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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Therapeutic Effects of Transgenic Canine Adipose Derived Mesenchymal Stem Cells in Canine Spinal Cord Injury

개 척수 손상에 대한 형질전환 개 지방유래 중간엽줄기세포의 치료 효과

by

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MAJOR IN VETERINARY SURGERY

DEPARTMENT OF VETERINARY MEDICINE

GRADUATE SCHOOL OF

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Supervised by

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ABSTRACT

The primary spinal cord injury (SCI) causes mechanical damage to the neurons and blood vessels. It leads to secondary SCI which activates multiple pathological pathways, which expand neuronal damage at the injury site. It is characterized by vascular disruption, ischemia, excitotoxicity, oxidation, inflammation and apoptotic cell death. It causes nerve demyelination and disruption of axons which perpetuate into a loss of impulse conduction through the injured spinal cord. It also leads to the production of myelin inhibitory molecules which with a concomitant formation of an astroglial scar,

impede axonal regeneration. The pivotal role regarding the neuronal necrosis is played by oxidation and inflammation. During an early stage of spinal cord injury there occur an abundant expression of reactive oxygen species (ROS) due to defective mitochondrial metabolism and abundant migration of phagocytes (macrophages, neutrophils). ROS cause lipid peroxidation of the cell membrane, and cell death. Abundant migration of neutrophils, macrophages, and lymphocytes collectively produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1beta (IL-1β), matrix metalloproteinase, superoxide dismutase and myeloperoxidases which synergize neuronal apoptosis. Therefore, it is crucial to control inflammation and oxidation injury to minimize the nerve cell death during secondary spinal cord injury. Therefore, in response to oxidation and inflammation, heme oxygenase-1 (HO-1) is induced by the resident cells to ameliorate the milieu. In the meanwhile, neurotrophic factors are induced to promote neuroregeneration. However, it seems that anti-stress enzyme (HO-1) and neurotrophic factor (BDNF) do not significantly combat the pathological events during secondary spinal cord injury. Therefore, an optimum healing can be induced if anti-inflammatory and neurotrophic factors are administered in a higher amount through an exogenous source.

In the 1st chapter, I selectively targeted the inflammation and neuroregeneration. I co-transplanted HO-1 expressing MSCs (HO-1 MSCs) and BDNF expressing MSCs (BDNF MSC) in one group (combination group)

of dogs with subacute spinal cord injury to selectively control the expression of inflammatory cytokines by HO-1 and induce neuroregeneration by BDNF. We compared the combination group with HO-1 MSCs group, BDNF MSCs group, and GFP MSCs group. We found that the combination group showed significant improvement in functional recovery. It showed increased expression of neural markers and growth associated proteins (GAP-43) than in other groups, which depicts enhanced neuroregeneration/neural sparing due to reduced expression of pro-inflammatory cytokines such as TNF-alpha, IL-6 and COX-2; and increased expression of anti-inflammatory markers such as IL-10 and HO-1. Histopathological study revealed a reduced intra-parenchymal fibrosis in the injured spinal cord segment in the combination group than in other groups. Thus it was concluded that selectively targeting the inflammation and neuronal growth with the combined use of HO-1 MSCs and BDNF MSCs more favorably promote healing of the SCI. HO-1 MSCs play a role in controlling the inflammation which favors the BDNF induced neuroregeneration at the injured spinal cord segment of dogs.

In the 2nd chapter, I compared the effects of freshly cultured HO-1 MSCs, GFP MSCs and frozen-thawed HO1 MSCs (FT-HO1 MSCs) after IV administration in dogs with acute SCI. All cell types were migrated to the injured spinal cord segment. Fresh HO-1 MSCs selectively controlled the expression of inflammatory cytokines (TNF alpha, IL-6, IL-1b) by upregulating the expression of HO-1 and IL-10. HO-1 MSCs group showed

increased neuronal sparing depicted by significantly upregulated expression of neurofilament proteins (NF-M) and reduced astroglial scar formation depicted by a significantly reduced expression of glial fibrillary acidic proteins (GFAP). HO-1 MSCs group showed reduced intra-parenchymal fibrosis and myelin degradation compared to GFP MSCs and FT-HO1 MSCs. The frozen-thawed HO-1 MSCs were used because they are readily available to administer immediately after SCI. I found similar results for FT-HO1 MSCs and GFP MSCs groups regarding the expression of NF-M, IL-6, IL-1β. Similarly, the degree of fibrosis and demyelination was also similar between them. Together, these data suggest that HO-1 MSCs could improve hind limb function by increasing the anti-inflammatory reaction, leading to neural sparing. Further, we found similar results between GFP MSCs and FT-HO-1 MSCs, which suggests that FT-HO-1 MSCs could be used as an emergency treatment for SCI.

Keywords: HO-1 MSCs, BDNF MSCs, neuroregeneration, inflammation, anti-inflammation, spinal cord injury, dogs

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CONTENTS

CHAPTER II

Therapeutic effects of intravenous injection of fresh and frozen-thawed HO-1 overexpressed Ad-MSCs in dogs with acute spinal cord injury

LIST OF ABBREVIATIONS

Ad-MSCs	Adipose derived mesenchymal stem cells
BDNF	Brain-derived neurotrophic factor
COX-2	Cyclooxygenase 2
cBBB score	canine Basso, Beattie, and Bresnahan score
FT	Frozen-thawed
FT-HO1 MSCs	Frozen-thawed HO-1 overexpressed Ad-MSCs.
FT MSCs	Frozen-thawed mesenchymal stem cells
GALC	Galactosylceramidase
GAP43	Growth Associated Proteins
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
HO-1	Heme Oxygenase 1
H & E stain	Hematoxylin and Eosin Stain
HO-1 MSCs	Freshly cultured HO-1 overexpressed Ad-MSCs.
IL-6	Interleukin 6
IL-1β	Interleukin 1beta
IL-10	Interleukin 10
MMP2	Matrix metalloproteinase 2
NF-M	Neurofilament medium
NSE	Neuron specific enolase
OCT	Optimal cutting temperature
PBS	Phosphate buffer saline
PVDF	polyvinylidene difluoride

pSTAT-3	Phosphorylated signal transducer activator of transcription 3
SCI	Spinal cord injury
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tuj-1	Beta 3 tubulin
β-actin	Beta-actin

LIST OF FIGURES

GENERAL INTRODUCTION

Primary spinal cord injury causes neuronal and vascular disruption which proceed towards secondary spinal cord injury. Secondary spinal cord injury is further divided in to acute, subacute and chronic phases. The acute phase begins 2hr after spinal cord injury which lasts for 48hr. It is mainly characterized by vascular disruption, infiltration of leukocytes, inflammation, oxidative cell injury, excitotoxicity, edema, demyelination and apoptosis. It is followed by a subacute phase which lasts for 14 days. In this phase the edema is resolved. The macrophages and astrocytes start infiltration to make a glial scar (Siddiqui et al. 2015). It is followed by a chronic phase which is comprised of a mature scar and wallerian degeneration. The scar is composed of chondroitin sulfate proteoglycan and myelin inhibitory proteins which inhibit the neuronal regeneration (Rowland et al. 2008).

Traumatic spinal cord injury causes severe hemorrhages and edema, which decrease the blood flow and exacerbates tissue ischemia (Leonard et al. 2015). Ischemia provokes anaerobic metabolism and decreases ATP production which consequently depresses ATP associated Na/K pump causing increase intracellular accumulation of Na^{+} and Ca^{++} ions, leading to severe intracellular edema (Mortazavi et al. 2015). Increase intracellular Ca^{++} ions level cause mitochondrial dysfunction which perpetuates in increasing

production of reactive oxygen species (ROS) (H2O2, O, superoxide, hydroxyl group) (Fatima et al. 2015). ROS cause lipid peroxidation of the nerve cell membrane and protein degradation to produce neurotoxic compounds such as 4-hydroxynenonal (4HNE) protein carbonyl and nitrotyrosine compounds (3NT) (Hall 2011).

After SCI, neutrophils as the first line of defense start entry in 24hr, where they produce pro-inflammatory cytokines, matrix metalloproteinase, superoxide dismutase and myeloperoxidase (Siddiqui et al. 2015). It is followed by the migration of monocytes which also produce inflammatory cytokines, maintain the state of inflammation and promote lymphocytic infiltration (McMahill et al. 2015). In dogs, there is an abundant expression of proinflammatory cytokines (TNF alpha, IL6, IL8) which create an inhibitory environment for neuronal regeneration (Spitzbarth et al. 2011) and activate apoptotic pathways (Rust and Kaiser 2017). Therefore during early SCI, anti-oxidant and anti-inflammatory interventions have been made to mitigate their deleterious effects. Methylprednisolone sodium succinate (MPSS) has been injected during early SCI to control the production of ROS and pro-inflammatory cytokines; however, its side effect of causing the severe gastrointestinal hemorrhages have limited its use (Evaniew et al. 2016; Olby et al. 2016). Thus as a safe alternative, the adipose derived stem cells (Ad-MSCs) have been found to reduce oxidation and inflammation during early stage of SCI (Y. Kim et al. 2015). There are various studies in dogs which

demonstrated that transplantation of Ad-MSCs has improved healing of spinal cord injury by mitigating the inflammatory environment and intraparenchymal fibrosis. The dogs showed significant improvement in functional recovery and conduction velocity through a somatosensory evoked potential (S. H. Lee et al. 2015; Lim et al. 2007; Park et al. 2011; Park et al. 2012; Ryu et al. 2009).

