axonal re-growth and limiting cellular infiltration from the periphery. Overall, scaffolds are expected to allow the modification of the inhibitory environment at the lesion site and, consequently, contribute to the process of regeneration (Fig. 2)(Sasaki et al. 2009, Park et al. 2012, Pires and Pego 2015). However, molecules and guidance cues present in the developing nervous system are not preserved throughout adulthood have different functions, resulting in an insufficient stimulation of axonal growth and



**Figure 2.** Injured spinal cord therapeutic approaches currently under investigation (Pires and Pego 2015).

limited means to direct axons towards appropriate targets. Several therapeutic strategies have been utilized that modulates the microenvironment and enhances replacement of neurons and glial cells through digestion of CSPGs by chondroitinaseABC (chABC), application of supplementary neurotrophic factors, and grafting of mesenchymal stromal cells (MSCs).

### **1) Transplantation of mesenchymal stromal cells**

MSCs are used in clinical studies for treat the SCI due to their immunomodulatory effect, neuroprotective properties, secretion of nerve regeneration related factors and ability of self-renewal and differentiation. The most common delivery method of MSCs is direct injection into the injured site, which allows many cells to be transplanted effectively. However, the advantages of MSCs therapy in SCI are a result of indirect environmental modification rather than direct trans-differentiation to functional neurons of applied MSCs, and MSCs have insufficient integration with host spinal cord. Most transplanted cells died within the first few days because of oxidative stress, hypoxia, and immune response (Uemura et al. 2010). Furthermore, improvement of functional recovery after MSCs transplantation in the canine SCI model was limited to non-weight bearing conditions (Lim et al. 2007, Ryu et al. 2009, Park et al. 2011). Strategies of MSCs transplantation require a safe and efficient method of cellular delivery. Therefore, less invasive and more effective methods for cell delivery have been investigated and intravenous

administration has been identified as an ideal and preferable minimally invasive method for delivering cell transplants for clinical translation (Park et al. 2012, Kim et al. 2015, Rittfeld et al. 2015). The grafted cells were detected in the lung, spleen, and injured spinal cord, but there were no such cells in the uninjured spinal cord after intravenous administration. These mean that the MSCs can probably migrate into the injured spinal cord through the broken blood–spinal cord barrier. Intravenous injection of MSCs shows peripheral immune-regulatory properties through inhibition of T-cell activities and through modulating the host systemic and central nervous system inflammatory response (Rabinowitz et al. 2008, Osaka et al. 2010, Kim et al. 2015).

## **2) Application of chABC**

The glial scar is mainly an astrocytic tissue consisting of hyperfilamentous astrocytes, with processes tightly packed gap and tight junctions and limited extracellular space. The scar is formed to isolate the injury and prevent the spreading of excitotoxicity and cytotoxic molecules. However, the glial scar represents a mechanical barrier for axonal re-growth, being also a source of chemical inhibitors for regeneration. CSPGs produced by astrocytes are present in the extracellular matrix in the CNS and are the major component of the glial scar. In this context, CSPGs have been targeted in SCI therapeutics. Generally, CSPGs in the glial scar are upregulated in the lesion site for at least 2 weeks

after the injury and maintained for 4 weeks or more (Iseda et al. 2008). chABC is a bacterial enzyme that digests the chondroitin sulfated glycosaminoglycan side chains on the protein core of CSPGs, which promotes axonal regeneration and reduces the inhibitory effects on neurite growth. Various therapeutic approaches, using chABC were on various type of intraspinal cell-based transplants and scaffolds (Lee et al. 2010). Treatment with this enzyme is likely to be advantageous even 7 days after SCI, establishing this strategy particularly concerning for non-acute spinal cord lesions. However, the origin of the enzyme as well as the degradation products formed, have been issue of concern due to the possibility of triggering a specific immune response. Moreover, these degradation products can exert some inhibitory influence on the growth of spinal axons. However, chABC treatment did not affect the proliferation of cells and protein synthesis, in addition, single injection of chABC brings sustained partial decrease in CSPGs for 8-12 weeks. Moreover, chABC is safe based on animal experiments, and topically applied chABC has no adverse effects on nerves (Olmarker et al. 1996). Application of chABC was administrated through intrathecally, directly into injured spinal cords, or using implanted materials. Several studies have reported repeated surgical treatments involving stem cell transplantation after pre-treatment with chABC (Olmarker et al. 1996, Karimi-Abdolrezaee et al. 2010, Jefferson et al. 2011, Milbreta et al. 2014). However, these methods had critical risks, those are several surgical interventions, anesthesia and infection by using implanted materials. To reduce



the number of surgical intervention, it is necessary to design combinational treatment with canine adipose tissue derived mesenchymal stromal cells (cADMSCs) and chABC at one time.

### **3) Application of neurotrophins**

Recently, in the context of cell therapy for SCI, genetically-modified cells have been used to improve functional recovery after SCI. This therapeutic approach enables the injured spinal cord to be modified. MSCs could differentiate into neuronal cell types, although MSCs express neuronal proteins when grafted into injured spinal cord microenvironment, but they were not often found to differentiate into cells with a neuronal phenotype. Thus, it seems likely that MSCs differentiation into phenotypic neurons was not required by these cells to provide a functional benefit. Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), Neurotrophin-3 (NT-3) were reported to be produced by MSCs (Weishaupt et al. 2012). The neurotrophins are molecules with interest in the context of SCI due to their important role in neural development, survival and regeneration. Injection of BDNF, NGF and NT-3 was performed in SCI animal models with different degrees of success. Sympathetic neurons and sensory neurons depend on NGF for survival, and surprisingly, robust growth of responsive axons is observed after NGF delivery by gene transfer into the injured spinal cord even

long after the injury. BDNF has been shown to reverse atrophy of rubrospinal neurons after axotomy (Xu et al. 1995, Ritfeld et al. 2015). Neurotrophin-3 (NT-3) has been shown to prevent cell death and reduce atrophy in spinal cord projection neurons. BDNF and NT-3 have also been shown to reduce axonal degeneration and induce sprouting of corticospinal axons (Dougherty et al. 2000). Thus, the release of such factors from transplanted MSCs within the host spinal cord may have contributed to the recovery of function following SCI. It also allows neurotrophic factors to be delivered to the lesion site and enhance intrinsic neuronal growth responses (Jin et al. 2002, Lu et al. 2005, Taylor et al. 2006).

After SCI, pro-apoptotic factors are typically up-regulated in axotomized neurons. BDNF may tip the scale toward survival and rescue neurons that may then take part in the restructuring of remaining neuronal circuitry. BDNF act as a promoter of cell survival and neurite outgrowth in treating spinal cord injury, and damaged neurons may be rescued from apoptotic cell death and degenerative atrophy when treated with BDNF. That makes the treatment of BDNF can promote axonal regeneration and rewiring of injured nerve fibers (Weishaupt et al. 2012). A large-scale animal study indicated that the local application of BDNF can induce neuroprotection if applied at high doses and shortly after trauma. Most of the generally desirable effects of BDNF in SCI are mediated by signaling through the high affinity tropomyosin-related kinase B (TrkB) receptor, a receptor that binds BDNF with high specificity and affinity.

Furthermore, BDNF can also interact with the low-affinity pan-neurotrophin receptor p75, resulting in signaling effects that often counteract TrkB activation. Naturally, these pathways are not entirely segregated and may overlap and complement each other in function (Weishaupt et al. 2012, Ritfeld et al. 2015). While there is abundant evidence for BDNF's ability to rescue severed rubrospinal neurons from lesion-induced degenerative changes, other descending or ascending tracts have also been reported to respond to BDNF. BDNF has great potential for up-regulating the number of surviving neurons after axotomy, but the reliability and extent of its action depends on the individual neuronal population among other factors. The prerequisite for a helpful effect is the availability of TrkB receptors on the responding cell, which can vary greatly among different cell types and compartments (Hollis et al., 2009; Kwon et al., 2004; Lu et al., 2001). Due to the differential expression of Trk receptors, a combinatory approach that uses other neurotrophins together with BDNF might further increase efficacy in neuroprotection after SCI (Lu et al. 2005, Weishaupt et al. 2012, Ritfeld et al. 2015). Currently, the use of neurotrophic factors appears to be particularly relevant when combined with drug/gene delivery strategies and/or cell-based therapies (McCall et al. 2012, Luo et al. 2013, Enomoto 2016).

To improve the limited functional recovery after SCI, I need to develop more advanced methods to better utilize MSCs. Lentiviral based BDNF gene therapy systems that offer prolonged gene expression and which were aimed at

continuous secretion of the factors that stimulate axonal growth and replacement of neurons throughout the lesion areas. If more cells survived in the lesion, it would provide a favorable environment for the ingrowth of endogenous neuronal cells. In addition, combinational treatment of chABC can modulate the microenvironment of injured spinal cord.

This study was performed to investigate strategies for enhancing the recovery after chronic SCI. The purposes of this study were to (1) investigate the effect of chABC in combination with cADMSCs, and (2) verify the cADMSCs secreting BDNF can enhance the functional recovery after chronic SCI.

# **CHAPTER I**

## **Effect of the combination of mesenchymal stem cells and chondroitinaseABC on chronic spinal cord injury**

### **ABSTRACT**

Transplantation of MSCs has been identified as a potential therapeutic modality for treating SCI. Degradation of CSPGs using the enzyme chABC can promote functional recovery after SCI. The effect of the simultaneous administration of MSCs and chABC on chronic SCI was investigated. Sixteen dogs were assigned to one of the following four groups: cADMSCs, chABC, cADMSCs + chABC, and control. Treatments were carried out 3 weeks after SCI; cADMSCs ( $1 \times 10^7$  cells suspended in 150  $\mu$ L of PBS), chABC (5 U/mL, 150  $\mu$ L), cADMSCs + chABC ( $1 \times 10^7$  cells suspended in 150  $\mu$ L of chABC), or PBS (150  $\mu$ L) groups was injected into the spinal cord at 3 locations to a depth of 3 mm using a 30-gauge needle. The spinal cord was harvested 8 weeks after transplantation. In a behavioral assessment, dogs treated with cADMSCs + chABC and cADMSCs showed significantly better functional recovery 8 weeks

after transplantation compared with the control and chABC groups ( $p < 0.05$ ). In addition, the combination of cADMSCs and chABC increased the expression of digested CSPGs (2B6),  $\beta$ 3-tubulin, and neurofilament microtubule (NF-M). However, the levels of cyclooxygenase2 (COX2) ( $p < 0.05$ ) and tissue necrosis factor  $\alpha$  (TNF $\alpha$ ) were higher in the treatment groups than in the control. In conclusion, transplantation of cADMSCs + chABC was more effective in improving clinical signs and neural regeneration, but a strategy for anti-inflammation after the treatment for chronic SCI would be needed for further improvement.

Keywords: chondroitinaseABC, chronic spinal cord injury, dog, mesenchymal stem cells

## INTRODUCTION

The regeneration of injured spinal cord is limited due to multiple factors including extensive cell loss, axonal disruption, and growth inhibitory molecules in the scar (Lee et al. 2010, Sharma et al. 2012). In particular, a major impediment of regeneration is the astroglial scarring at the site of injury in chronically injured spinal cords. Therefore, chronic SCI require multifaceted strategy for treatment (McKeon et al. 1999, Karimi-Abdolrezaee et al. 2010).

Previous studies have shown that CSPGs are one of the inhibitory molecules deposited in the glial scar after SCI and contribute to regenerative failure (Lemons et al. 1999, McKeon et al. 1999). CSPGs form boundaries as barriers to axon growth during central nervous system (CNS) development and are expressed in gliotic regions surrounding injury sites in the CNS (Silver 1994, Asher et al. 2000). Generally, CSPGs are upregulated in the lesion site for at least 2 weeks after the injury and maintained for 4 weeks or more (Iseda et al. 2008). ChABC is a bacterial enzyme that digests the chondroitin sulfated glycosaminoglycan side chains on the protein core of CSPGs. ChABC promotes axonal regeneration and reduces the inhibitory effects on neurite growth (Lee et al. 2010). Moreover, chABC is safe based on animal experiments, and topically applied chABC has no adverse effects on nerves (Olmaker et al. 1996).

Many therapeutic approaches to SCI have been focused on various types of intraspinal cell-based transplants and chABC. Investigators have applied chABC through intrathecally (Fouad et al. 2005, Karimi-Abdolrezaee et al. 2010), directly into injured spinal cords (Iseda et al. 2008, Jefferson et al. 2011), or using implanted materials (Lee et al. 2010). Several studies have reported repeated surgical treatments involving stem cell transplantation after pre-treatment with chABC (Karimi-Abdolrezaee et al. 2010, Imagama et al. 2011). However, these methods had critical risks which were associated with several surgical interventions, anesthesia, and infection.

In the current study, to reduce the number of surgical interventions, a combinational therapy of cADMSCs and chABC was designed. I investigated the effects of chABC in combination with cADMSCs on the functional recovery and mechanisms associated with the restoration of chronic SCI including decreased glial scarring and enhanced neuronal extension, as well as the facilitation of endogenous neuronal regeneration.



## **MATERIALS AND METHODS**

### **1. Isolation and culture of cADMSCs**

cADMSCs were obtained according to the methods described in previous articles (Lim et al. 2007). Briefly, adipose tissues were aseptically collected from the subcutaneous fat of 2-year-old experimental dogs during general anesthesia. This procedure was conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University (SNU-120306-5). Tissues were washed extensively with phosphate-buffered saline (PBS), minced, and digested with collagenase type I (1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) for 2 hours at 37°C. The tissue samples were washed with PBS solution and then centrifuged at  $300 \times g$  for 10 minutes. The resulting pellet (i.e., the stromal vascular fraction) was resuspended, filtered through a 100- $\mu\text{m}$  nylon mesh, and incubated overnight in medium with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) at 37°C with 5% humidified CO<sub>2</sub>. Unattached cells and residual non-adherent red blood cells were removed after 24 hours by washing with PBS solution. The medium was changed at 48-hour intervals until the cells became confluent. After cells reached 90% confluence, they were trypsinized and stored in liquid nitrogen or subcultured.

## **2. Viability of cADMSCs in the presence of chABC**

When the cells reached 80% confluence, attached cells were harvested with trypsin-EDTA and then immersed in a 96-well plate. Five U/ mL chABC (chABC, Sigma, St. Louis, MO, USA) was added to the plate in chABC group and not in control. After 2 days of culture, the medium was removed and washed with PBS to remove loosely adherent cells. The number of cells were measured by the 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method using a commercially available kit (CellTiter 96<sup>®</sup> Aqueous, Promega, Madison, WI, USA). Briefly, phenazine methosulfate (PMS) solution was added to MTS solution immediately before the mixture was added to the cell culture plate. The cells were incubated at 37°C with 5% humidified CO<sub>2</sub> for 90 minutes. Plates were read on a microplate spectrophotometer (Versa<sub>max</sub>, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 492 nm.

## **3. Duration of chABC activity on CSPGs**

From 2 hours to 7 days, 5 U/mL chABC were added to chondroitin sulfate (CS, chondroitin sulfate sodium salt from shark cartilage, Sigma) to evaluate the degradation ability of chABC at 37°C and PBS was added as a control. Total

sulfated glycans were measured by a sulfated GAG assay (Blyscan™, Biocolor Ltd., Newtownabbey, Northern Ireland) (Bhattacharyya et al. 2014).