Following spinal cord injury, the expression of important neurotrophin i.e. BDNF is increased by astrocytes, microglia and oligodendrocytes (Dougherty et al. 2000) which promotes neuronal survival, growth, and maturation (Wollen 2010). BDNF has anti-oxidant and anti-inflammatory properties. It decreases lipid peroxidation of cell membrane and promotes neuronal survival (Joosten and Houweling 2004). Therefore, BDNF has been used for therapeutic purpose to promote the healing of injured neurons. Mesenchymal stem cells have been stimulated to deliver BDNF in a higher amount through their gene editing (W. Ji and Qiu 2016) or culturing in a specified growth medium (Kingham et al. 2013). BDNF overexpressed mesenchymal stem cells (BDNF MSCs) showed higher proliferation and differentiation capability. They produced increase amount of BDNF and expressed higher nerve cell markers i.e. Nestin, Neuron-specific enolase and GFAP (Q. Liu et al. 2015b). In a rat spinal cord injury (SCI), improved healing in terms of increased neuronal sparing and blood vessel density was observed with the transplantation of BDNF overexpressed MSCs (BDNF MSCs) (Ritfeld et al. 2015). Similarly, an enhanced axonal sprouting with an

improved functional recovery was observed in acute SCI of rats after transplantation of BDNF MSCs (Sasaki et al. 2009). In dogs with chronic SCI, BDNF MSCs injected dogs showed significant improvement in functional recovery with a higher expression of neural markers due to a decreased expression of inflammatory cytokines (S. H. Lee et al. 2016a).

Another important factor which is induced during SCI is a hemeoxygenase 1 (HO-1). Its expression is induced in both gray and white matter, primarily in glial cells due to the stimulation by heme, hypoxia, oxidative stress and pro-inflammatory cytokines (IL-1, IL-6, Interferon-g) (Y. Liu et al. 2002; A. Mautes et al. 1998). HO-1 is an anti-stress enzyme which has antioxidant and anti-inflammatory properties (Abraham et al. 2007; Choi and Alam 1996; Loboda et al. 2008). Because of its potent antioxidant ability, it protects from cell death, increases cell viability as well as it promotes angiogenesis and stem cell differentiation (Kozakowska et al. 2014). HO-1 attenuates neutrophil infiltration (Yamauchi et al. 2004) and CD-8 T-cell accumulation (Chora et al. 2007). It increases the production of IL-10 and blocks the production of TNF-α from macrophages (Piantadosi et al. 2011). Owing to the potent anti-oxidant and anti-inflammatory properties of HO-1, it has been studied in a variety of clinical disorders. HO-1 expressing MSCs (HO-1 MSCs) have been transplanted in rats with myocardial infarction. The infarct size was reduced with improved cardiac functions due to a reduced expression of inflammatory cytokines such as IL-6, TNF-alpha, and IL-1b,

and increased expression of IL-10, FGF and VEGF (Zeng et al. 2008). HO-1 MSCs showed increased viability in vivo after transplantation in rats with experimental acute kidney disease. An improved kidney function was observed owing to the anti-inflammatory and anti-oxidant functions of the transplanted HO-1 MSCs (N. Liu et al. 2015a). The dogs with subacute SCI were injected with HO-1 MSCs. They showed improved functional recovery and neuronal sparing due to a reduced astrocyte infiltration and decreased expression of inflammatory cytokines such as IL-6, TNF-α, and COX2 (S. H. Lee et al. 2017).

The stem cells are usually administered through invasive procedures; however, this could injure spinal cord tissues due to rough surgical manipulation (Taylor-Brown et al. 2015). Therefore, to avoid complication associated with invasive procedures, an intravenous (IV) route could be the best alternative. This route is simple and rapid. The MSCs injected through this route migrated to an injured spinal cord segment, which with a concomitant systemic immune suppression suppressed inflammation of the injured spinal cord segment by upregulating the expression of an anti-inflammatory cytokine such as IL-10 (Badner et al. 2016; Ohta et al. 2017). The recovery of SCI is rapid when the MSCs are injected during the early stage of disease. The dogs with acute SCI showed significant improvement in their functional recovery and a higher expression of neural markers after 1-week of IV transplantation of Ad-MSCs (Y. Kim et al. 2015). The problem with the cultured MSCs is that they are not

readily available to deliver immediately at the time of spinal cord injury, for this purpose frozen-thawed cells could be the best alternate option. The freeze-thawing procedures do not affect their morphology, proliferation, and differentiation characteristics. The frozen-thawed MSCs were positive for stem cells markers such as CD90, CD105, CD73 and negative for hematopoietic cells associated markers such as CD34, CD19, CD45, CD14. Similarly, the markers for multi-cell line differentiation such as Nanog, Rex-1, Sox-2, and Oct-4 were not negatively affected due to freeze-thawing procedures; however, the viability and immunosuppressive capability were reduced (Yong et al. 2015). After IV transplantation the freeze-thawing procedures also affect their biodistribution, homing and gene expression in vivo (Chinnadurai et al. 2014; Hoogduijn et al. 2016). HO-1 gene editing in MSCs enables them to cope with environmental stress (Hamedi-Asl et al. 2012). It suppressed apoptosis and increased viability of stem cells (Bai et al. 2012). Thus it will also improve their viability, proliferation rate, antioxidant capacity and mRNA expression of growth factors during freeze-thawing procedures. Hence FT-HO1 MSCs could be used as an alternative to freshly cultured MSCs for the emergency treatment of SCI.

In the present study, I compared the therapeutic effects of different types of transgenic MSCs after intraparenchymal injection and IV injection in dogs with SCI. Considering the essential role of HO-1 as an anti-inflammation and BDNF as a neuroregeneration, the two different types of transgenic stem

cells such as HO-1 MSCs and BDNF MSCs were combined and injected in to the injured spinal cord segment to selectively mitigate the inflammatory microenvironment by HO-1 MSCs and induce neuroregeneration by BDNF MSCs (chapter 1). Considering the higher resistance of HO-1 MSCs to the external environment both freshly cultured HO-1 MSCs and FT-HO-1 MSCs were injected IV in dogs with acute SCI with an assumption that they will migrate to the injured spinal cord segment and will mitigate the oxidation and inflammation at the injury site more than the MSCs.

CHAPTER I

Improved healing after the co-transplantation of HO-1 and BDNF overexpressed mesenchymal stem cells in the sub-acute spinal cord injury of dogs

ABSTRACT

Abundant expression of proinflammatory cytokines after a spinal cord injury (SCI) creates an inhibitory microenvironment for neuroregeneration. The mesenchymal stem cells help to mitigate the inflammation and improve neural growth and survival. For this purpose, I potentiated the function of adipose derived mesenchymal stem cells (Ad-MSCs) by transfecting them with brain derived neurotrophic factor (BDNF) and heme oxygenase-1 (HO-1), through a lentivirus, to produce BDNF overexpressed Ad-MSCs (BDNF MSCs), and HO-1 overexpressed Ad-MSCs (HO-1 MSCs). Dogs with SCI were randomly assigned into four treatment groups. We injected both HO-1 and BDNF-overexpressed MSCs, as a combination group, to selectively control inflammation and induce

neuroregeneration in SCI dogs, and compared this with BDNF MSCs, HO-1 MSCs, and GFP-MSCs injected dogs. The groups were compared in terms of improvement in canine Basso, Beattie, and Bresnahan (cBBB) score during 8-weeks of experimentation. After 8-weeks, spinal cords were harvested and subjected to western blot analysis, immunofluorescent staining, and hematoxylin and eosin (H & E) staining. The combination group showed a significant improvement in hindlimb functions, with a higher BBB score, and a robust increase in neuroregeneration, depicted by a higher expression of Tuj-1, NF-M, and GAP-43 due to a decreased expression of the inflammatory markers interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and an increased expression of interleukin-10 (IL-10) ($p \leq 0.05$). H & E staining showed more reduced intraparenchymal fibrosis in combination group, than in other groups ($p \leq 0.05$). It was thus suggested that the cotransplantation of HO-1 and BDNF MSCs is more effective in promoting the healing of spinal cord injury. HO-1 MSCs reduce inflammation, which favors BDNF-induced neuroregeneration in SCI of dogs.

INTRODUCTION

Primary SCI inflict neuronal damage and vascular disruption in spinal cord tissues. This proceeds towards a secondary SCI, which, with a series of inflammatory and other pathological events, initiates an expanding zone of destructed tissue with a concomitant inhibition of neuroregeneration (Mortazavi et al. 2015; Rowland et al. 2008).

Following SCI, the expression of brain derived neurotrophic factor is increased in astrocytes, microglia and oligodendrocytes (Dougherty et al. 2000) which is responsible for neuronal survival, growth and maturation (Wollen 2010). Simultaneously, HO-1 expression is induced after SCI, both in gray and white matter, primarily in microglial cells and other glial cells (Y. Liu et al. 2002; A. E. Mautes et al. 2000; Vargas et al. 2005) which have antioxidant and anti-inflammatory properties (Abraham et al. 2007; Choi and Alam 1996), attenuate neutrophil infiltration (Yamauchi et al. 2004) and CD-8 T-cell accumulation (Chora et al. 2007).

Allogenic adipose derived mesenchymal stem cells (Ad-MSCs) have been found to induce neuroregeneration, and neuronal differentiation with improved functional outcomes in dogs with SCI (Ryu et al. 2009). The Ad-MSCs are further potentiated by lenti-virus mediated gene editing with neurotrophic factors, for more robust neural regeneration and functional

outcomes (X.-C. Ji et al. 2015). BDNF MSCs produce an excessive amount of BDNF, which promotes neural regeneration by upregulating neural markers such as Tuj-1, nestin and NF-M in dogs with SCI (S. H. Lee et al. 2016a). Similarly, dogs with SCI showed improved functional recovery with intrathecal injections of HO-1 MSCs, due to a decreased expression of inflammatory cytokines, such as TNF-α, IL-6, cyclooxygenase-2 (COX-2) as well as decreased astrogliosis, and augmented neuroregeneration (S. H. Lee et al. 2016b).

Upon considering the essential role of BDNF and HO-1 in recovering from SCI, I hypothesize that BDNF and HO-1 selectively control inflammation and induce neuroregeneration with more favorable, functional clinical outcomes. Therefore, I produced and used, in combination, the two different types of Ad-MSCs highly expressing BDNF and HO-1.

MATERIALS AND METHODS

1. Selection of Animal

The experiment was performed on 16 male dogs between the ages of 1 to 2 years, with an average weight of 10.5 ± 1.6 kg. The dogs were randomly divided into 4 groups, comprising 4 dogs each. The groups were assigned according to the type of cells used. BDNF-MSCs group: only BDNF overexpressed Ad-MSCs were used. HO-1 MSCs group: only HO-1 overexpressed Ad-MSCs were used; Combination group: both BDNF and HO-1 overexpressed Ad-MSCs were used, in combination; GFP-MSCs group: only Ad-MSCs expressing green fluorescent protein (GFP) were injected at the injury site and were used as control. The dogs were kept in accordance with the guidelines laid by the Institute of Laboratory Animal Resources, Seoul National University, Korea. The research was approved by Animal Care and Use Committee of Seoul National University (SNU-160720-13). All the dogs were clinically sound, and showed no neurological abnormality.

2. Isolation and culture of ADSCs

The gluteal subcutaneous fat was aseptically collected from a healthy beagle dog of age 1.5 year, under general anesthesia. The tissues were washed with phosphate buffer saline (PBS), minced in 6 well plate, and digested with collagenase type-1 (1mg/mL, Sigma, St. Louis, MO, USA), for 2 hrs under shaking incubation. The suspension was strained through a nylon mesh of pore size 100 μm and centrifuged, to obtain the stromal vascular fraction (SVF). The SVF was resuspended and cultured in commercially-available low-glucose Dulbecco's modified Eagle's medium (DMEM; GenDEPOT, USA), supplemented with 10% FBS (FBS, Gibco BRL, Grand Island, USA), and 1% penicillin and streptomycin (PS), for 24 hrs in humidified incubator at 37°C and 5% CO_2. After 24 hrs, the Ad-MSCs were washed with PBS to remove the cells debris, and the medium was changed. Next time the medium was changed after an interval of 48hrs, until the cells reached 80-90% confluence. The cells were either stored or subcultured, and further processed for the experiment (Neupane et al. 2008).

3. Preparation of transgenic lenti-virus and Ad-MSCs transduction

Ad-MSCs were transduced with lentivirus vectors encoding green fluorescent protein (GFP), canine BDNF with GFP, or canine HO-1 with GFP, depending on the group to which the cells belonged. For the preparation of the

edited plasmid with the desired gene, the canine HO-1 and BDNF genes were cloned by referring to the pub-med database. The genes encoding Flag-tagged BDNF, HO-1 and GFP, were amplified by Phusion DNA Polymerases with the cDNA of canine peripheral blood (Thermo Scientific, Pittsburgh, PA, U.S.A.). The canine specific primer set for BDNF (forward, *5′-ATGACCATCCTTTTCCTTAC-3′;* reverse, *3′-GATAGAAGGGGAGAATTACC-5′*), and HO-1 (forward, *5′-GACAGCATGCCCCAGGAT-3′;* reverse, *3′-TCACAGCCTAAGGAGCCAGT-5′*), with restriction enzymes EcoRI and BamHI, were inserted into a pCDH-EF1-MCS-pA-PGK-copGFP-T2A-Puro vector (System Biosciences). Dharmacon Trans-Lentivirus packaging system was used to prepare the transgenic viruses. First, the HEK293T cells were cultured in a 100 mm culture plate using a high-glucose medium (DMEM), containing 10% non-heat-inactivated FBS, and 1% PS, until the cell density of 5.5×10^6 cells in 14 mL was reached. The suspension of DNA transfer-vectors encoding GFP, BDNF, and HMOX-1, the lentiviral packaging mix (Fisher Scientific Cat #14-432-23) encoding viral proteins Gag-Pol, Rev, and VSV-G, and $CaCl_2$, was made and added, drop-wise, to the cultured cells for transfection. The cells were incubated for 10-16 hrs, at 37°C and 5% CO_2. The medium was changed and the viral particles were collected from the culture medium after 48 hrs of incubation. Successful transfection was confirmed by observing GFP expression during incubation, under a fluorescent microscope. Finally, three types of lentiviruses, Lenti-BDNF-GFP,

Lenti-HO-1-GFP, and lenti-GFP, were obtained for gene delivery into the Ad-MSCs. The concentration of viral particles was determined according to the manufacturer guidelines. The MSCs were transduced at Passage 1, during 50 to 60% confluence, by the lentivirus at MOI=100 (McGinley et al. 2011), and were sub-cultured. The transduced cells were cultured with 3 μg/mL puromycine to get a higher percentage of transduced stem cells with a GFP expression of 90%. We obtained three types of cells: Ad-MSCs overexpressing BDNF (BDNF MSCs), Ad-MSCs overexpressing HO-1 (HO-1 MSCs), and Ad-MSCs expressing only GFP (GFP-MSCs). The modified stem cells were sub-cultured up to 3 passages and were subsequently used for in-vivo study.

4. Induction of SCI

The SCI was induced by balloon compression method under general anesthesia (Lim et al. 2007). A mini hemi-laminectomy was performed by creating 3-5 mm hole at the 4th lumbar vertebral arch (L4) using a high speed pneumatic burr. A 6-french embolectomy catheter (Sorin, Biomedica, Italy) was inserted, and advanced to the cranial margin of 1st lumbar vertebra (L1), through fluoroscopic guidance. The balloon was distended with contrast agent (Omnipaque; Amersham Health, Ireland) diluted in normal saline (50:50) at a dose rate of 50 μL/kg body weight. The catheter was removed after 12 hr.

This technique was found to be reliable and repeated with satisfactory outcomes in our previous studies (T.-S. Lee and Chau 2002; S. H. Lee et al. 2016a; Lim et al. 2007; Park et al. 2012; Ryu et al. 2009). As found by computed tomography, this SCI model occludes 80% of the spinal canal which inflict compression injury to the spinal cord (Lim et al. 2007; Ryu et al. 2009). All the dogs were kept under close supervision during the experimental period. Each dog was placed in a big cage with a soft bedding comprised of 12 mm thick soft mats to prevent from bed sores. The food and water was provided ad libitum. A manual bladder compression was performed three times a day to assist urination.

5. Transplantation of overexpressed Ad-MSCs

After 1 week of SCI induction, BDNF MSCs, HO-1 MSCs and GFP-MSCs were injected. The injured segment of the spinal cord at L1 was exposed through dorsal laminectomy, and about 1×10^7 cells dilute in 150 μL PBS were injected 2-3 mm deep into the spinal cord parenchyma. Three injections, 50 μL each were injected: 2 at the epicenter and 1 in the center of injured spinal cord segment. The cells were injected slowly, within the interval of 5 min. In the combination group, we mixed 5×10^6 BDNF MSCs with 5×10^6 HO-1 MSCs to make a full dose of 1×10^7 cells. The percentage

of GFP expression before the cell transplantation was 90% ± 2, 88% ± 3, 91% ± 21, 89 ± 3 for GFP MSCs, HO-1 MSCs, BDNF MSCs and mixed BDNF and HO-1 MSCs respectively. After Ad-MSCs transplantation, the dogs were kept for 8 weeks and observed for functional improvement.