#### **4. Induction of SCI**

SCI was induced using a balloon compression method previously described (Lim et al. 2007). Briefly, the dogs were medicated and anesthetized with intravenous cefazolin sodium (40 mg/kg, Cefazoline; Chong Kun Dang Pharm, Korea), tramadol (4 mg/kg, Toranzin; Sam Sung Pharm. Ind. Co., Korea), zolazepam hydrochloride (5 mg/kg, Zoletil 50; Virbac, SA., Carros, France), and subcutaneous atropine sulfate (0.05 mg/kg, Atropine; Jeil Pharm., Seoul, Korea). Anesthesia was maintained with 2% isoflurane inhalation (Aerane; Ilisung, Korea) in oxygen. The multiparameter anesthetic monitor Datex-Ohmeda (Microvitec Display, Bradford, UK) was used to monitor physiological measures including rectal temperature, oxygen saturation, end tidal CO<sub>2</sub>, and pulse rate during anesthesia. Following anesthetic stabilization, the dogs were placed in a ventral recumbent position and a hemilaminectomy was performed through a left paramedian approach at the fourth lumbar segment (L4). A 3–5-mm hole was made in the left vertebral arch at L4 using a high-speed pneumatic burr and a 3-French embolectomy catheter (SORIN Biomedica, Sallugia, Italy) inserted into the hole at L4. A balloon catheter was advanced under fluoroscopic guidance at the cranial margin of first lumbar

segment (L1) and inflated at the region with 50  $\mu\text{L}/\text{kg}$  of a contrast agent (Omnipaque; Amersham Health, Cork, Ireland), diluted 50:50 with saline. The balloon catheter was fixed with a Chinese finger trap suture and removed after 12 hours. According to a previous study (Lim et al. 2007), the SCI model used occludes more than 80% of the spinal canal as confirmed by computed tomography. Following SCI, the soft tissues and skin were closed through standard methods. After the operation, dogs were bandaged, monitored in an intensive care unit. The dogs were fed with balanced nutrition twice a day, and if needed, manual bladder expression was performed at least three times daily until voluntary urination was established.

## **5. Transplantation of cADMSCs and chABC into the injured sites**

Injection of PBS, cADMSCs, and chABC was performed at 3 weeks after experimentally-induced SCI. The dogs were anesthetized using the same methods described for induction of the SCI. For the control group, the injured site was exposed by dorsal laminectomy and 150  $\mu\text{L}$  of PBS was injected into the L1 spinal cord at 3 locations to depths of 3 mm using a 30-gauge needle (middle of the injury site, proximal and distal margins). For the cADMSCs group ( $1 \times 10^7$  cells suspended in 150  $\mu\text{L}$  of PBS), chABC group (150  $\mu\text{L}$ ) and cADMSCs + chABC group ( $1 \times 10^7$  cells suspended in 150  $\mu\text{L}$  of chABC) was

transplanted at the SCI site in the same way as for the control group.

## **6. Behavioral assessments**

In order to evaluate the functional recovery of the hindlimbs, behavioral assessments were performed before the operation and weekly after the operation. Each dog was videotaped for a minimum of 10 steps from both sides and behind when walking on the floor. Dogs that could not bear weight on their hindlimbs were also videotaped while supported by holding the base of their tail. Data was recorded using the Basso, Beattie, and Bresnahan (BBB) scores (Table 1)(Barros Filho and Molina 2008), as well as the revised Tarlov scale and modified Tarlov scale (Table 2)(Rabinowitz et al. 2008). The dogs' gaits were scored independently from the videotapes by two individuals blinded to the experimental conditions. A mean score was calculated every week after the SCI until the 11-week study period ended.

## **7. Histopathological and immunofluorescence analyses**

**Table 1. BBB scores**

Score	Physical findings
0	No observable movement of the hindlimbs
1	Slight movement of one or two joints, usually hip/knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of the other
3	Extensive movement of two joints
4	Slight movement of all three joint of the hindlimbs
5	Slight movement of two joints and extensive movement of the third joint
6	Extensive movement of two joints and slight movement of the third joint
7	Extensive movement of the three joints in the hindlimb
8	Sweeping without weight bearing or plantar support of the paw without weight bearing
9	Plantar support of the paw with weight bearing only in the support stage or occasional, frequent or inconsistent dorsal stepping with weight bearing and no plantar stepping
10	Plantar stepping with occasional weight bearing and no forelimb-hindlimb co-ordination
11	Plantar stepping with occasional weight bearing and occasional forelimb-hindlimb co-ordination
12	Plantar stepping with occasional weight bearing and frequent forelimb-hindlimb co-ordination
13	Plantar stepping with frequent to consistent weight bearing and frequent forelimb-hindlimb co-ordination
14	Plantar stepping with consistent weight support, consistent forelimb-hindlimb co-ordination and predominantly rotated paw position (internally or externally) during locomotion both at the instant of initial contact with the surface as well as before moving the toe at the end of the support stage or frequent plantar stepping, consistent forelimb-hindlimb co-ordination and occasional dorsal stepping
15	Consistent plantar stepping, consistent forelimb-hindlimb co-ordination

- and no movement of the toes or occasional movement during forward movement of limb; predominant paw position is parallel to the body and the time of initial contact
- 16 Consistent plantar stepping and forelimb-hindlimb co-ordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the time of initial contact and curved at the instant of movement
  - 17 Consistent plantar stepping and forelimb-hindlimb co-ordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the time of initial contact and at the instant of movement of the toes
  - 18 Consistent plantar stepping and forelimb-hindlimb co-ordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the time of initial contact and curved during movement of toes
  - 19 Consistent plantar stepping and forelimb-hindlimb co-ordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the instant of contact and at the time of movement of the toes, and the animal presents a downward tail some or all of the time
  - 20 Consistent plantar stepping and forelimb-hindlimb co-ordination during gait and movement of the toes occurs frequently during forward movement of the limb; the predominant paw position is parallel to the body at the instant of contact and at the time of movement of the toes, and the animal presents a consistent elevation of the tail and trunk instability
  - 21 Consistent plantar stepping and co-ordinated gait, consistent movement of the toes; paw position is predominantly parallel to the body during the whole support stage; consistent trunk stability; consistent tail elevation
-

**Table 2. Revised and modified Tarlov scores**

Physical finding	Revised score	Physical finding	Modified score
Flaccid hindlimbs	1	Paralysis in hindlimb	1
Tone in hindlimbs	2		
Purposeful hindlimb motion	3	Movement at all joints and assisted gait	2
Stands with assistance	4		
Stands unassisted	5	Movement at all joints with ataxic gait unassisted	3
Limited ambulation	6		
Full ambulation	7	Full ambulation and incompleted climb a 20° incline ramp	4
Climbs a 20° incline ramp half way	8		
Climbs 20° incline ramp	9	Full ambulation and climbs a 20° incline ramp	5



All of the dogs were euthanized eight weeks after transplantation and the spinal cord from the eleventh thoracic segment (T11) to the third lumbar segment (L3) was extracted by dissection. Each sample was fixed in 10% sucrose/PBS at 4°C for 12 hours and immersed in 20% sucrose solution overnight at 4°C. The dura was removed with scissors, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek<sup>®</sup>, Sakura, Torrance, CA, USA), frozen, and cut longitudinally into two sections. One half of each section was immediately frozen with liquid nitrogen for a western blot analysis and the other half was cut into 10- $\mu$ m sections with a cryomicrotome. These sections were mounted on silane-coated glass slides and stained with hematoxylin and eosin (H&E) to detect fibrosis. Primary antibodies were used against chondroitin sulfate (CS56; Abcam, Cambridge, UK), Cdk2 (2B6; Abcam, Cambridge, UK), glial fibrillary acidic protein (GFAP),  $\beta$ 3-tubulin, NF-M, pSTAT3, galactosylceramidase (GalC, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for immunofluorescence determination. The sections were fixed and permeabilized for 10 minutes with 0.1% (v/v) Triton X-100 and washed, then pre-incubated with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 30 minutes to decrease nonspecific antibody binding. Sections were incubated with the primary antibodies for 24 hours at 4°C, and then incubated for 60 minutes with fluorescein isothiocyanate (FITC)-conjugated secondary antibody at room temperature, washed, and mounted on slides. DAPI (4,6-diamidino-2-penylindole) was used for nuclear staining. Histomorphometric

analysis in H&E and CS56 was performed at four site in each samples using imagine analysis software (Image J version 1.47; National Institutes off Health, USA).

## **8. Western blot analysis**

The frozen half of each spinal cord section 0.25cm from the epicenter of the injured region was used for Western blot analysis. Briefly, the spinal cord tissue was washed twice with PBS and frozen at -150°C. Then the tissue was homogenized with a sonicator (three 20 sec bursts; Branson Sonicator 250; Branson Ultrasonic Corp., Danbury, CT, USA) in lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/mL aprotinin, 1mM phenylmethylsulfonylfluoride and 0.5 mM sodium orthovanadate) for 30 min on ice. Lysates were cleared by centrifugation (10 min at  $1,500 \times g$ , 4°C) and protein concentrations were determined using the Bradford method.(Bradford 1976) Equal amounts of spinal cord protein (20 µg) were resolved by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membrane blots were washed with TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), blocked with 5% skim milk for 1 hour, and incubated with the appropriate primary antibodies at the recommended dilutions. The membranes were washed

and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The antibodies used included: anti-actin (Sigma, MO, USA), Cdk2 (2B6), chondroitin sulfate (CS56) NF-M (Abcam, Cambridge, UK),  $\beta$ 3-tubulin, COX2, TNF $\alpha$ , phosphorylated-signal transducer and activator of transcription 3 (pSTAT3), GalC, GFAP (Santa Cruz Biotechnology)

## **9. Statistical analysis**

Data are presented as medians and quartiles. A statistical analysis was performed using SPSS version 21.0. In all experiments, Kruskal-Wallis tests were followed by Mann-Whitney U tests to compare the four groups.  $p < 0.05$  was considered significant.

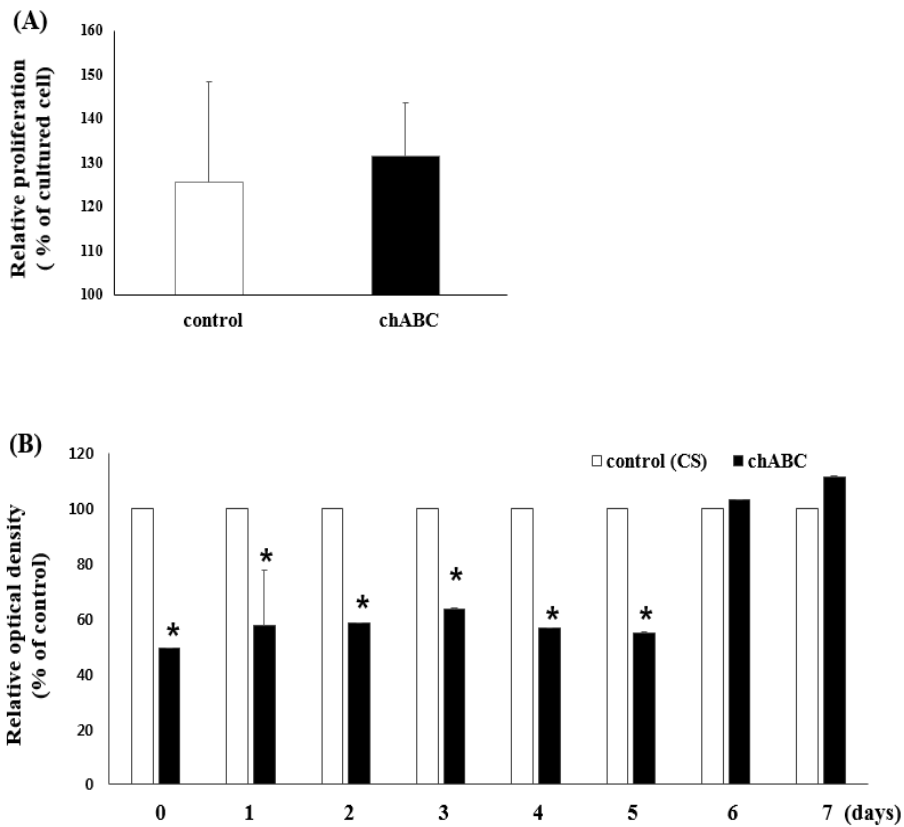
## **RESULTS**

### **1. Effect of chABC on cell viability**

After 2 days of passage 3 cADMSC culture in the presence of chABC I identified the number of viable cells. There was no significant difference in the proliferation rate between the normal conditioned culture group and chABC group (Fig. 3A). The results revealed that 5 U/mL chABC did not have a harmful effect on the viability of cADMSCs.

### **2. Degradation effect of chABC over time**

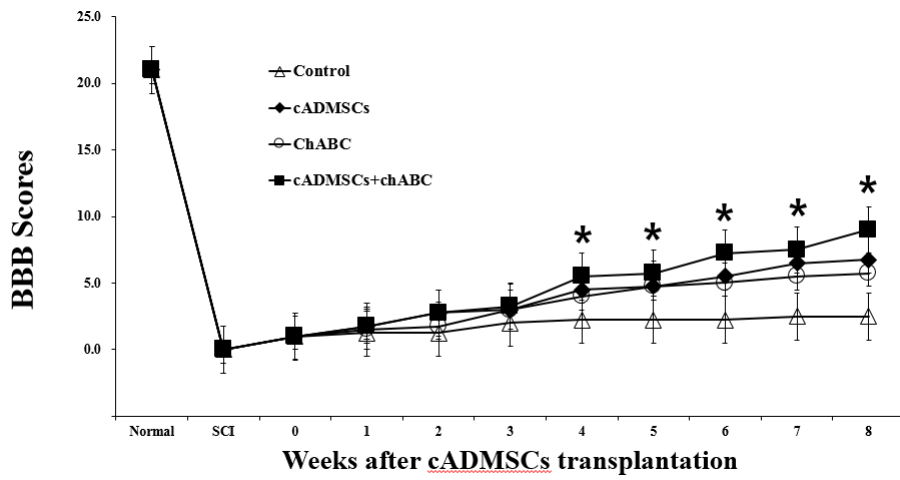
Until 5 days after chABC treatment, the degradation rate of CS was significantly different between the control and chABC groups. At 37°C, the degradation capacity of the chABC was maintained for 5 days. Six days after treatment, the degradation rate of CS was equal for the two groups and a significant difference was not observed (Fig. 3B).



**Figure 3.** (A) Proliferation of cultured cells in the presence of chABC condition and normal cultured condition. No significant differences of proliferation between the normal conditioned culture group and chABC group. (B) Degradation effect of chABC over time. Until 5 days after treatment of chABC, degradation rate of CSPG was significantly different between control and chABC group ( $*p < 0.05$ ). Six days after treatment, the degradation of CS showed same degree between two groups and there was no significant difference.