6. Behavioral assessment

All the dogs were evaluated for gait analysis before SCI and after Ad-MSCs injection. The motor activity was assessed by the 19 points scoring system of canine Basso, Beattie, and Bresnahan (cBBB) (Song et al. 2016). All the dogs were observed for improvement in the functional recovery on daily basis, when they were resting in the cage or set free during cleaning and washing of the cage. On weekly basis, the improvement in the cBBB score was recorded. Each dog was allowed to move freely in a contained area for 4-5 min to observe improvement in locomotion, ability of weight bearing and hind limbs coordination. The movement of the joints and muscle contraction was recorded when the dog's hindquarter was lifted with the base of the tail while standing, and flexion of the joints was recorded while the dog was lifted up by an attendant. For deep nociception, the phalanges were pinched with an artery forceps and the degree of pain perception was evaluated by the degree of limb protraction, lip licking, vocalization, and facial expressions. The gait

evaluation was performed by three persons blind to the study and the average values were presented for each dog and group.

7. Western Blot Analysis

At the end of the experimental period, each dog was euthanized and the spinal cords were extracted through dorsal laminectomy, from 12th thoracic vertebra (T12) to the 3rd lumbar vertebra (L3). The spinal cord segments were immersed for 12 hrs in 10% sucrose at 4°C, followed by 20% sucrose at 4°C for the next 24 hrs. The duramater was carefully removed and the spinal cord segments were embedded and frozen in the optimal cutting temperature (OCT) compound (Rica Biosystems, Richmond, USA). The spinal cord segments were longitudinally incised into two equal halves; one half was kept for western blot analysis and the other half was employed for cryosectioning, for performing immunofluorescent staining and histopathological assessment.

For western blotting, the entire injured segment of the spinal cord tissue was properly minced and incubated with 300 μL Ripa Lysis Buffer (Gen Depot, USA), and 3 μL proteinase inhibitor solution (Gen depot, USA) for 30 min on ice, followed by cold centrifugation (4°C, 10 min, 13000 rpm). In the case of transgenic cells, for the confirmation of successful expression of

the transfected gene, the cells of each treatment group were harvested at 90% confluence from three 150 mm petri dishes, pelleted, incubated with Ripa lysis buffer, and centrifuged (4°C, 10 min, 13000 rpm). The supernatant was collected and the protein concentration was determined by the Bradford assay. About 20 μg of protein of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk for 1 hr, followed by overnight incubation with primary antibodies. The following primary antibodies were used: for neural markers, Tuj-1 (sc69966, β-III-tubulin, immature neurons), neurofilament (NF-M, mature neurons, sc-398532), GAP-43 (growth associated proteins for neuro-regeneration, ab-12274), GFAP (Astrocytes, sc65343), nestin (Neural differentiation, Neural Stem Cells, ab7695); for inflammatory markers, COX2 (cyclooxygenase-2, sc19999), TNF-α (sc1350), IL-6 (interleukin-6, ab-6672), Phosphorylated STAT3 (sc1008R); for anti-inflammatory markers, IL-10 (interleukin-10, R&D, MAB7352), HO-1 (ab-13243), and for neurotrophic factor, BDNF (ab-101747). The membrane was incubated for 1 hr at 4°C with anti-mouse secondary antibody (ab6728), and anti-rabbit secondary antibody (ab6721). The β-actin antibody (sc-47778) was used as an internal reference. Protein bands were visualized using Cyanagen WESTAR chemiluminescent substrates (Cyanagen, Italy), through the image quant LAS 4000 mini system (GE Health care biosciences, Sweden).

8. Immunohistological and histopathological assessments

For Immunofluorescent staining, the spinal cord segment was sliced at 10 µm, by the cryomicrotome, at -20ºC. The sections were mounted on silane coated slides, fixed, and permeated for 10 min with 0.1% v/v Triton X-100, followed by an incubation with 1% bovine serum albumin in PBS for 30 min (BSA; Sigma-Aldrich, St. Louis, MO, USA). The slides were incubated with primary antibodies of neural marker Tuj-1, NF-M, GFAP, and nestin for 24 hrs at 4ºC. This was followed by 2 hrs incubation of fluorescein iso-thio-cyanate conjugated anti-mouse (Alexa flour, ab-150111), and anti-rabbit secondary antibodies (Flamma 648). DAPI was used to stain the nuclei, and a coverslip was placed over the sections. The slides were examined under the microscope (EVOS FL imaging system, USA). Cells positive for specific markers were counted from the 5 randomly selected areas at the injury site, on a slide, through the integrated cell counting system in the microscope. The values were expressed as a percentage, which represented the average of each slide, and the relative group.

In order to measure the degree of fibrosis, hemorrhages and vacuoles, slides were stained with hematoxylin and eosin. The images were obtained to include the entire injured area of the spinal cord i.e. rostral, caudal and middle

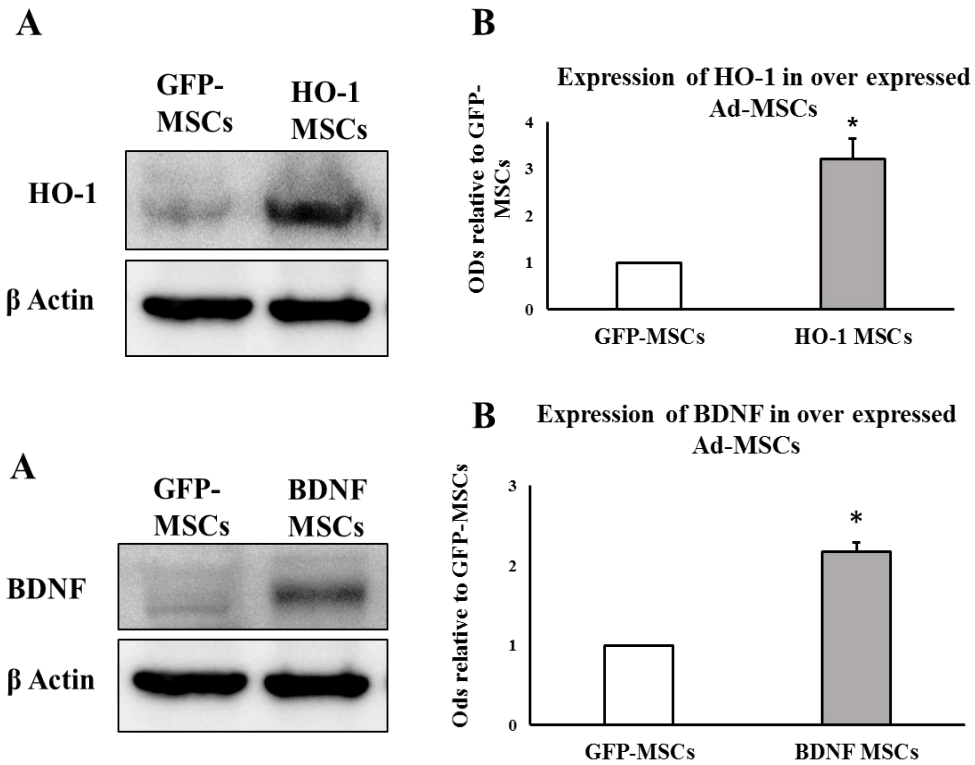
segments of the injured spinal cords (Ninomiya et al. 2015). The lesions were measured for degree of fibrosis by computer-associated image analysis system (Image-J Version 1.37; National Institute of Health, USA). Average values were presented for each slide and group.

9. Statistical Analysis

Results are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS v.21.0 software (IBM Corp., USA). Group means were compared with the Kruskal-Wallis tests followed by Mann-Whitney U tests. A p value of less than 0.05 was considered statistically significant.

RESULTS

Ad-MSCs transfected with BDNF and HO-1 genes were successfully expressed, as shown by the expression of GFP and western blot analysis (Fig 1.1). Western blot showed significant increase in the expression of BDNF and HO-1 in transfected Ad-MSCs, relative to GFP-MSCs (Fig 1.1, B $*p \leq 0.05$). Corresponding to the type of cells used in vivo, BDNF was highly upregulated, in the combination and the BDNF MSCs group, than in the HO-1 MSCs and GFP-MSCs groups (Fig 1.2, $*p \leq 0.05$). It also showed upregulation in HO-1 group compared to GFP-MSCs group (Fig 1.2, $\#p \leq 0.05$). Regarding in-vivo expression of HO-1, it was upregulated in combination and HO-1 groups compared to the BDNF and GFP-MSCs groups (Fig 1.2, $*p \leq 0.05$).


A
GFP-MSCs
HO-1 MSCs
HO-1
β Actin
B
Expression of HO-1 in over expressed Ad-MSCs
ODs relative to GFP-MSCs
4
3
2
1
0
*
GFP-MSCs
HO-1 MSCs
A
GFP-MSCs
BDNF MSCs
BDNF
β Actin
B
Expression of BDNF in over expressed Ad-MSCs
Ods relative to GFP-MSCs
3
2
1
0
*
GFP-MSCs
BDNF MSCs

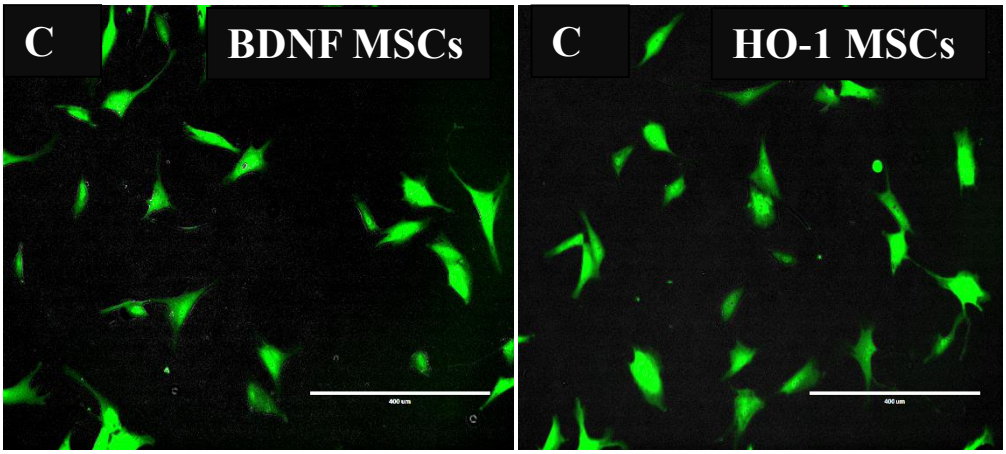

C
BDNF MSCs
C
HO-1 MSCs

Figure 1.1. Expression of transfected HO-1 and BDNF genes in Ad-MSCs. A, represent the densities of HO-1 and BDNF proteins in HO-1, BDNF and GFP MSCs. B, quantitative data obtained by densitometry showing optical densities (ODs) relative to GFP-MSCs, bar represent average of three samples and standard error, * indicate significance ($p \leq 0.05$). C, represent the flourescence of GFP depicting the successful expression of transfected gene.

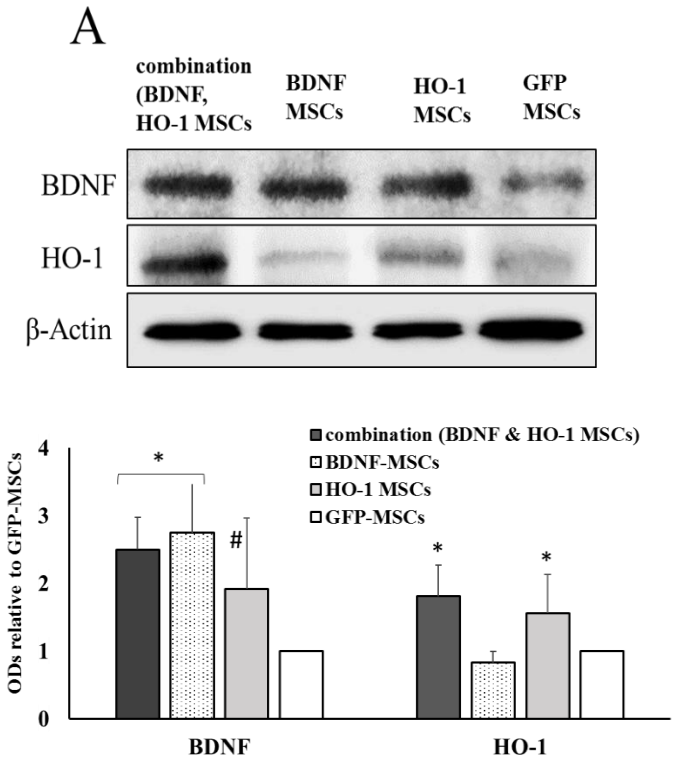


Figure 1.2. In-vivo expression of HO-1 and BDNF, 8 weeks after transplantation of HO-1 and BDNF MSCs. BDNF was highly upregulated in combination and BDNF groups (*$p \leq 0.05$) compared to HO-1 and GFP-

MSCs groups, while it was also upregulated in HO-1 group compared to GFP-MSCs group (#$p \leq 0.05$). HO-1 was upregulated in combination and HO-1 groups (*$p \leq 0.05$).

1. Behavioral assessment

The functional outcomes of the use of modified Ad-MSCs was assessed using the cBBB score. All the dogs were physically normal with a cBBB score of 19 before the SCI. After SCI, the score for all the dogs was zero. The hindquarter of all the dogs was completely paralyzed, showing no deep pain. After 1 week of SCI, Ad-MSCs were injected (intrathecal), and at the time, most of the dogs exhibited few reflexes. The cBBB score recorded at the time of Ad-MSCs transplantation was 0.25 ± 0.5 for combination, BDNF and GFP MSCs groups, and 0 for HO-1 MSCs group. During the course of experimental period the combination, BDNF and HO-1 MSCs groups showed improvement in their BBB score upto an intermediated stage of improvement i.e. 8-13, however GFP-MSCs group remained in the early stage of BBB score improvement i.e. 0-7. The combination group showed a rapid improvement in functional recovery after 5 weeks of Ad-MSCs injections (Fig 1.3, *$p \leq 0.05$), and this remained significantly high up to 8 weeks, compared to the GFP-MSCs group (Fig 1.3, *$p \leq 0.05$). After 7-8 weeks the combination

group also attained a significantly higher BBB score than the BDNF (Fig 1.3, +p ≤ 0.05) and HO-1 MSCs groups (Fig 1.33, +p ≤ 0.05). Comparing the BDNF group with the HO-1 group, both showed similar pattern of improvement in the BBB score (Fig 1.3, ns); whereas, comparing the BDNF MSCs group with the GFP-MSCs group, a significantly higher BBB score was found after 7-8 weeks (Fig 1.3, $p ≤ 0.05). A significant improvement in the score of the HO-1 MSCs group was seen (Fig 1.3, #p ≤ 0.05) after 8 weeks compared to GFP MSCs group. In the combination group, 3 out of 4 dogs were able to stand and support their body weight; they were able to support their own body weight when forced to walk as well, however the coordination between legs was weak. In the BDNF and HO-1 groups, only 1 out of 4 dogs was able to bear its weight. In the GFP-MSCs group, the dogs were only able to bear their weight when support was provided at the tuber coxae. Collectively the average BBB score obtained after 8 weeks was 10 ± 0.8, 8.5 ±1, 8.25 ± 1.2 and 6.75 ± 0.5 for combination, BDNF, HO-1 and GFP-MSCs groups respectively. Dogs that were able to support their body weight showed stronger limb protraction upon phalangeal pinching however no dog vocalized during pinching process, which may indicate spinal reflex action with weak recovery of pain perception. After SCI, all the dogs failed to urinate voluntarily. At the end of experimental period, the voluntary urination recovered in 2 dogs in the combination group, 1 dog each in the BDNF and HO-1 groups, and none in the GFP-MSCs group, after 8 weeks of Ad-MSCs

injection. The defecation was normal in all the dogs.