### 3. Behavioral observations

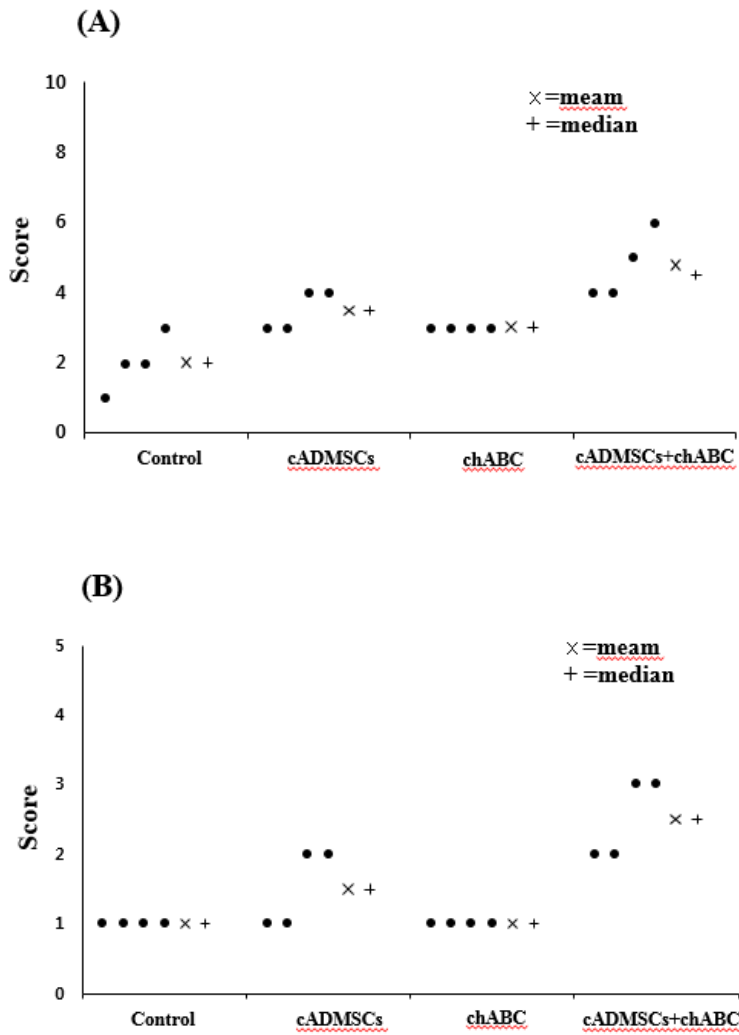
All injured dogs showed complete pelvic limb paralysis after SCI, with BBB scores prior to SCI of 21, and afterwards of 0. At the time of transplantation, the mean scores for all groups were 1, because slight movement of a minimum of one joint was detected. BBB scores were obtained every week until 8 weeks after transplantation. In all groups, the scores gradually increased during the study period, but the improvement rate decreased after 4 weeks of transplantation. The BBB scores of the cADMSCs + chABC group, cADMSCs group, and chABC group significantly improved 8 weeks after transplantation compared with the control group ( $p < 0.05$ , Fig. 4). Among the treatment groups, the cADMSCs + chABC and cADMSCs groups improved significantly more than the chABC group ( $p < 0.05$ ). But there was no significant difference between cADMSCs + chABC and cADMSCs groups. Moreover, Improvement of hindlimb locomotion in the cADMSCs group and chABC group was limited by the inability of the dogs to bear their own weight. On the other hand, three dogs in the cADMSCs + chABC group and one dog in the cADMSCs group were able to support their body weight when help was occasionally provided to stand. To evaluate the behaviors qualitatively, I used a revised and modified Tarlov scores to assess neurologic recovery 8 weeks after transplantation. According to this grading system, I found significantly higher motor recovery



**Figure 4.** Behavioral analysis results using BBB scores. BBB scores during 8 weeks. Post-transplantation, the BBB scores of cADMSCs + chABC group, cADMSCs group, and chABC group significantly improved in 8 weeks after transplantation compared to control group ( $*p < 0.05$ ).

in the cADMSCs + chABC group than the control and chABC groups (Figs. 5A and B).



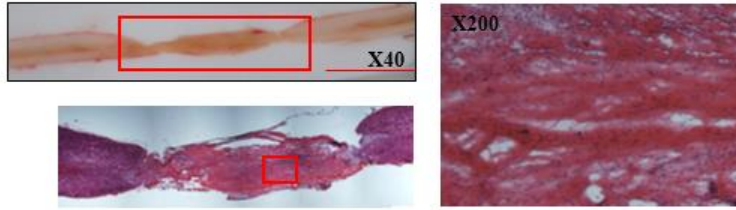


**Figure 5.** Behavioral analysis results using Tarlov scores. Hindlimb locomotion at 8 weeks after transplantation was determined by two grading system, (A) revised Tarlov scores and (B) modified Tarlov scores. X, mean; +, median

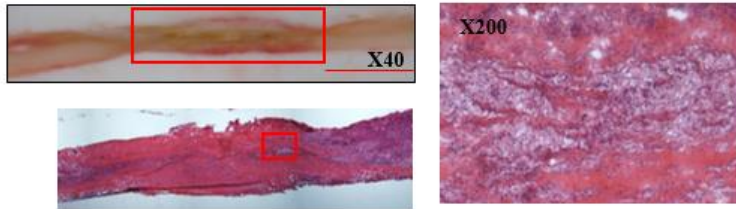
#### **4. Histopathological and immunohistochemical assessments**

Atrophic and fibrotic changes in the injured regions were detected in all groups 8 weeks after transplantation (Fig. 6). The sizes of the injured regions (cm) in the PBS, cADMSCs, chABC and cADMSCs + chABC groups were 2.88, 2.23, 2.58, and 2.18, respectively. In order to assess the longitudinal cross-section of SCI samples in detail, the samples were cut and embedded in OCT so that the spinal cord parenchyma could be compared among groups. In the PBS group (Fig. 6A), spinal cord sections revealed expanded parenchymal fibrosis. In addition, a histopathological analysis with H&E staining showed severe pathologic changes in the parenchyma. Most of the samples in the PBS and chABC groups (Figs. 6A and C) had fibrotic changes and vacuolar formation. Upon magnification of the injury epicenter, severe fibroblast-like cell proliferation because of chronic inflammation was detected. In the cADMSCs + chABC group (Fig. 6D), the spinal cord lesions were limited to the compression region, and a greater reduction in fibrotic changes was observed compared with the other groups. Moreover, upon magnification, a reduction in fibroblast-like cell proliferation was observed. Through the quantification of fibrotic tissue in H&E staining, there were significant differences between control and ADMSCs including groups ( $p < 0.01$ ) and between chABC and cADMSCs ( $p < 0.05$ ). Immunohistochemistry revealed that expression of the

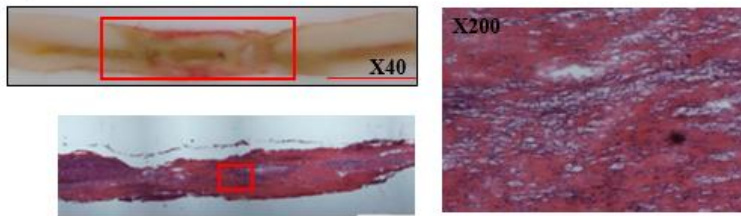
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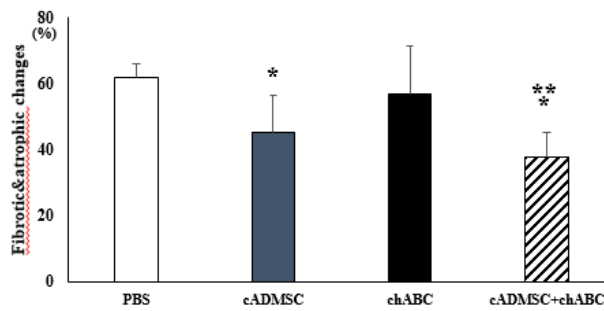
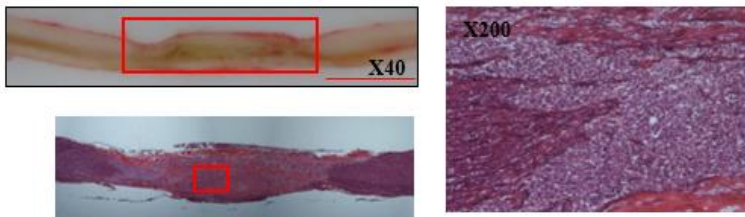
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(C)



(D)



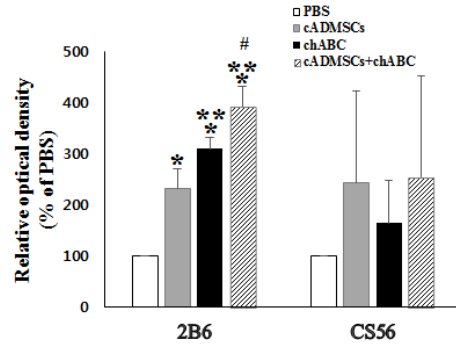
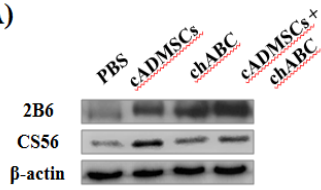
**Figure 6.** Histological analysis of spinal cord lesions stained with H&E. Most of the samples in the PBS and chABC groups had damaged tissues and vacuolar formation. Upon magnification of the injury epicenter, severe fibroblast-like cell proliferation as a result of chronic inflammation was detected. In the cADMSCs + chABC group, the spinal cord lesion was limited to the compression region and a greater reduction in fibrotic changes was observed compared with other groups. Also, upon magnification a reduction in fibroblast-like cell proliferation was observed. The scale bar indicates 1 cm. Quantification of fibrotic and atrophic changes was showed significant difference among the groups ( $*p < 0.01$  compared to PBS group,  $**p < 0.05$  compared to chABC group).

CSPG digestion marker, 2B6, was higher in all treatment groups than in the control but, there were significant differences among the groups. The cADMSCs + chABC, chABC, and cADMSCs groups, in order, expressed increasingly higher levels of 2B6. The expression of intact CSPGs was different among groups but there were no significant differences (Figs. 7A and 8).

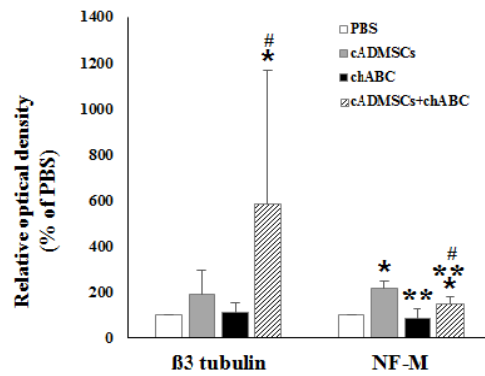
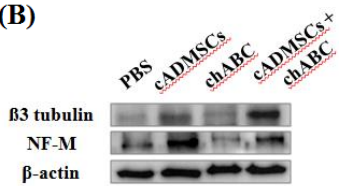
Expression of  $\beta$ 3-tubulin in the cADMSCs + chABC group was significantly higher than it was in the PBS and chABC groups, but not higher than it was in the cADMSCs group (Figs. 7B and 9). NF-M in the cADMSCs group was significantly higher than in the other groups, and the cADMSCs + chABC group showed a significant increase in NF-M expression relative to the control and chABC groups ( $p < 0.05$ , Figs. 7B and 9).

Expression of GFAP was significantly lower in all treatment groups than in the control. Significantly higher levels of GalC were observed in the cADMSCs and chABC groups than in the control, and significantly higher pSTAT3 was observed in the cADMSCs, chABC and cADMSCs + chABC groups than in the control (Figs. 7C and 9). The level of COX2 was higher in all treatment groups than in the control but did not differ among the treatment groups (Fig. 7D). In the case of  $\text{TNF}\alpha$ , there were no significant differences among groups.

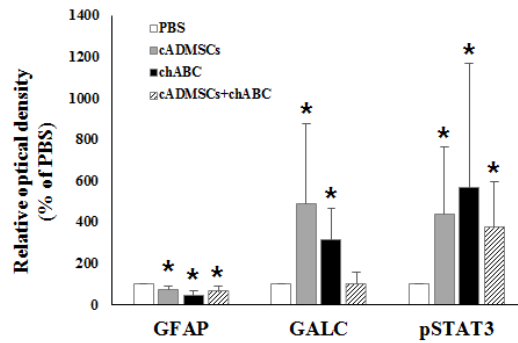
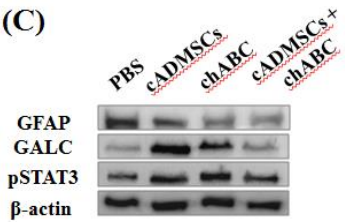
(A)



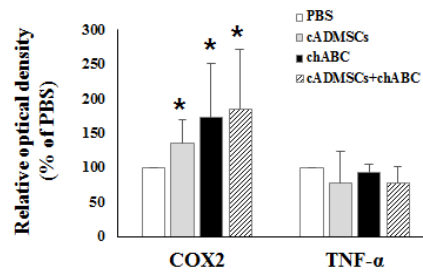
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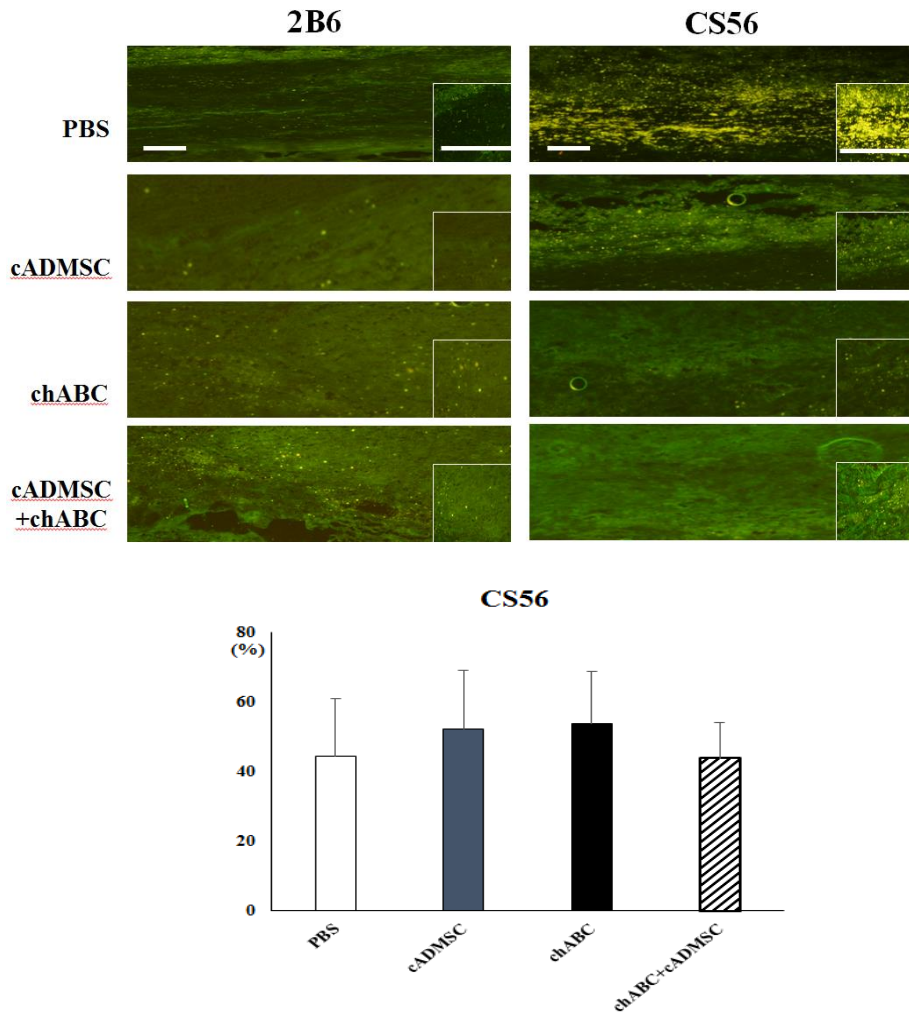
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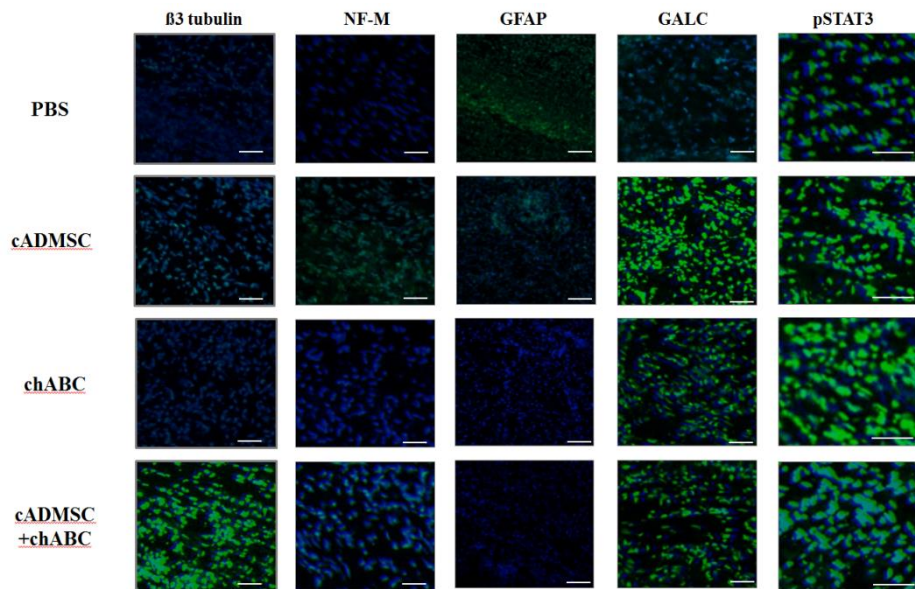