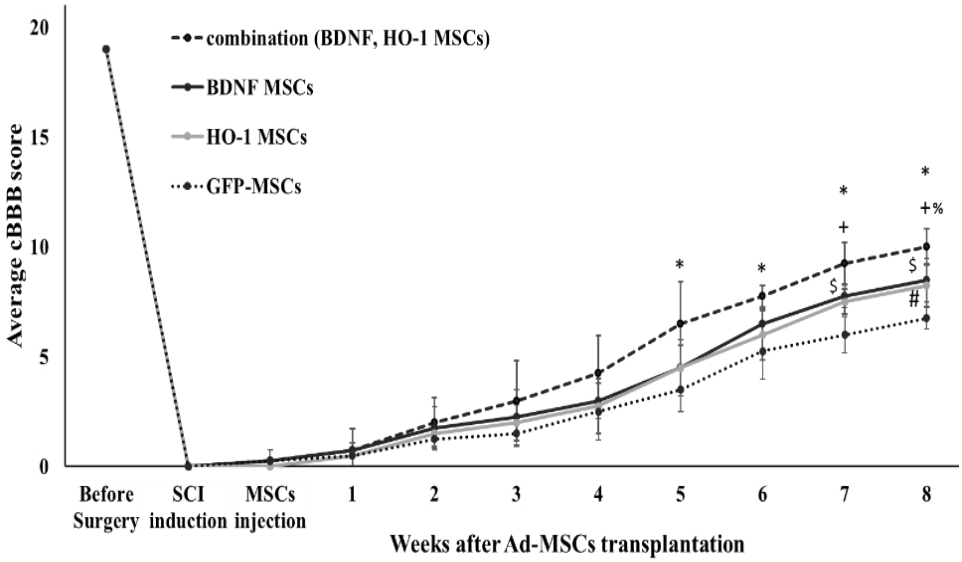
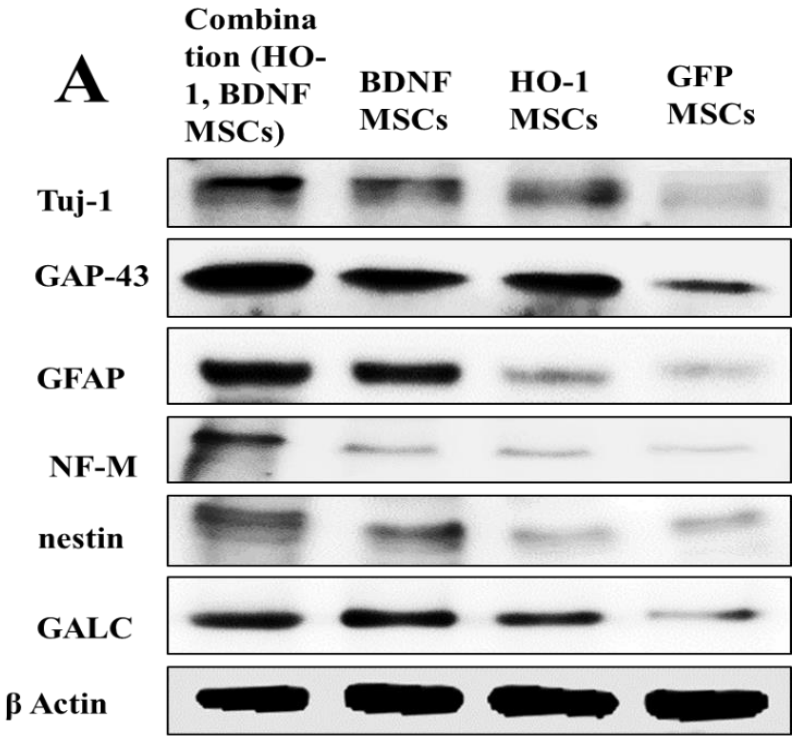


Figure 1.3. Changes in cBBB score until 8 weeks after transplantation of MSCs. The BBB score of combination group, BDNF MSCs group and HO-1 MSCs group was significantly high. *p ≤ 0.05, Combination *vs* GFP-MSCs; %p ≤ 0.05, Combination *vs* BDNF MSCs; +p ≤ 0.05, Combination *vs* HO-1 MSCs; $p ≤ 0.05, BDNF MSCs *vs* GFP-MSCs; #p ≤ 0.05, HO-1 MSCs vs GFP-MSCs. Each group represent the average score of four dogs (n=4).

2. Neuro regeneration and differentiation

The expression of Tuj-1, GAP-43, and NF-M was the highest in the combination group, than in other groups (Fig 1.4, *$p \leq 0.05$). It was the same in the BDNF and HO-1 groups, although higher than in the GFP-MSCs group (Fig 1.4, #$p \leq 0.05$). The expression of nestin, GFAP, and GALC was the same in the combination group and the BDNF MSCs group, although significantly higher than in the HO-1 and GFP-MSCs groups (Fig 1.4, *$p \leq 0.05$). The expression of GALC was higher in the HO-1 MSCs group than in the GFP-MSCs group (Fig 1.4, #$p \leq 0.05$). Collectively, the process of neuroregeneration might be upregulated in the combination group, evidenced by the highest expression of Tuj-1, GAP-43, and NF-M, since an equal level of upregulated expression of nestin, GALC, and GFAP, was found in the combination and BDNF groups.


A
Combination (HO-1, BDNF MSCs)
BDNF MSCs
HO-1 MSCs
GFP MSCs
Tuj-1
GAP-43
GFAP
NF-M
nestin
GALC
β Actin

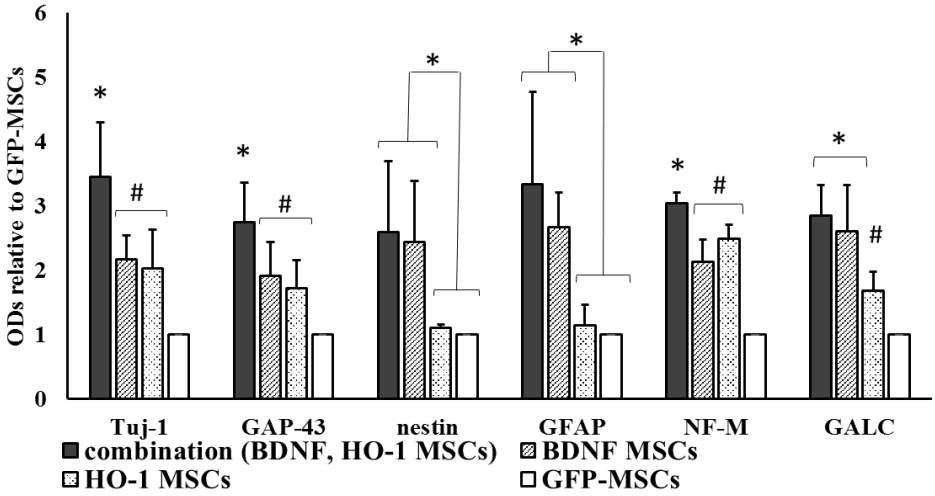

B
ODs relative to GFP-MSCs
0
1
2
3
4
5
6
Tuj-1
GAP-43
nestin
GFAP
NF-M
GALC
combination (BDNF, HO-1 MSCs)
BDNF MSCs
HO-1 MSCs
GFP-MSCs

Figure 1.4. Expression of neural markers. A, represent the densities of neural markers. B, quantitative data obtained by densitometry showing optical densities (ODs) relative to GFP-MSCs. The expression of Tuj-1, GAP-43 and NF-M was significantly increased in combination group compared to other groups (*$p \leq 0.05$) while it was same between BDNF and HO-1 MSCs groups however significantly higher than GFP-MSCs group (#$p \leq 0.05$). The expression of nestin, GFAP and GALC was same in combination and BDNF groups although significantly higher than HO-1 and GFP-MSCs group (*$p \leq 0.05$). GALC was also significantly higher in HO-1 group compared to GFP-MSCs groups (#$p \leq 0.05$). The graph represents mean ± SE of four dogs per groups, as determined by densitometry relative to β-actin.

3. Expression of inflammatory and anti-inflammatory markers

The expression of TNF-α and IL-6 was reduced in the combination and HO-1 MSCs groups, compared to the BDNF MSCs and GFP-MSCs groups. Additionally, the expression was not different between the BDNF MSCs group and the GFP-MSCs group (Fig 1.5, *$p \leq 0.05$). COX-2 represented similar expression within the combination, BDNF MSCs and HO-1 MSCs groups; however, this was lower than the expression in the GFP-MSCs group (Fig 1.5, *$p \leq 0.05$). pSTAT3 was upregulated in the combination and HO-1 groups, compared to the BDNF MSCs and GFP-MSCs groups (Fig 1.5, *$p \leq 0.05$). Within the BDNF MSCs and GFP-MSCs groups, a higher expression in the BDNF MSCs group was evidenced than in the GFP-MSCs group (Fig 1.5, #$p \leq 0.05$). IL-10, as an anti- inflammatory marker, showed upregulation in both the combination and HO-1 groups, but remained downregulated in the BDNF and GFP-MSCs groups (Fig 1.5, *$p \leq 0.05$).

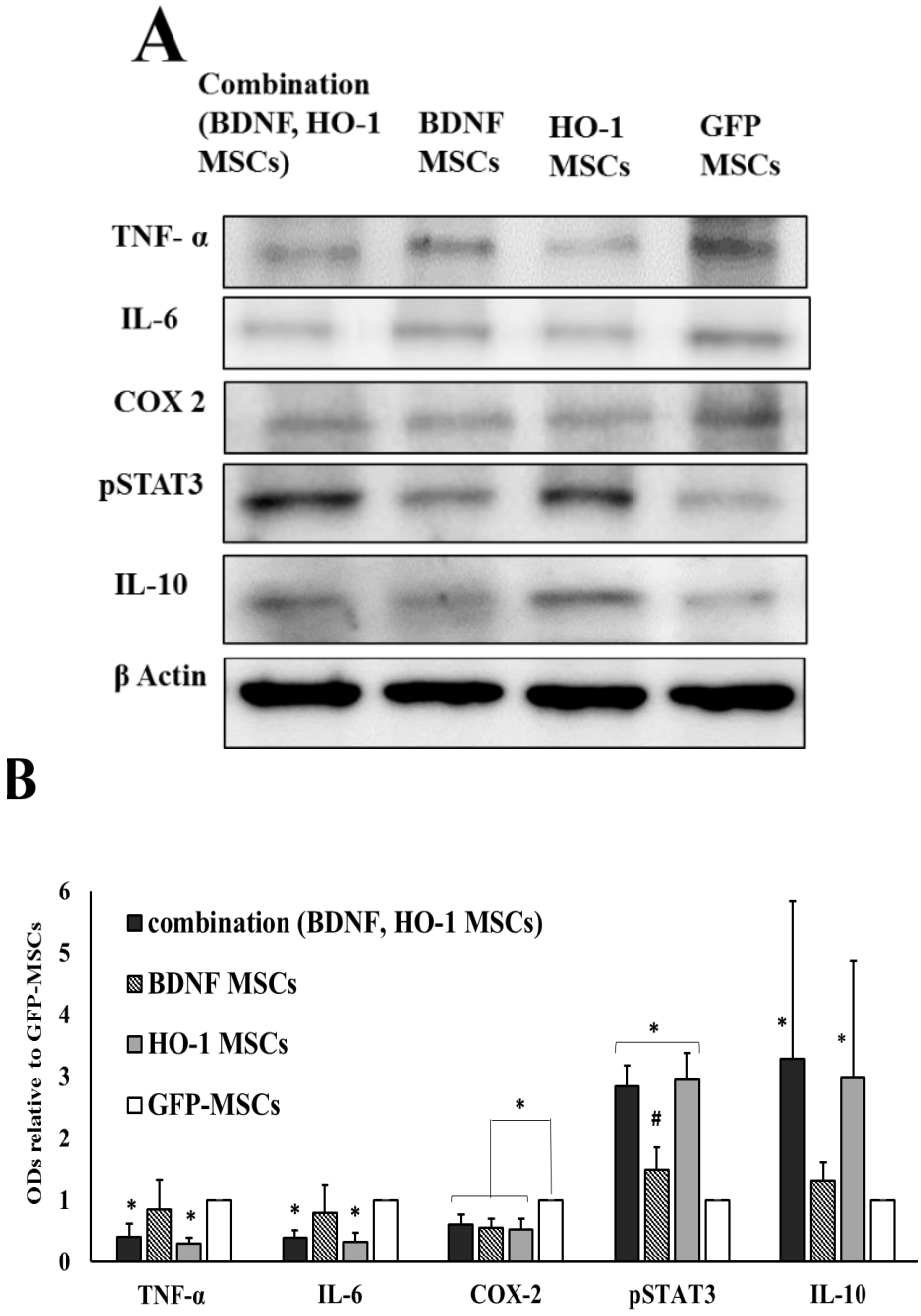


Figure 1.5. Expression of inflammatory and anti-inflammatory markers. A,

represent the densities of inflammatory and anti-Inflammatory markers. B, quantitative data obtained by densitometry showing optical densities (OD) relative to GFP-MSCs. The expression of TNF-α and IL-6 was significantly low in combination and HO-1 MSCs groups compare to BDNF and GFP-MSCs groups (*$p \leq 0.05$). The expression of COX-2 was significantly decreased in all combination, BDNF and HO-1 MSCs groups, compared to GFP-MSCs group (*$p \leq 0.05$). pSTAT3 was upregulated in both combination and HO-1 MSCs group, compared to BDNF and GFP MSCs groups (*$p \leq 0.05$), its expression was also higher in BDNF group compared to GFP-MSCs group (#$p \leq 0.05$). IL-10 was significantly higher in combination and HO-1 groups (*$p \leq 0.05$) compared to BDNF and GFP-MSCs groups. The graph represents mean ± SE of four dogs per groups, as determined by densitometry relative to β-actin.

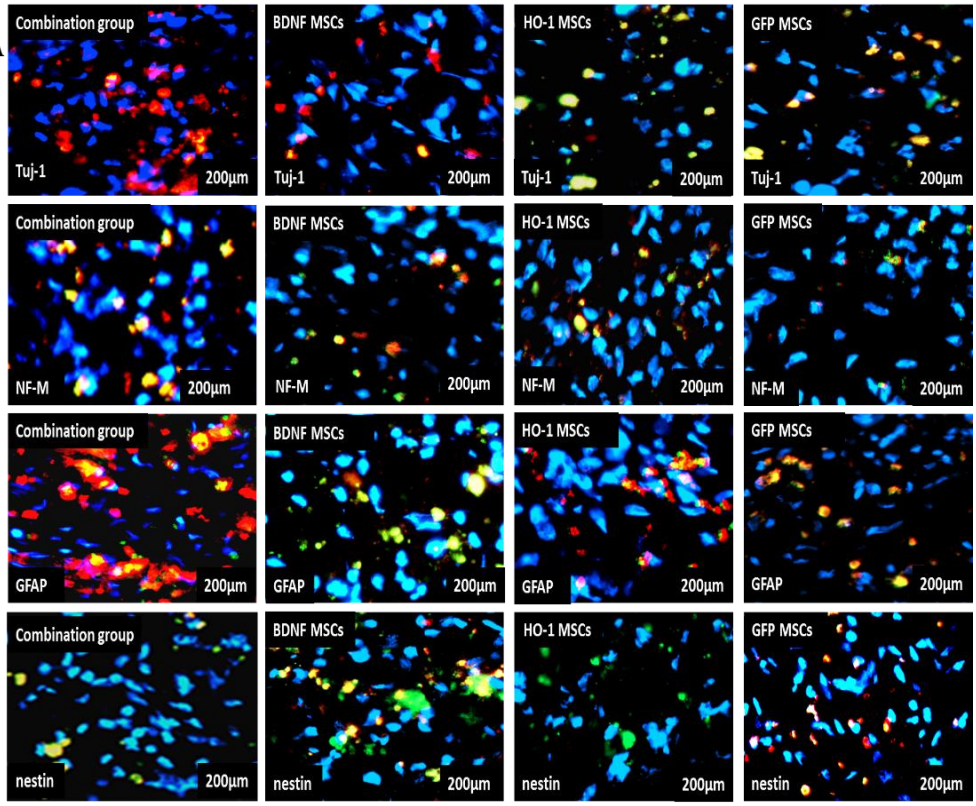
4. Immunohistochemistry

Immunohistochemistry was performed to evaluate the expression of neural markers Tuj-1, GFAP, NF-M, and nestin in transplanted MSCs and host cells at the injured segment of the spinal cord. The transplanted cells survived until 8 weeks. They were evenly distributed in the injured spinal cord segment. Based upon the presence of GFP expression, the percentage of live transplanted cells observed after 8 weeks was 14.9 % ± 5.8, 11 ± 4.36, 14.1 ± 4.65, 10.89 ± 5.28 for combination, BDNF MSCs, HO-1 MSCs and GFP MSCs groups respectively. The cells differentiated into neural and glial cells evidenced by the positive expression of neural and glial cells markers. No clustering of the transplanted cells observed. The cells were found together with endogenous neurons and glial cells, because GFP positive cells shown by green and blue color were found among the cells which were negative for GFP and positive for blue and red color of the neural marker.

Regarding the expression of neural markers, we found a similar pattern of expression, compared to that found in western blot results. We obtained the highest percentage of Tuj-1 positive cells, at 16.78% ± 2.14, in the combination group (Fig 1.6; $*p \leq 0.05$). The percentage value was not significantly different between the BDNF and HO-1 MSCs groups, at 10.17% ± 3.378 and 7.13% ± 2.86, respectively although it was higher than that of the

GFP-MSCs group, at 4.34% ± 0.30 (Fig 1.6; #p ≤ 0.05). The percentage expression of GFAP was similarly high in the combination group, at 16.87% ± 3.30, compared to the BDNF group, at 14.25% ± 1.21 (Fig 1.6; *p ≤ 0.05). The percentage expression in the BDNF group was higher than that of the HO-1 and GFP-MSCs groups, at 4.9% ± 2.1 and 8.40% ± 5.40, respectively. In case of NF-M positive cells, the percentage was the highest in the combination group, at 13.50% ± 5.50 (Fig 1.6; *p ≤ 0.05), followed by the BDNF and HO-1 MSCs groups, at 9.70% ± 1.03, and 7.91% ± 0.30, respectively, subsequently followed by the GFP-MSCs group, at 6.27% ± 1.68 (Fig 1.6; #p ≤ 0.05). Nestin, as a differentiation and stem cells marker, was high only in the combination and BDNF MSCs groups, at 13.07% ± 0.47, and 11.33% ± 3.23, respectively. These values were significantly higher than those of the HO-1 and GFP-MSCs groups, at 3.23% ± 1.50, and 4.80% ± 3.10, respectively (Fig 1.6; *p ≤ 0.05).