**Figure 7.** Protein expression at 8 weeks after transplantation. (A) 2B6 and CS56, (B) neuronal markers were evaluated by western blotting.  $\beta$ 3-tubulin and NF-M (C) intracellular signal molecule of astrogliosis; GFAP, GalC, pSTAT3, and (D) inflammatory markers; COX2 and TNF $\alpha$ . Data represent means  $\pm$  SE of three independent experiments; \* $p$  < 0.05 compared to PBS group, \*\* $p$  < 0.005 compared to cADMSCs group, and # $p$  < 0.05 compared to chABC group. The graph and bars depict means  $\pm$  SE of four dogs per groups, as determined by densitometry relative to  $\beta$ -actin.



**Figure 8.** Cleavage of CSPGs glycosaminoglycan side chains and intact CSPGs immunofluorescence in the spinal cord lesion were detected (using antibody 2B6 and CS56). In contrast to the strong 2B6 labeling observed in treated groups, the control group showed weak expression of 2B6. The scale bar indicates 50um. Quantification of CS56 was showed no significant difference among the groups.





**Figure 9.** Immunofluorescence staining at 8 weeks each groups after transplantation. Injured spinal cord lesions were immunostained for  $\beta 3$ -tubulin, NF-M, GFAP, GalC, and pSTAT3 (green), and each nucleus was stained with DAPI (blue). Each figure was shown in representative of three experiments. The Scale bar indicates 50um.

## DISCUSSION

The objective of this study was to develop a combined strategy that allows application of cADMSCs simultaneously with chABC for the treatment of chronic SCI. Treatments with cADMSCs only or with chABC 3 weeks after SCI resulted in significantly improved clinical signs quantified by BBB scores and Tarlov scores when compared to the control group. The degradation of CSPGs and expression of  $\beta$ 3-tubulin in the cADMSCs + chABC group were better (i.e., higher) than they were in the other groups. The level of NF-M in the cADMSCs group showed more increase than other groups. The levels of COX2 and pSTAT3 in the treatment groups were higher than in the control group.

The *in vitro* results of the current study revealed that chABC treatment did not influence the proliferation of the cultured cells. In a previous report, chABC treatment did not affect the proliferation of cells and protein synthesis *in vivo* (Jo et al. 2006). The CSPGs degradation capacity of chABC is reliable for approximately 8 weeks after transplantation (Wang et al. 2012). Brückner (Brückner et al. 1998) also showed that a single injection of chABC results in a sustained partial decrease in CSPG levels for 8–12 weeks. However, based on the results of *in vitro* study, the CS degradation capacity of chABC was sustained for approximately 1 week. Delivery of chABC for 1 week before mesenchymal stem cell (MSC) transplantation reduced CSPGs, indicating

successful enzymatic degradation (Ikegami et al. 2005, Karimi-Abdolrezaee et al. 2010). I designed an experiment for the simultaneous injection of cADMSCs and chABC because of the lack of harmful effects of chABC on cADMSCs, and the possibility of their synergistic effect.

The antibody for CS56 recognizes an epitope located on various intact chondroitin sulfate glycosaminoglycan chains. The antibody 2B6 recognizes an epitope created after chABC degradation of chondroitin-4 sulfate and does not identify the intact form (Lee et al. 2013).

The beneficial effects of CSPGs degradation by chABC are the modification of extracellular matrix (ECM) organization and the extension of motile astrocytic processes towards the lesion epicenter (Milbreta et al. 2014). In quantitative histological analyses, degraded CSPGs were higher in the cADMSCs group than in the control group, and significantly more degradation of CSPGs was detected in the cADMSCs + chABC group. MSCs constitutively expressed matrix metalloproteinases (MMPs) and migrated into tissues in response to inflammatory stimuli (Ries et al. 2007). MMPs can degrade all ECM proteins including the core proteins of CSPGs (Cua et al. 2013). In particular, the CSPGs degraded by MMPs contribute to the growth-promoting properties of degenerated nerves (Ferguson and Muir 2000). More degradation of CSPGs in the cADMSCs + chABC group than the cADMSCs and chABC groups suggested a synergic effect of the combination treatment.

The combination of stem cell transplantation and chABC injection has been shown to stimulate axonal growth after CNS damage (Fouad et al. 2005, Zhang et al. 2010). These treatment could promote axonal sparing and plasticity in the chronically injured spinal cord (Karimi-Abdolrezaee et al. 2010), which in turn improves the survival microenvironment of transplanted cells. Eight weeks after transplantation is sufficient for axonal regeneration, theoretically (Steward et al. 2003). The individual effects of MSCs and chABC on axonal regeneration can be combined resulting in further improvement (Wang et al. 2012). In the present study, the recovery of hindlimb function in the treatment groups was better than in the control. My results showed higher levels of neuronal markers in the treatment groups;  $\beta$ 3-tubulin was especially higher in the combination treatment group and NF-M in the cADMSCs group than in other groups. The treatment groups that include cADMSCs had higher expression of neuronal markers than the groups that do not include the cADMSCs treatment. It was reported that improvement of clinical function by transplantation of cADMSCs might be due to paracrine effects or differentiation into neuronal cells by implanted cells (Park et al. 2012). Although in the present study transplanted cells were not tracked the implanted cADMSCs could survive until 8 weeks. Survived cADMSCs might affect the increase of neuronal cells (Ryu et al. 2011). Enhanced function of endogenous neurons by engraft cells may relay signals from disrupted fibers, including local circuit interneurons or ascending fibers that are present in the dorsal column (Bregman et al. 1993). However,

there was no difference in the functional recovery among treatment groups. Unlike the *in vitro* results, this might indicate that the chABC affected the viability or differentiation ability of cADMSCs *in vivo*.

The result of present study revealed that the reactive astrocyte marker GFAP was significantly lower in the transplantation groups than in the control group. Within the entire CNS, astrocytes are highly differentiated glial cells involved in numerous essential functions. Astrocytes respond by a process of reactive astrogliosis that varies with the nature and severity of the insult; in particular, eliminating reactive astrocytes reduces scar formation at the injury site (Milbreta et al. 2014). Therefore, decreased expression of GFAP in the treatment groups indicates reduced scar formation, as indicated by the degradation of CSPGs.

All treatment groups showed significantly increased expression of COX2. Furthermore, pSTAT3 and GalC expression were higher than in the control. pSTAT3 is upregulated after CNS insults and the activation of STAT3 is good evidence for certain aspects of astrogliosis. In previous studies, MSCs were implanted 1 week after SCI (Ryu et al. 2011, Park et al. 2012) and the results show the downregulation of inflammatory markers and increase in the expression of neuronal markers. The functional recovery in the present study improved, but inflammation markers such as COX2 and pSTAT3 were expressed at higher levels. In the present study, implantation of MSCs and/or

chABC was performed at 3 weeks after SCI, when scar tissue including CSPGs was formed. Higher expression of inflammation markers in the treatment groups might be due to the degraded CSPGs, which stimulate macrophages to migrate into injured sites. Further studies would be needed to reduce the inflammation that occurred after treatment with MSCs and chABC.

## **CHAPTER II**

### **Combinatorial transplantation of chondroitinase ABC with BDNF-expressing mesenchymal stromal cells, along with intravenous injection of MSCs in a dog model of chronic spinal cord injury**

#### **ABSTRACT**

The microenvironment of the chronically-injured spinal cord does not allow for axonal regeneration due to glial scarring. To ameliorate that circumstance, several therapeutic strategies have been utilized. I investigated whether combinatorial transplantation of chABC and MSCs genetically modified to secrete BDNF with intravenous administration of MSCs can promote recovery of hindlimb function after chronic SCI. Canine BDNF-expressing MSCs were generated using a lentivirus packaging protocol. Twelve beagle dogs with experimentally-induced chronic SCI were divided into chABC/MSC-GFP, chABC/MSC-BDNF, and chABC/MSC-BDNF/IV groups. The MSCs ( $1 \times 10^7$  cells) and chABC were transplanted 3 weeks after SCI in all groups and intravenous injection of MSC-GFP ( $1 \times 10^7$  cells) was performed 1 and 2 weeks

after MSC transplantation in the chABC/MS-C-BDNF/IV group. Spinal cords were harvested 8 weeks after transplantation. The dogs in the chABC/MS-C-BDNF included groups had significantly improved functional recovery 8 weeks after transplantation compared to those in the chABC/MS-C-GFP group. The animals in the chABC/MS-C-BDNF/IV group showed significant improvements in functional recovery at 6, 7, and 8 weeks compared to those in the chABC/MS-C-BDNF group. Fibrotic changes were significantly decreased in the chABC/MS-C-BDNF/IV group. I also observed significant decreases in the expression levels of TNF $\alpha$ , interleukin-6 (IL-6), COX2, GFAP, and GalC and increased expression levels of BDNF,  $\beta$ 3-tubulin, NF-M, and nestin in the chABC/MS-C-BDNF/IV group. I suggest that combinatorial transplantation of chABC and BDNF-expressing MSCs, along with intravenous injection of MSCs is the best therapy for chronic SCI.

Keywords: chondroitinase ABC, brain-derived neurotrophic factor, mesenchymal stromal cells, intravenous injection, chronic spinal cord injury



## INTRODUCTION

Regeneration of axons after chronic SCI occurs at very low levels spontaneously. However, suitable growth factors and substrates are known to enable lesioned axons to regenerate (Jin et al. 2002). The microenvironment of the damaged spinal cord prevents the regeneration of axons due to glial scarring, growth inhibitory molecules, and inflammation (Yick et al. 2000, Lu et al. 2005). Furthermore, the environment of the injured spinal cord facilitates stem cells differentiation to glial lineages, such as astrocytes and oligodendrocytes (Rossi and Keirstead 2009, Park et al. 2012).

To ameliorate these circumstances, several therapeutic strategies have been utilized, including digestion of CSPGs by chABC, grafting of MSCs, and the application of supplementary neurotrophic factors (Yick et al. 2000, Jin et al. 2002, Sasaki et al. 2009, Park et al. 2012, Kim et al. 2015). Recently, in the context of cell therapy for SCI, genetically-modified cells have been used to improve functional recovery after SCI. This therapeutic approach enables the injured spinal cord to be modified. It also allows neurotrophic factors to be delivered to the lesion site and enhance intrinsic neuronal growth responses (Jin et al. 2002, Ritfeld et al. 2015).

Fewer studies have been carried out to investigate chronic SCI as opposed to

acute or subacute spinal injury. Much of the current research on SCI does not focus on combining therapeutic methods to obtain better effects. It is suggested that a 3-week delay between injury and treatment represented chronic SCI, the glial scar is established at the lesion site by this time (Lu et al. 2007, Bowes et al. 2012, Lee et al. 2015). I have previously reported the effects of chABC and MSC transplantation in the 3-weeks-delayed SCI model. This combinatorial therapy leads to an improvement of functional recovery. However, CSPG degradation by chABC and the MSC may increase the expression of inflammatory cytokines at the injured site (Lee et al. 2015). Systemic administration of MSCs may reduce the levels of inflammatory cytokines at the injured spinal cord (Osaka et al. 2010, Kim et al. 2015).

I hypothesized that the degradation of the glial scar by chABC combined with the presence of neurotrophic factors released by of the transplanted MSCs secreting BDNF can enhance functional recovery after chronic SCI. Additionally, intravenous administration of MSCs may reduce inflammation at the injured spinal cord. Based on my hypothesis, the effects of chABC combined with those of BDNF-expressing MSCs (MSC-BDNF) transplanted into the injured spinal cord and the systemic delivery of MSCs were evaluated. I measured clinical outcomes and markers such as the degradation of CSPGs, neuronal regeneration, and immunomodulation.

## **MATERIALS AND METHODS**

### **1. Isolation and culture of cADMSCs**

CADMSCs were obtained according to the methods described in a previous study (Lim et al. 2007). Briefly, adipose tissue was aseptically collected from the gluteal subcutaneous fat of 2-year-old beagle dogs under general anesthesia. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-151027-4), Korea. The fat tissue was washed with PBS and digested with collagenase type I (1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) for 2 hours at 37°C. The samples were then washed with PBS and centrifuged at  $300 \times g$  for 10 minutes. The pellet (i.e., the stromal vascular fraction) was resuspended, filtered through a 100- $\mu\text{m}$  nylon mesh, and incubated in medium with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) at 37°C with 5% humidified CO<sub>2</sub>. The medium was changed at 48-hour intervals until the cells became confluent. After cells reached 90% confluence, they were trypsinized and stored in liquid nitrogen or subcultured. The cells were used for the following experiments at passage 3.

### **2. Lentiviral packing and transfection with green fluorescence**

## **protein (GFP) labeled BDNF**

CADMSCs were transduced with lentivirus vectors encoding GFP and canine BDNF with GFP. The transfected cells expressed GFP and had upregulated BDNF production in the reference to the gene database (GenBank: AB105074.1). pPACK Packaging Plasmid Mix (System Biosciences, Mountain View, CA, USA) was used for lentiviral packaging. In brief, the genes encoding Flag-tagged BDNF and GFP were amplified from a cDNA library of canine peripheral blood using Phusion DNA Polymerases (Thermo Scientific, Pittsburgh, PA, USA). The canine BDNF cDNA was amplified by PCR using the 5' BDNF primer ATGACCATCCTTTTCCTTAC, which contains an EcoRI site and the 3' BDNF primer GATAGAAGGGGAGAATTACC, which contains a BamHI site (Fig. 10, System Biosciences, San Diego, CA, USA). HEK293T cells (Thermo Scientific, Waltham, MA, USA) were maintained in 10% FBS and 1% penicillin/streptomycin in Dulbecco's Modified Eagle's medium (DMEM) at 37 °C and 5% CO<sub>2</sub>. Twenty-four hours before transfection,  $4 \times 10^6$  HEK293 cells were seeded onto a 100-mm dish. The following day, 20  $\mu$ L of lentiviral packaging mix (System Biosciences, San Diego, CA, USA) encoding the viral proteins Gag-Pol, Rev, and VSV-G, as well as 2  $\mu$ g of the lentiviral transgene plasmids were transfected into cells for lentivirus production using TurboFect (Thermo Scientific, Waltham, MA, USA). GFP-expressing virus particles and GFP-labeled BDNF-expressing virus particles were collected and



**Figure 10.** Construction of lentiviral vector. Lentiviral vectors contain an EF-1 $\alpha$  promoter, BDNF, copGFP, and puromycin gene. RSV: Rous sarcoma virus U3, LTR: long terminal repeat, RRE: Rev-responsible element, cPPT: central polyprine tract, WPRE: woodchuck hepatitis virus post-transcriptional regulatory element.

transduced into cADMSCs at passage 1. After the cADMSCs reached 90% confluence, I performed a selection step was using puromycin (3 µg/ml, Gibco BRL, Grand Island, NY, USA). Approximately 30 to 40% of the cells were successfully transduced after the puromycin selection step. The cADMSCs were subcultured and passage 3 cells were used for the following experiments. All procedures were performed in accordance with the Seoul National University Institutional Biosafety Committee (SNUIBC) (SNUIBC-R150716-1-1). A fluorescence microscope was used to detect GFP fluorescence in cADMSCs transduced with GFP (MSC-GFP) and those transduced with GFP-labeled BDNF (MSC-BDNF).

### **3. Viability of gene-modified cADMSCs and the effect of chABC**

Lentiviral transduction of cADMSCs in culture was confirmed by GFP expression for 5 passages. After cADMSCs, MSC-GFP, and MSC-BDNF reached 90% confluence, they were trypsinized and cell viability was measured using the AO/PI cell viability kit (F23001, Logos Biosystems, Annandale, VA, USA) and a dual-fluorescence cell counter (Luna-FL, Logos Biosystems, Annandale, VA, USA). Cell viability was examined 4 independent times.

When the cells reached 80% confluence, attached cells were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) and transferred to a 96-well plate. Five U/ mL chABC (chABC, Sigma, St. Louis, MO, USA) was added to

wells containing cADMSCs, MSC-GFP, and MSC-BDNF. Control wells did not receive chABC. After 2 days of culture, the medium was removed and washed with PBS to remove loosely adherent cells. After the cells reached 90% confluence, they were trypsinized and cell viability was measured using the AO/PI cell viability kit and a dual fluorescence cell counter.

#### **4. Western blot analysis of cADMSCs**

CADMSCs, MSC-GFP, and MSC-BDNF were prepared for western blot analysis. The cell lysates were cleared and protein concentrations were determined using the Bradford method (Bradford 1976). Equal amounts of the samples (20 µg) were resolved by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membrane blots were washed with TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), blocked with 5% skim milk for 1 hour, and incubated with the appropriate primary antibodies at the recommended dilutions. The membranes were washed and the primary antibodies were detected using goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG conjugated to horseradish peroxidase. The secondary antibodies were diluted 1:2,000. Reactive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The primary antibodies used and their final dilutions are as follows: anti-actin (Sigma-

Aldrich, St. Louis, MO, USA) at 1:1,000, anti-BDNF (Abcam, Cambridge, UK) at 1:2,000, and anti-GFP (Thermo Scientific, Waltham, MA, USA) at 1:1,000. Western blot analysis was performed for 4 samples per group, and all experiments were performed in triplicate.

## **5. Flow cytometry analysis**

Cell surface protein expression of cADMSCs, MSC-GFP, and MSC-BDNF was evaluated using flow cytometry. I used a FACS Calibur flow cytometer (BD Biosciences, NJ, USA) and Cell Quest Pro software (BD Biosciences, NJ, USA). The cells were stained for flow cytometry using specific antibodies following the supplier's protocol (BD Biosciences, NJ, USA). In brief, cultured cells were washed twice with PBS and trypsinized. The harvested cells were then washed with PBS and divided into groups for antibody staining. Each aliquot contained approximately  $1 \times 10^5$  cells. The following antibodies were used to detect cell surface protein expression: mouse anti-dog CD34, rat anti-dog CD45 (AbD Serotec, Oxford, UK), mouse anti-dog CD44, mouse anti-human CD73, mouse anti-dog CD90, and mouse anti-human CD105 (Abcam, Cambridge, UK). All antibodies were conjugated with fluorescein isothiocyanate or phycoerythrin. The cells were incubated in the dark for 30 minutes at room temperature. After incubation, the cells were washed two times



with PBS and resuspended in 500  $\mu$ L of PBS.

## **6. Induction of SCI**

Twelve healthy 2-3-year-old male beagles, weighing an average of  $8.87 \pm 1.65$  kg were used for the present study. All dogs were clinically judged to be in good health, were neurologically normal, and had their own admission number from the Institute of Laboratory Animal Resources, Seoul National University (SNU-151027-4). All dogs were cared for in accordance with Animal Care and Use Guidelines (Institute of Laboratory Animal Resources, Seoul National University, Korea) during the experiment. The dogs were assigned, four per group, to one of three groups: 1) transplantation of MSC-GFP with chABC (chABC/MSC-GFP), 2) transplantation of MSC-BDNF with chABC (chABC/MSC-BDNF), and 3) combined therapy use of MSC-BDNF with chABC transplantation and MSC-GFP intravenous injection (chABC/MSC-BDNF/IV).

Experimental SCI was induced using the balloon compression method as previously described (Lim et al. 2007). Briefly, the dogs were treated with intravenous cefazolin sodium (40 mg/kg, Cefazoline; Chong Kun Dang Pharm., Seoul, Korea), tramadol (4 mg/kg, Toranzin; Sam Sung Pharm. Ind. Co., Seoul,

Korea), Alfaxalone (2 mg/kg, Alfaxan; Jurox Inc., MO, USA), and subcutaneous atropine sulfate (0.05 mg/kg, Atropine; Jeil Pharm., Seoul, Korea). Anesthesia was maintained by inhalation of 2% isoflurane in oxygen (Aerrane; Ilisung, Korea). The anesthetic monitor Datex-Ohmeda (Microvitec Display, Bradford, UK) was used to monitor physiological parameters, including rectal temperature, oxygen saturation, end tidal CO<sub>2</sub>, and pulse rate, during anesthesia. Hemilaminectomy was performed at the fourth lumbar segment (L4). A 3-French embolectomy catheter (SORIN Biomedica, Salluggia, Italy) was inserted into the hole at L4. A balloon catheter was advanced under fluoroscopic guidance at the cranial margin of the first lumbar segment (L1) and inflated using 50 μL/kg of the contrast agent (Omnipaque; Amersham Health, Cork, Ireland) diluted 50:50 with saline. The balloon catheter was fixed with a Chinese finger trap suture and removed after 12 hours. Following SCI, the soft tissues and skin were closed using standard methods. After the operation, the dogs were bandaged and monitored in an intensive care unit. The dogs were fed a balanced diet twice a day, and if needed, manual bladder expression was performed at least three times daily until voluntary urination was established.

## **7. Direct transplantation of cADMSCs and chABC into the injured sites and intravenous administration of cADMSCs**

Direct transplantation of MSC-GFP and MSC-BDNF with chABC was performed 3 weeks after SCI in the chABC/MS-GFP, chABC/MS-BDNF, and chABC/MS-BDNF/IV groups. The dogs were anesthetized using the same methods as those described for the induction of the SCI. For direct transplantation into the injured site, the L1 spinal cord was exposed by dorsal laminectomy. A total number of ten million cells suspended in 150  $\mu$ L of chABC were injected at the SCI site in three locations (middle of the injury site, and the proximal and distal margins) at a depth of 3 mm using a 30-gauge needle. One and two weeks after direct transplantation, a suspension of ten million MSC-GFP in 10 ml lactated Ringer's buffer was administered intravenously to dogs in the chABC/MS-BDNF/IV group over the course of 20 minutes (Kim et al. 2015).

## **8. Behavioral assessments**

To evaluate the functional recovery of the hindlimbs, behavioral assessments were performed before the operation and then weekly for 11 weeks after the operation. Each dog was videotaped for a minimum of 10 steps from both sides and from behind when walking. Dogs that were unable to bear weight on their hindlimbs were also videotaped while being supported by an experimenter

holding the base of their tail. Data were obtained using BBB scores (Table 1) (Park et al. 2012), as well as the revised Tarlov and modified Tarlov scales (Table 2) (Rabinowitz et al. 2008). Two individuals blinded to the experimental conditions scored the dogs' gaits independently after observing the videotapes. A mean score was calculated every week following the SCI until the end of the 11-week study period.

## **9. Histopathological and immunofluorescence analyses**

All of the dogs were euthanized eight weeks after direct transplantation and the spinal cord from the eleventh thoracic segment (T11) to the third lumbar segment (L3) was extracted by dissection. Each sample was fixed in 10% sucrose/PBS at 4°C for 12 hours and immersed in 20% sucrose solution overnight at 4°C. The dura was removed with scissors, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek<sup>®</sup>, Sakura, Torrance, CA, USA), frozen, and cut longitudinally into two sections. One half of each section was immediately frozen for western blot analysis using liquid nitrogen and the other half was cut into 10- $\mu$ m sections using a cryomicrotome. These sections were mounted on silane-coated glass slides and stained with H&E to detect fibrosis. Primary antibodies used in the immunofluorescence studies were against chondroitin-4-sulphate (2B6), chondroitin sulfate (CS56), BDNF

(Abcam, Cambridge, UK), GFAP,  $\beta$ 3-tubulin (Tuj-1) (Santa Cruz Biotechnology, Texas, USA), NF-M (Abcam, Cambridge, UK), and nestin (Santa Cruz Biotechnology, Texas, USA). The sections were fixed and permeabilized for 10 minutes using 0.1% (v/v) Triton X-100, washed, and pre-incubated in 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 30 minutes to decrease nonspecific antibody binding. Sections were incubated with the primary antibodies overnight at 4°C and incubated for 60 minutes at room temperature with anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa 647 (Abcam, Cambridge, UK). The secondary antibodies were diluted 1:2,000. DAPI (4,6-diamidino-2-penylindole) was used for nuclear staining. Histomorphometric analyses of H&E-, 2B6-, and CS56-stained sections were performed at four sites in each sample using image analysis software (ImageJ version 1.47; National Institutes of Health, USA).

## **10. Western blot analysis**

The frozen half of each spinal cord section 0.25cm from the epicenter of the injured region was used for western blot analysis. Briefly, the spinal cord tissue was washed twice with PBS and frozen at -150°C. The tissue was then homogenized using a sonicator (three 20-second bursts; Branson Sonicator 250; Branson Ultrasonic Corp., Danbury, CT, USA) in lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 1

mg/mL aprotinin, 1mM phenylmethylsulfonylfluoride, and 0.5 mM sodium orthovanadate) for 30 minutes on ice. Lysates were cleared by centrifugation (10 minutes at  $1,500 \times g$ ,  $4^{\circ}\text{C}$ ) and protein concentrations were determined using the Bradford method (Bradford 1976). Equal amounts of spinal cord protein (20  $\mu\text{g}$ ) were resolved by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membrane blots were washed with TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), blocked with 5% skim milk for 1 hour, and incubated with the appropriate primary antibodies at the recommended dilutions. The membranes were washed and the primary antibodies were detected using goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The antibodies used included: anti-actin (Sigma, MO, USA), 2B6, CS56, matrix metalloproteinases-2 (MMP-2), COX2, IL-6 (Abcam, Cambridge, UK), TNF $\alpha$  (Santa Cruz Biotechnology, Texas, USA), anti-GFP (Thermo Scientific Waltham, MA, USA), BDNF (Abcam, Cambridge, UK), GFAP, GalC, Tuj-1 (Santa Cruz Biotechnology, Texas, USA), NF-M (Abcam, Cambridge, UK), and nestin (Santa Cruz Biotechnology, Texas, USA). The western blot analysis was performed for 4 samples per group ( $n = 12$ ) and all experiments were performed in triplicate.

## **11. Statistical analysis**

In all quantification procedures, observers were blind to the nature of the experimental manipulation. Data are presented as medians and quartiles. Statistical analysis was performed using a commercially available statistical software program (SPSS Statistics, version 21.0, IBM Corp., NY, USA). In all experiments, Kruskal-Wallis tests were followed by Mann-Whitney U tests to compare the three groups.  $p < 0.05$  was considered significant.

## RESULTS

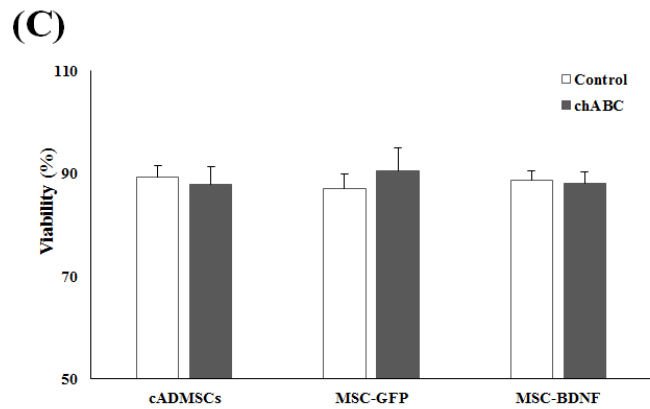
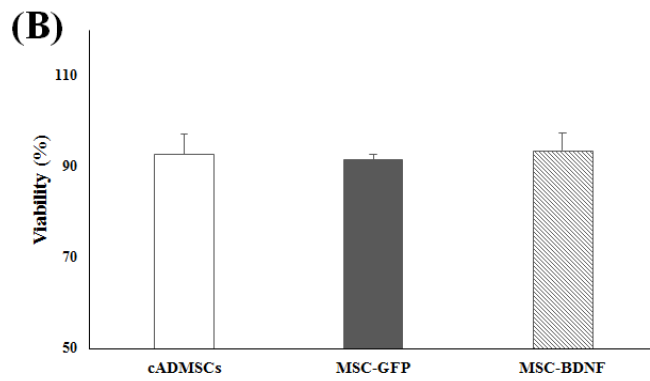
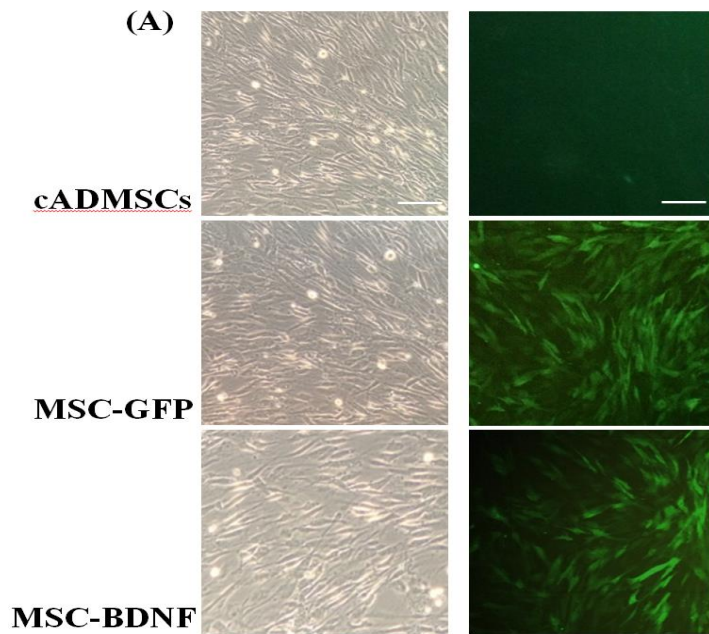
### 1. GFP expression and viability of BDNF-expressing cADMSCs

MSC-GFP and MSC-BDNF expressed green fluorescence, as observed using a fluorescence microscope (Fig. 11A). Cell viabilities of cADMSCs, MSC-GFP, and MSC-BDNF were  $92.75 \pm 4.61\%$ ,  $91.59 \pm 1.32\%$ , and  $93.65 \pm 3.82\%$ , respectively, under normal culture conditions. No significant differences in viability were observed among the cells (Fig. 11B).

### 2. Effect of chABC on cell viability

The viabilities of cADMSCs, MSC-GFP, and MSC-BDNF in the presence of chABC were investigated. There were no significant differences among the cells (Fig. 11C). The results indicate that 5 U/ml chABC does not affect the viabilities of the 3 kinds of MSCs.





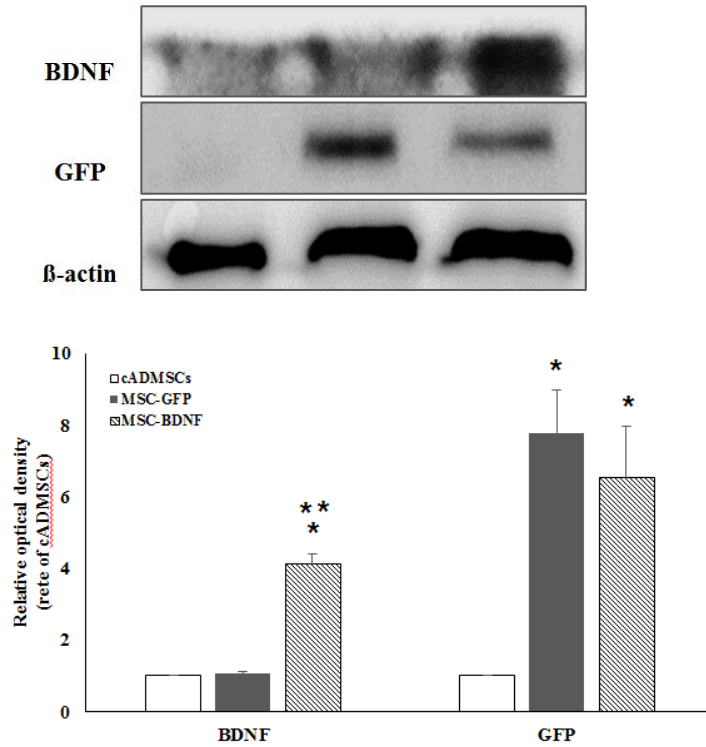
**Figure 11.** GFP expression, viability of gene-modified cADMSCs, and the effects of chABC **(A)** GFP expression of gene-modified cADMSCs. GFP expression was identified in MSC-GFP and MSC-BDNF using a fluorescence microscope. **(B)** Viability of the cells. There were no significant differences in viability between the groups. **(C)** Viability of combination culture with chABC. Proliferation of cultured cells in the presence of chABC and under normal culture conditions for cADMSCs, MSC-GFP, and MSC-BDNF. No significant differences in proliferation were observed between cells cultured under normal conditions and those cultured in the presence of chABC. The scale bar indicates 50  $\mu\text{m}$ .

### **3. Western blot analysis of the cADMSCs**

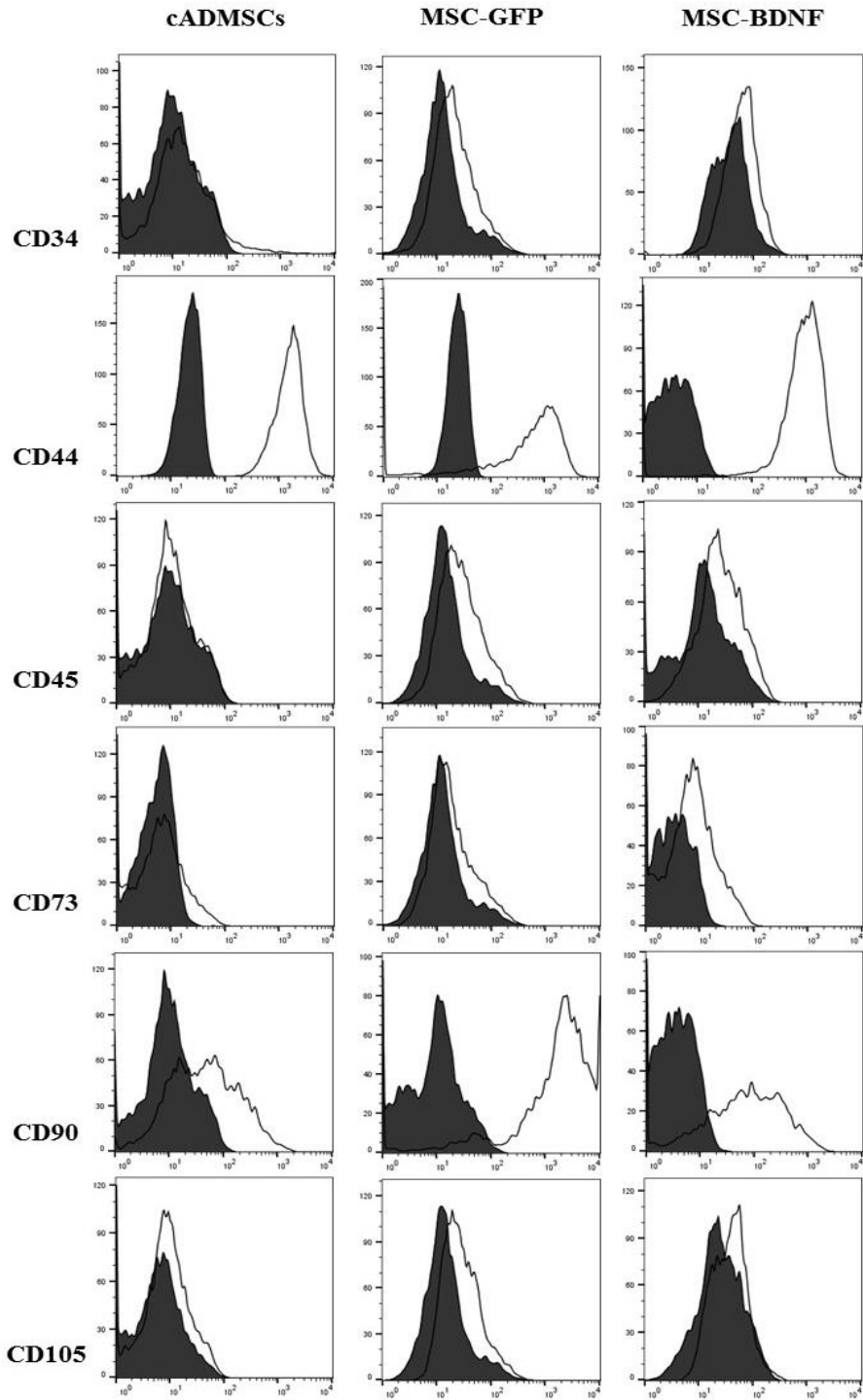
Protein expression of BDNF in MSC-BDNF was significantly higher than that of the cADMSCs and MSC-GFP. Furthermore, MSC-GFP and MSC-BDNF had a significant increase in GFP expression compared to the cADMSCs ( $p < 0.05$ ). However, no differences were observed between MSC-GFP and MSC-BDNF (Fig. 12).

### **4. Characteristics of gene-modified cADMSCs**

I characterized the transfected cADMSCs in vitro, which were found to have stable GFP expression for at least five passages in MSC-GFP and MSC-BDNF. The morphological features of the gene modified MSCs were adherent spindle-like shape and cell surface markers were identified similar with cADMSCs; CD34 (-), CD44(+), CD45 (-), CD73 (-), CD90 (+), and CD105 (-) (Fig. 13). Which was characteristic of cADMSCs and consistent with previous studies (Vieira et al. 2010, Lee et al. 2011). Transduction of cADMSCs with GFP or GFP-labeled BDNF did not affect the presence of typical cADMSC surface markers.



**Figure 12.** Western blot analysis of gene-modified cADMSCs. BDNF expression was significantly higher in the MSC-BDNF than in the cADMSCs or MSC-GFP (\*,\*\* $p < 0.05$ ). The MSC-GFP and MSC-BDNF had a significant increase in GFP expression compared to the cADMSCs (\* $p < 0.05$ ). There were no differences between the MSC-GFP and MSC-BDNF. (\* $p < 0.05$  compared to cADMSCs, \*\* $p < 0.05$  compared to MSC-GFP).



**Figure 13.** Flow cytometric analysis of gene-modified cADMSCs. Cell surface protein expression of cADMSCs, MSC-GFP, and MSC-BDNF was evaluated using flow cytometry. The cell surface markers of gene-modified cADMSCs were similar to those of cADMSCs. The cells were CD34(-), CD44(+), CD45(-), CD73(-), CD90(+), and CD105(-).

## 5. Behavioral observations

After the induction of SCI, all experimental dogs showed complete pelvic limb paralysis. Their BBB were 21 prior to the SCI and were down to 0 following SCI. BBB scores were obtained every week until 8 weeks after direct transplantation of cells. In all groups, the scores gradually increased during the study period. However, the chABC/MSC-BDNF group and the chABC/MSC-BDNF/IV group showed higher rates of improvement during the first week after direct transplantation, while the chABC/MSC-GFP group showed improvements 3 weeks after transplantation. The BBB scores of dogs in the chABC/MSC-BDNF group were significantly more improved 2, 3, and 8 weeks after direct transplantation compared to those in the chABC/MSC-GFP group ( $p < 0.05$ ). The dogs in the chABC/MSC-BDNF/IV included groups were showed significantly higher improvements in their BBB scores 6, 7, and 8 weeks after direct transplantation compared to those in the chABC/MSC-BDNF group ( $p < 0.05$ , Fig. 14A). Improvements in hindlimbs locomotion in all dogs in the chABC/MSC-BDNF/IV group and in three dogs in the chABC/MSC-BDNF group enabled them to support their own body weight occasionally. On the other hand, only three dogs in the chABC/MSC-GFP group had a limited ability to support their body weights when aided by experimenters. To evaluate these behaviors qualitatively, I used the revised and

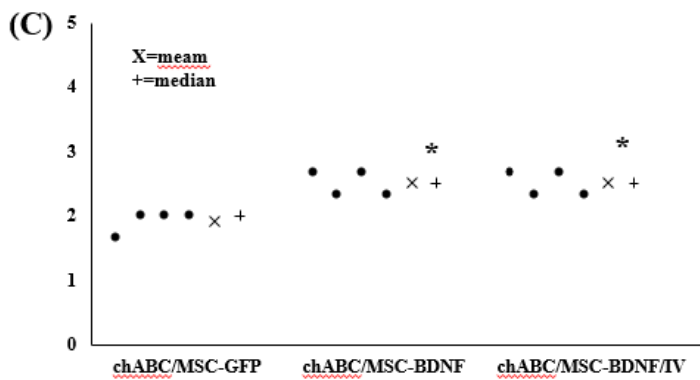
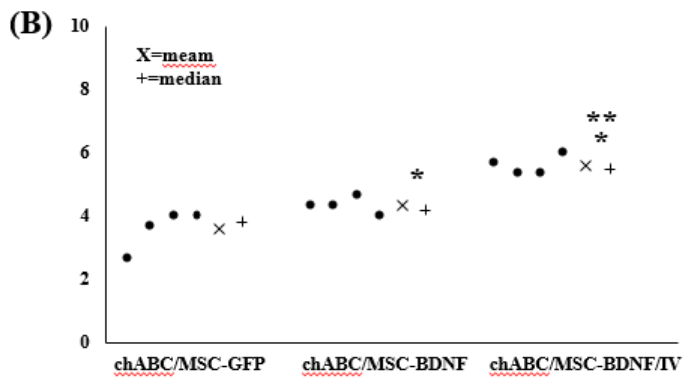
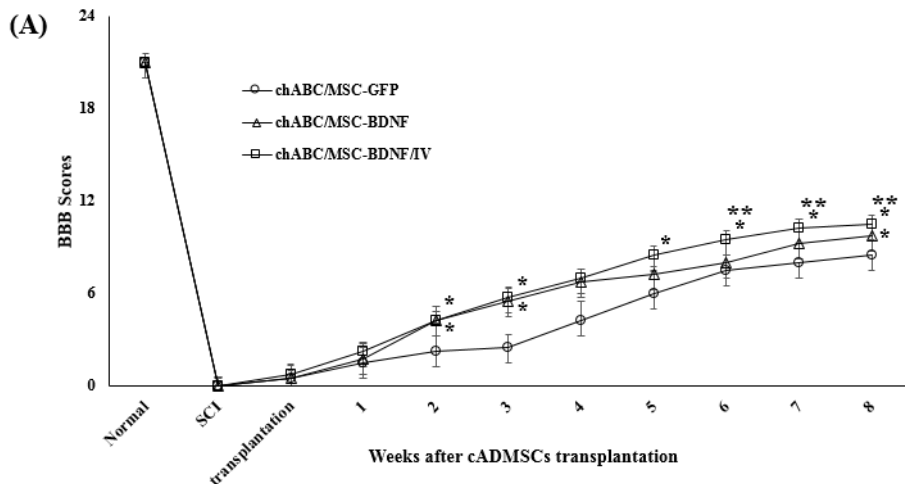
modified Tarlov scales to assess neurologic recovery 8 weeks after transplantation. I found significantly higher motor recovery in the chABC/MSC-BDNF/IV group than in the chABC/MSC-GFP and chABC/MSC-BDNF groups ( $p < 0.05$ , Figs. 14B and 14C).

## **6. Histopathologic assessments**

Gross findings indicate that the sizes of the injured regions in the chABC/MSC-GFP, chABC/MSC-BDNF, and chABC/MSC-BDNF/IV groups were  $1.35 \pm 0.17$ ,  $1.17 \pm 0.13$ , and  $1.15 \pm 0.14$  cm, respectively. I found no significant differences in this measure among the different groups. Fibrotic and atrophic changes in the injured regions were detected in most of the samples in from the three groups 8 weeks after transplantation. Histopathological analysis using H&E staining showed pathological changes in the parenchyma. However, in the chABC/MSC-BDNF/IV group, the spinal cord lesions were limited to the compression region, and a reduction in fibrotic changes was observed in comparison to the chABC/MSC-GFP group. Using high-power microscopy, I found that hemorrhages, fibroblast-like cell proliferation, and infiltration of microglial cells into the injured region were also reduced in the chABC/MSC-BDNF/IV group. Quantification of fibrotic tissue in H&E-stained sections

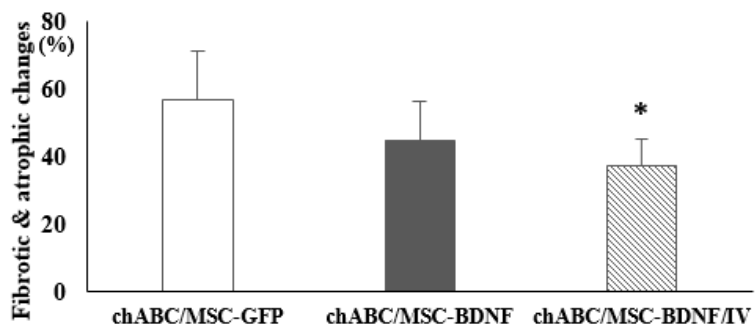
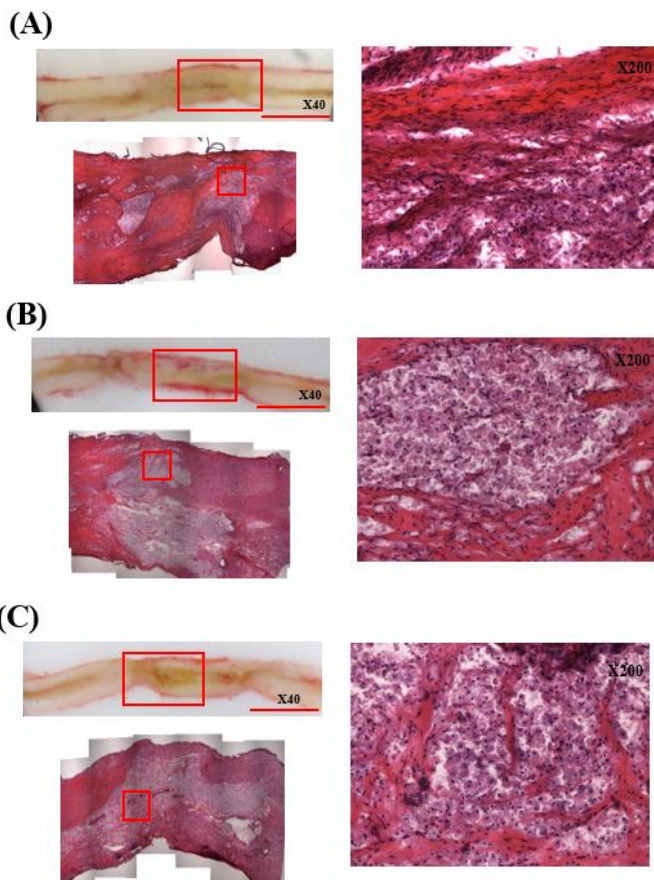


indicated significant differences between the chABC/MSC-GFP and chABC/MSC-BDNF/IV groups ( $p < 0.05$ , Fig. 15).



**Figure 14.** Behavioral analysis using BBB scores and Tarlov scales

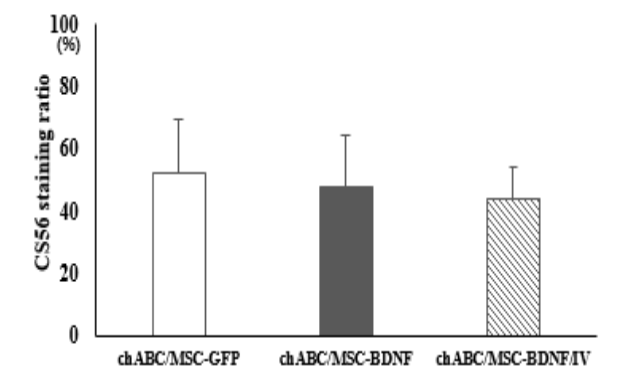
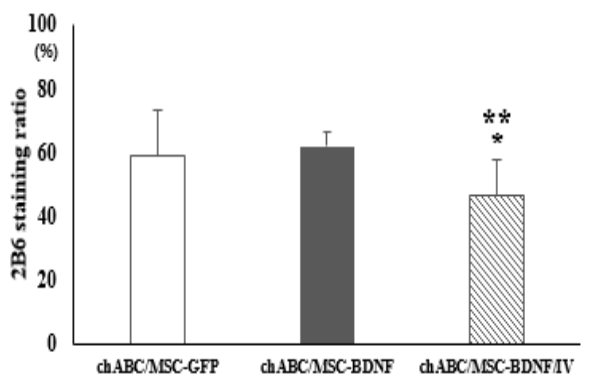
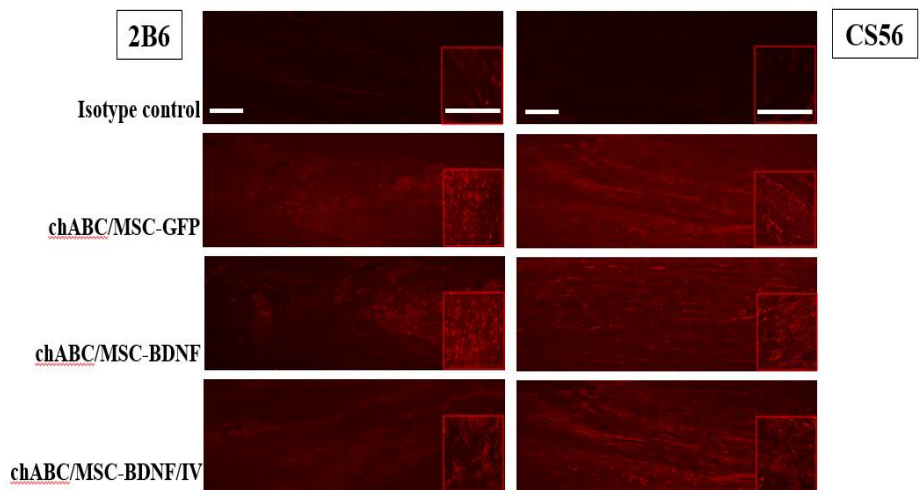
(A) The scores of all groups gradually increased during the study period. The chABC/MSC-BDNF group and the chABC/MSC-BDNF/IV group showed high rates of improvement one week after direct transplantation and the chABC/MSC-GFP group displayed rapid improvements 3 weeks after transplantation. The BBB scores of the chABC/MSC-BDNF group showed significant improvements 2, 3, and 8 weeks after direct transplantation compared to those of the chABC/MSC-GFP group ( $*p < 0.05$ ). The BBB scores of the chABC/MSC-BDNF/IV groups were significantly more improved 2, 3, 5, 6, 7, and 8 weeks after direct transplantation compared to those of the chABC/MSC-GFP group ( $*p < 0.05$ ). The chABC/MSC-BDNF/IV group showed more significant improvements in the BBB score at 6, 7, and 8 weeks after direct transplantation compared to the ch/MSC-BDNF group ( $**p < 0.05$ ). Hindlimb locomotion 8 weeks after transplantation was measured using two grading systems: the revised Tarlov scale (B), and the modified Tarlov scale (C). Higher motor function recovery was observed in the chABC/MSC-BDNF/IV group compared to the chABC/MSC-GFP and chABC/MSC-BDNF groups ( $*p < 0.05$  compared to chABC/MSC-GFP group,  $**p < 0.05$  compared to chABC/MSC-BDNF group). X, mean; +, median.



**Figure 15.** Histopathological analysis using H&E staining. Histological analysis of spinal cord lesions stained with H&E. **(A)** chABC/MSC-GFP group, **(B)** chABC/MSC-BDNF group, and **(C)** chABC/MSC-BDNF/IV group. Fibrotic and atrophic changes in the injured regions were detected in all groups. Fibroblast-like cell proliferation was detected in the chABC/MSC-GFP group upon magnification of the injury epicenter. In the chABC/MSC-BDNF group, the spinal cord lesions were limited to the compression region and a reduction in fibrotic changes was observed in comparison to the chABC/MSC-GFP group. Under high power, hemorrhage, fibroblast-like cell proliferation, and infiltration of microglial cells into the injured region were also found to be reduced in the chABC/MSC-BDNF/IV group. Quantification of fibrotic tissue revealed significant differences between the chABC/MSC-GFP group and the chABC/MSC-BDNF/IV group ( $*p < 0.05$ ). The scale bar indicates 1 cm.

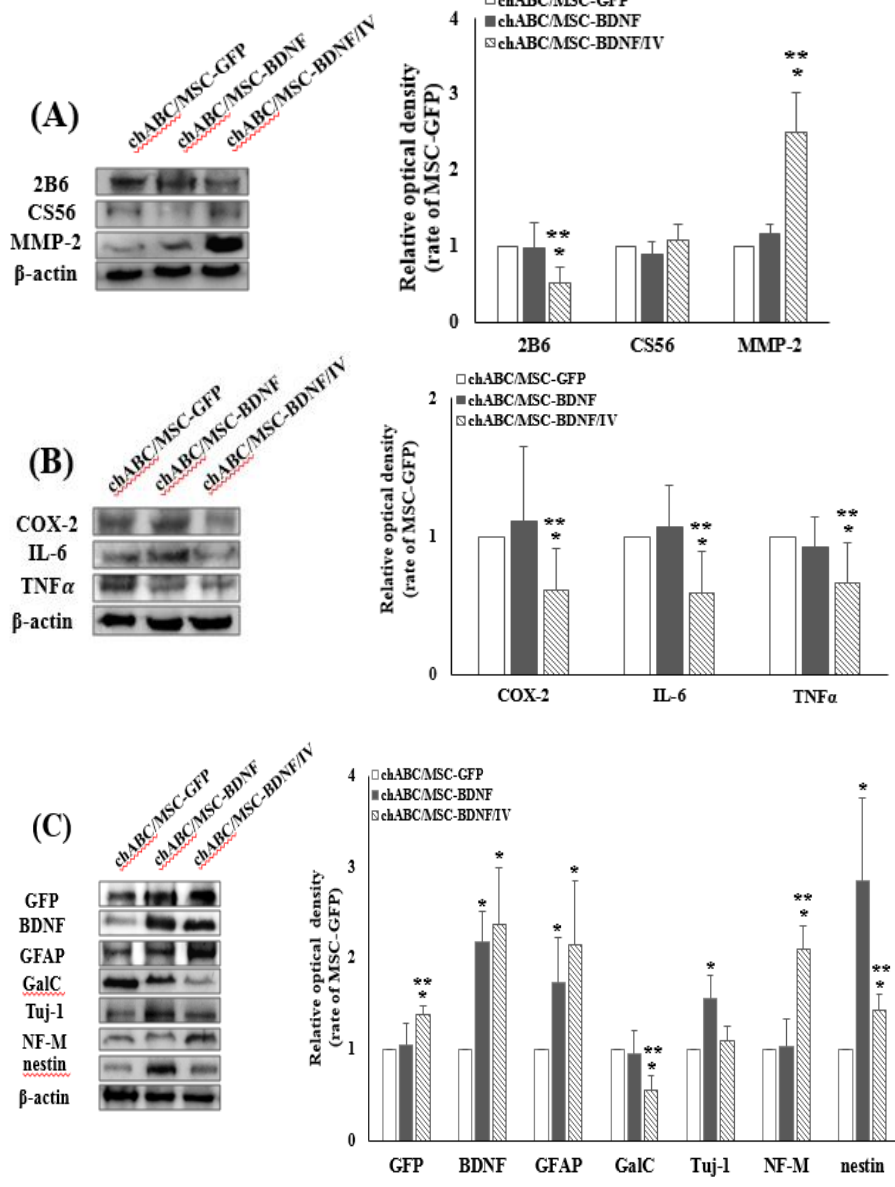
## 7. Immunohistochemical assessments

Immunohistochemistry revealed that the expression of the CSPG digestion marker, 2B6, was higher in the chABC/MSC-GFP and chABC/MSC-BDNF groups than in the chABC/MSC-BDNF/IV group. However, the expression of the intact CSPG marker, CS56, was similar among the groups. The levels of MMP-2 were significantly higher in the chABC/MSC-BDNF/IV group compared to the other two groups (Figs. 16 and 17A). The levels of COX2, IL-6, and TNF $\alpha$  were significantly lower in the chABC/MSC-BDNF/IV group than in the chABC/MSC-GFP and chABC/MSC-BDNF groups (Fig. 17B). Expression of GFP and NF-M was not different between the chABC/MSC-GFP and chABC/MSC-BDNF groups but was higher in the chABC/MSC-BDNF/IV group compared to the other two groups ( $p < 0.05$ ). Expression of BDNF, GFAP, and nestin was significantly higher in the chABC/MSC-BDNF included groups compared to the chABC/MSC-GFP group. The chABC/MSC-BDNF group had higher nestin expression compared to the chABC/MSC-BDNF/IV group ( $p < 0.05$ ). The level of GalC was decreased in the chABC/MSC-BDNF/IV group in comparison to the other groups. The chABC/MSC-BDNF group had higher expression levels of Tuj-1 than the chABC/MSC-GFP group ( $p < 0.05$ , Figs. 17C and 18).

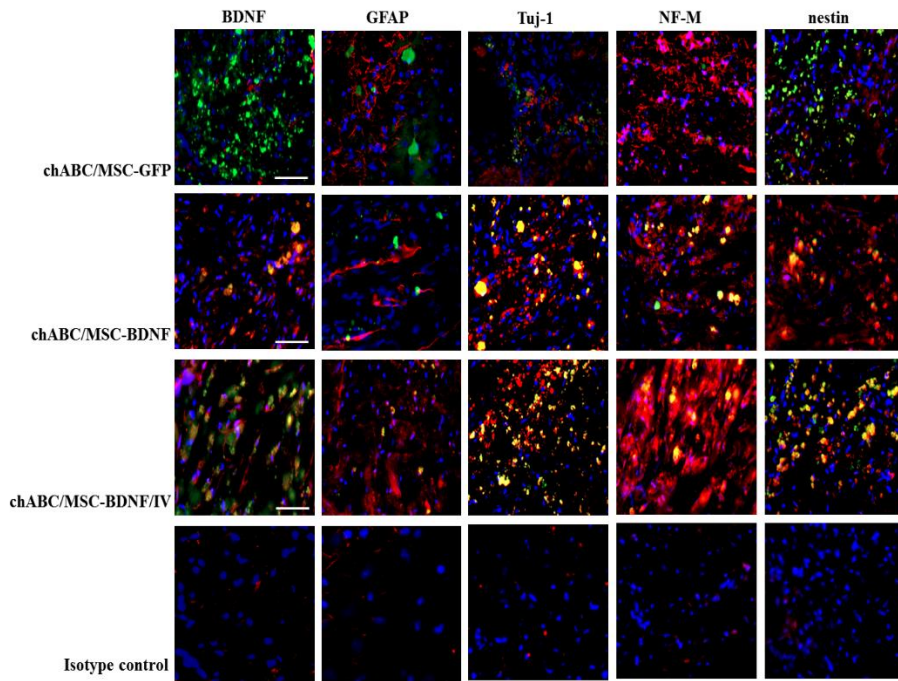


**Figure 16.** Immunohistochemical analysis of chondroitin-4-sulfate (2B6) and intact chondroitin sulfate (CS56). CSPGs and their cleavage products, glycosaminoglycan side chains, were detected using immunofluorescence in the spinal cord lesion (using antibodies 2B6 and CS56). Expression of the CSPG digestion marker, 2B6, was significantly higher in the chABC/MSC-GFP and chABC/MSC-BDNF groups than in the chABC/MSC-BDNF/IV group. However, levels of the intact CSPG marker, CS56, were similar among the three groups. Quantification of 2B6 indicated significantly lower levels in the chABC/MSC-BDNF/IV group than in the chABC/MSC-GFP and chABC/MSC-BDNF groups. CS56 levels were not significantly different among the three groups. (\* $p < 0.05$  compared to chABC/MSC-GFP group, \*\* $p < 0.05$  compared to chABC/MSC-BDNF group)





**Figure 17. (A)** 2B6, CS56, and MMP-2. Expression of the CSPG digestion marker, 2B6, was higher in the chABC/MSC-GFP and chABC/MSC-BDNF groups than in the chABC/MSC-BDNF/IV group. Expression of the intact CSPG marker, CS56, was similar among the three groups. MMP-2 had significantly higher levels in the chABC/MSC-BDNF/IV group than in the other groups. **(B)** Inflammatory markers COX2, IL-6, and TNF $\alpha$ . The levels of COX2, IL-6, and TNF $\alpha$  were significantly lower in the chABC/MSC-BDNF/IV group than in the chABC/MSC-GFP and chABC/MSC-BDNF groups, but did not differ between the chABC/MSC-GFP and chABC/MSC-BDNF groups. **(C)** GFP, neuronal markers, and intracellular astrogliosis markers; BDNF, GFAP, GalC, Tuj-1, NF-M, and nestin. Expression levels of GFP and NF-M were not different between the chABC/MSC-GFP and chABC/MSC-BDNF groups, but were higher in the chABC/MSC-BDNF/IV group compared to the other two groups. Expression levels of BDNF, GFAP, and nestin were significantly higher in the chABC/MSC-BDNF group compared to the chABC/MSC-GFP group. Nestin had higher expression levels in the chABC/MSC-BDNF group compared to the chABC/MSC-BDNF/IV group. The levels of GalC were decreased in the chABC/MSC-BDNF/IV group compared to the other two groups. The chABC/MSC-BDNF group had higher expression levels of Tuj-1 compared to the MSC-GFP group. \* $p < 0.05$  compared to the chABC/MSC-GFP group, \*\* $p < 0.05$  compared to the chABC/MSC-BDNF group. The graph and the bars depict means  $\pm$  SE for four dogs per group, as determined by densitometry relative to  $\beta$ -actin.



**Figure 18.** Immunohistochemical assessments. Immunofluorescence staining was performed at 8 weeks after transplantation. Injured spinal cord lesions were immunostained for BDNF, GFAP, Tuj-1, NF-M, nestin (red), direct transplantation or intravenous injection (green), and nuclei were stained with DAPI (blue). Each figure shown is representative of three experiments. The scale bar indicates 50  $\mu$ m.

## DISCUSSION

The present study indicates that the administration of chABC/MSC-BDNF improves hindlimb function more significantly than chABC/MSC-GFP, and that intravenously injected MSC-GFP has anti-inflammatory effects. Previously, I investigated the effect of treatment using a combination of cADMSC and chABC in a chronic SCI model. My results indicated improved hindlimb function, decreased glial scarring, and facilitated endogenous neuronal regeneration in treated animals (Lee et al. 2015). In this study, gene modification with BDNF or GFP did not affect the proliferation of cells and the presence of cADMSC surface markers (Ritfeld et al. 2015). In addition, using chABC in combination with the gene-modified cADMSCs did not affect the proliferation and viability of the cells, as in the previous study (Lee et al. 2015).

Only a few studies have investigated chronic SCI and the effects of cells genetically modified to express canine BDNF (Jin et al. 2002, Lu et al. 2005). The neurotrophic factor BDNF exhibits protective properties in the injured CNS and has anti-apoptotic effects, which promote cell survival after CNS damage. It also can improve functional recovery after SCI (Dougherty et al. 2000, Jin et al. 2002, Lu et al. 2005, Sasaki et al. 2009, Osaka et al. 2010). In the current study, the chABC/MSC-BDNF and chABC/MSC-BDNF/IV groups showed a more rapid improvement of functional recovery compared to the

chABC/MS-C-GFP group. It has been suggested that the degradation of CSPGs and the early release of BDNF from genetically-modified cADMSCs leads to significantly greater neuronal regeneration compared to treatment with unmodified cells. It is thus thought that treatment with the gene-modified cADMSCs is sufficient to improve the functional recovery of affected hindlimbs (Jin et al. 2002, Lu et al. 2005). The early application of neurotrophic factors to the injured spinal cord has been shown to promote axonal growth and to prevent the atrophy of neurons (Bregman et al. 1997, Bregman et al. 1998). In addition, exogenous transplantation of BDNF induces axonal regrowth at the site of SCI (Xu et al. 1995).

Reactive astrocytosis is a functional state of astrocytes that is adopted as a response to CNS injury and has opposing roles in SCI. Reactive astrocytes contribute to glial scar formation by forming a barrier while protecting the damaged nervous system (Fawcett and Asher 1999, Pekny and Nilsson 2005, Park et al. 2012). A proper environment in the injured spinal cord, modulated by the administration of neurotrophic factors, may change the properties of the reactive astrocytes and enable them to take important roles in neuroprotection and the secretion of neurotrophic factors (Lukovic et al. 2015). In this study, the groups that were transplanted with BDNF-expressing cADMSCs showed higher expression levels of GFAP and other neuronal markers. My results indicate that proper modification of the microenvironment in SCI by the administration of MSC-BDNF may promote the activation of beneficial

signaling pathways in reactive astrocytes.

In the current study, MSC-BDNF transplantation therapy combined with MSC-GFP intravenous injection led to significantly improved clinical outcomes after 8 weeks compared to the transplantation of MSC-GFP or MSC-BDNF. All dogs in the chABC/MSC-BDNF/IV group were able to support their weight occasionally without any help 8 weeks after transplantation. Moreover, dogs in the chABC/MSC-BDNF/IV group had low levels of inflammatory cytokines, which may have resulted from the inhibition of inflammatory responses and microglial activation. I have observed the fibrotic and atrophic changes of the spinal cord parenchyma through H&E staining, and which means that the inflammation has occurred. The infiltration of microglial cells indicates inflammatory reaction.

In my previous study, combination therapy using chABC and cADMSC transplantation led to high levels of inflammation and chondroitin-4-sulfate (C4S), which is an indicator of CSPGs degradation. These results indicate that the degraded CSPGs caused inflammation (Iida et al. 2007, Lee et al. 2015). In this study, the groups that were transplanted with BDNF-expressing cADMSCs showed higher expression levels of GFAP and other neuronal markers. My results indicate that proper modification of the microenvironment in SCI by the administration of MSC-BDNF may promote the activation of beneficial signaling pathways in reactive astrocytes. The results of this study indicate that

the levels of MMP-2 were higher in the chABC/MSC-BDNF/IV group than in other groups. Previous reports indicate that MSCs express MMPs constitutively and move along the injured tissue in response to inflammatory stimuli. Furthermore, systemically-administered cADMSCs are known to have immune-regulatory properties involved in systemic immune modulation and the inhibition of T-cell activities (Ries et al. 2007, Kim et al. 2015). It is suggested that the more significant improvements in clinical outcome in the chABC/MSC-BDNF/IV group compared to the chABC/MSC-BDNF group is due to the additional effects of MMP-2 and the immune modulation effects of intravenously-injected MSCs.

Previous reports indicate that most transplanted cells remain at the lesion site or migrate for short distances from the injured lesion into the host tissue (Jin et al. 2002, Lu et al. 2005, Ryu et al. 2011), which is consistent with my results. Furthermore, my studies of transplanted cell survival as indicated by GFP expression revealed no differences between the different transplanted cell types. However, the group that received the additional intravenous injection had significantly higher expression levels of GFP, which indicates that the intravenously administrated cADMSCs probably migrate to the injured spinal cord and survive for 8 weeks (Osaka et al. 2010, Kim et al. 2015). In this study, I did not use negative controls for ethical concerns, as my previous studies indicated limited improvements of hindlimbs function seen as slight joints movement in the negative control groups. In addition, the transplantation of

MSCs in previous studies supports a role for these cells in neuronal regeneration (Park et al. 2011, Park et al. 2011, Park et al. 2012, Kim et al. 2015, Lee et al. 2015). It is obvious that the recovery of the chronically injured spinal cord is not enhanced or sustained without support from additional neurotrophic factors. The release of BDNF from the transplanted MSC-BDNF or the anti-inflammatory effects of intravenously-administered MSC-GFP, along with changes in neurotrophic factors endogenously produced in the host spinal cord may contribute to functional recovery following chronic SCI.

My results demonstrate that the combination of chABC with BDNF secreted by genetically-modified cADMSCs, along with the intravenous injection of cADMSCs promotes clinical recovery in the chronically injured spinal cord via anti-inflammatory effects, microenvironment modification, and neuronal regeneration. Thus the combinatorial treatment used in this study can be the best therapy for chronic SCI.



## GENERAL CONCLUSION

This study was performed to investigate the therapeutic strategies by using gene-modified cADMSCs and chABC in chronic SCI. The results of the two experiments were as follows.

1. chABC and cADMSCs were administrated directly at the injured spinal cord to develop a combinatorial strategy that allows application of cADMSCs simultaneously with chABC for the treatment of chronic SCI. The groups that treated with cADMSCs and cADMSCs + chABC showed significantly improved clinical outcome, when compared to the control group. The degradation of CSPGs and expression of neuronal markers in the cADMSCs + chABC group were better than other groups.
2. Combinatorial transplantation of chABC and cADMSCs genetically modified to secrete BDNF with intravenous administration of cADMSCs were investigated. The chABC/MSC-BDNF/IV group showed significant improvements in functional recovery compared to those in the other groups. The chABC/MSC-BDNF group showed

significant decreases in the expression levels of inflammation and increased expression levels of neuronal markers.

On the basis of these results, the therapeutic strategy using chABC and BDNF expressing cAMDSCs might be essential for restoration after chronically injured spinal cord. The chABC could nurse the injured environment by degrading the CSPGs at the glial scars, the BDNF expressing cADMSCs could reinstate neuronal factors, and intravenously injected cADMSCs could ameliorate the injured environmental improvement. Thus the combinatorial treatment of direct and intraveonus injections of gene modified cADMSCs and chABC has a good therapeutic potential in the treatment of chronic SCI and this can be used as an alternative treatment modality in neuronal regeneration.

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## 국문초록

개의 만성 척수 손상에 대한 뇌유래신경인자발현  
중간엽줄기세포와 콘드로이치네이즈ABC의  
신경 재생 효과

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척수 손상 이후 신경조직의 성공적인 기능회복은 수의신경학에서 중요한 주제이다. 다분화능 중간엽유래 줄기세포의 적용은 신경재생에 있어서 다양한 가능성을 가지고 있다. 또한, 만성적 척수손상 치료에 콘드로이치네이즈ABC (chABC)와 뇌유래신경인자 (BDNF)와 같은 신경인자들이 널리 사용되고 있다. 신경친화효과를 위해 이러한 요소들의 단독 적용 또는 신경 재생을 방해하는 장애물로 작용하는 콘드로이친설페이트 프로테오글라이칸의 분해는 연구되어왔으

나, 이러한 요소들의 연합치료는 단독치료보다 효과적인 새로운 시도가 될 것이라 예상할 수 있다.

본 연구의 첫 번째 실험에서 개 지방유래 중간엽 줄기세포와 chABC의 병용이 만성적 개 척수 손상에서 기능회복과 신경재생에 미치는 영향을 확인하였고, 두 번째 실험에서는 chABC와 BDNF 발현유도 개 지방유래 중간엽 줄기세포의 효과를 개 지방유래 중간엽 줄기세포 정맥주사 병용과 함께 만성적 개 척수손상 모델에서 비교하였다.

chABC는 5 U/mL의 농도에서 개 지방유래 중간엽 줄기세포의 생존율에 영향을 미치지 않음을 확인하였다. 개 지방유래 중간엽 줄기세포와 chABC 병용군 그리고 개 지방유래 중간엽 줄기세포 단독 처치군은 대조군과 chABC 단독 처치군에 비해 직접주입 8주 후에 유의적으로 개선된 임상증상을 보였다. 그리고 개 지방유래 중간엽 줄기세포와 chABC 병용군에서는 2B6,  $\beta$ 3-tubulin, NF-M의 발현이 현저히 증가했다. 하지만 모든 실험군에서 대조군에 비해 COX2와 TNF $\alpha$ 의 발현이 증가한 것을 확인하였다. 개 지방유래 중간엽 줄기세포와 chABC 병용 처치는 보다 효과적으로 임상증상을 개선하고, 신경원성 인자들의 발현을 증가시킬 수 있지만, 만성 척수 손상의 치료 이후에 추가적인 항염증처치를 실시해야 한다.

염증 인자들의 높은 발현은 손상된 척수의 미세환경에 부정적인

영향을 미친다. 또한 개 지방유래 중간엽 줄기세포의 이식 이후에 신경원성인자들의 발현을 확인 할 수 없었다. 척수손상 이후의 임상 증상 개선을 향상하기 위해서는 염증을 감소시키고, 신경원성인자의 발현을 증가시키는 적절한 방법이 필요하다

두 번째 실험에서는 만성적 척수 손상에서 chABC와 BDNF 발현 개 지방유래 중간엽 줄기세포의 직접 주입 그리고 정맥을 통한 개 지방유래 중간엽 줄기세포의 적용 효과에 대해 확인하였다. BDNF 발현 개 지방유래 중간엽 줄기세포를 직접 주입한 두 군에서는 개 지방유래 중간엽 줄기세포를 주입한 군에 비해 주입 8주 후에 유의적인 임상증상의 개선을 보였으며, 정맥을 통한 개 지방유래 중간엽 줄기세포의 추가 적용을 실시한 군은 주입 6주 후부터 유의적인 기능 개선을 보였다. 조직학적인 결과에서도 섬유화의 확연한 감소를 확인할 수 있었으며 TNF $\alpha$ , IL-6, COX2, GFAP, GalC 발현의 현저한 감소를 BDNF,  $\beta$ 3-tubulin, NF-M, nestin 발현의 현저한 증가를 보였다. 이러한 결과는 chABC를 통한 신경손상 반흔의 분해와 주입한 줄기세포로부터의 신경인자 분비의 상승효과로 인한 개선으로 판단된다.

렌티바이러스 패키징을 이용한 유전자 조작 BDNF 발현 개 지방유래 중간엽 줄기세포의 생산은 안전하고 지속적인 방법이며, chABC와 줄기세포의 병용 그리고 정맥을 통한 줄기세포의 적용은

손상된 척수의 미세환경개선, 항염증 효과 그리고 신경 재생의 측면에서 임상적인 기능 개선에 효과가 있다. 결론적으로 BDNF 발현 중간엽 줄기세포와 chABC의 병용 치료는 신경 재생 효과의 향상을 통해 척수 손상 후 기능회복에 있어서 대체 치료 방법으로 사료된다.

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주요어: 중간엽 줄기세포, 개의 척수손상, 콘드로이치네이즈ABC,

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