A


Combination group
BDNF MSCs
HO-1 MSCs
GFP MSCs
Tuj-1
NF-M
GFAP
nestin
200µm

B

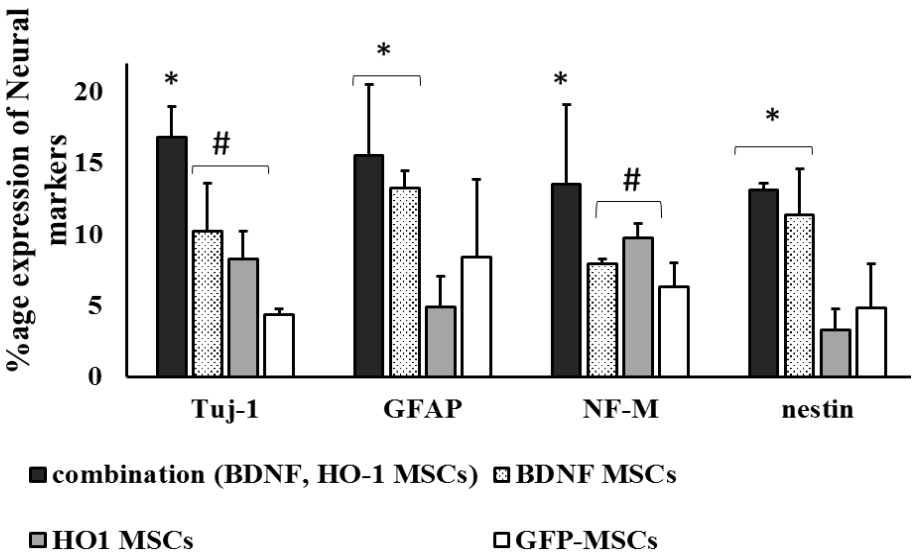
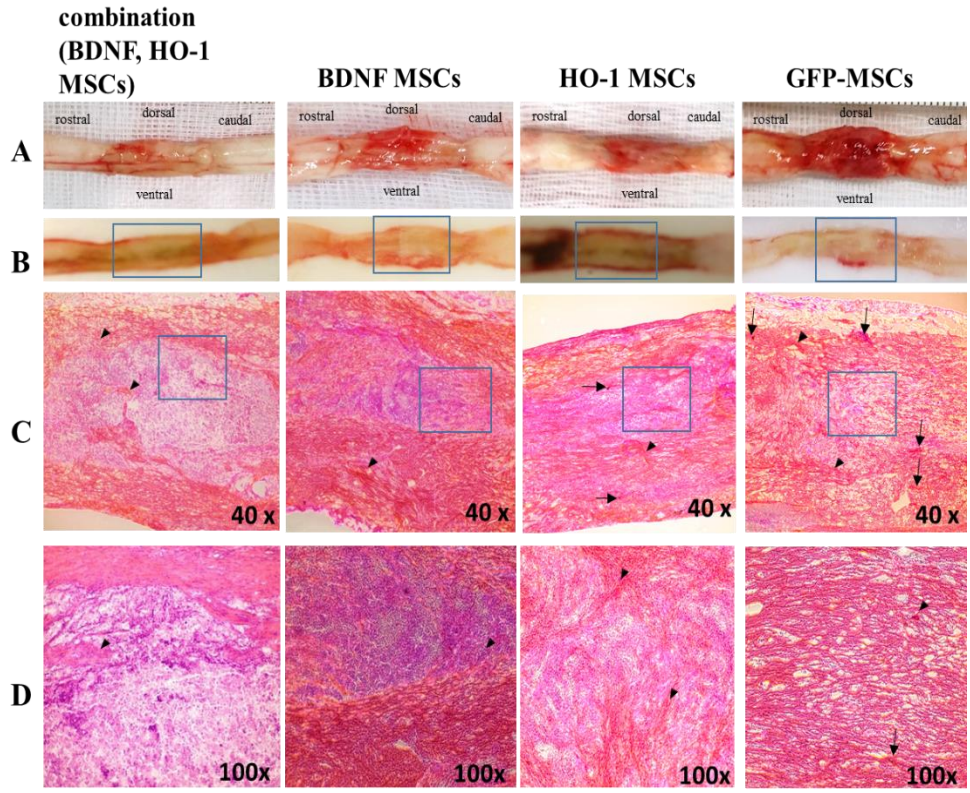

%age expression of Neural markers
0
5
10
15
20
*
#
Tuj-1
GFAP
NF-M
nestin
combination (BDNF, HO-1 MSCs)
BDNF MSCs
HO1 MSCs
GFP-MSCs

Figure 1.6. Immuno-fluorescent staining for the expression of neural markers. A, images of stained slides. The slides were stained with Tuj-1, NF-M, GFAP and nestin as red and nucleus was stained with DAPI as blue. Each image represents the four samples per group with a scale bar of 200um. B, %age expression of neural marker positive cells. The %age of cells positive for Tuj-1 and NF-M were highest in the combination group, followed by higher %age in BDNF and HO-1 MSCs groups compared to GFP-MSCs group (*#$p \leq 0.05$). While the cells positive for GFAP and nestin were similar between combination and BDNF MSCs groups, however higher than HO-1 and GFP-MSCs groups (*$p \leq 0.05$).

5. Histopathological assessment

The injured spinal cord showed atrophy, fibrosis, and hemorrhages (Fig 1.7, A). The degree of fibrosis was significantly the lowest in the combination group, compared to other groups (Fig 1.7 E, *$p \leq 0.05$). This was followed by higher fibrosis in the BDNF and HO-1 groups, although lower, compared to the GFP-MSCs (Fig 1.7 E, #$p \leq 0.05$). Based upon the visual examination, the combination group showed a well-organized parenchymal matrix with very few intervening fibrotic areas in the healthy area (Fig 1.7, C, D, arrowheads). The parenchymal matrix was comparatively less organized in the BDNF and HO-1 MSCs groups, with more intervening fibrotic areas in these groups than in the combination group. More robust fibrosis was observed in the GFP-MSCs group, with a more distorted parenchymal matrix (Fig 1.7, C, D). More hemorrhagic areas were observed in the GFP-MSCs group than in the BDNF, HO-1, and combination groups (Fig 1.7, C, D arrows).


combination (BDNF, HO-1 MSCs)
BDNF MSCs
HO-1 MSCs
GFP-MSCs
A
B
C
D
rostral
dorsal
caudal
ventral
40 x
100x

E

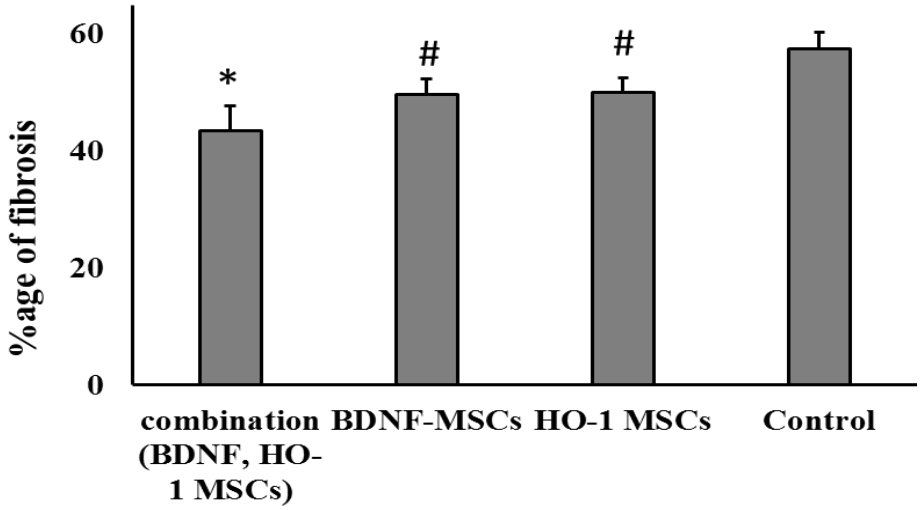

%age of fibrosis
60
40
20
0
*
#
#
combination (BDNF, HO-1 MSCs)
BDNF-MSCs
HO-1 MSCs
Control

Figure 1.7. Histopathology of spinal cord lesions stained with H & E stain. A, an injured spinal cord showed fibrosis, atrophy and hemorrhages. B, spinal cords sliced at injured segment. C, indicate the fibrotic (red & pink) and healthy areas (purple) at injured spinal cord section, at low magnification 40x, and D, at high magnification 100X, scale bar 200µm. E, %age expression of fibrotic area. There is a fibrosis at the epicenter which covers most of the healthy areas at the injured segment (C). The percentage fibrosis in combination group was lowest than in the other groups (E, *$p \leq 0.05$), followed by lower fibrosis in BDNF and HO-1 MSCs groups compared to GFP MSCs (E, #$p \leq 0.05$). Arrow head denote fibrotic areas while arrows indicate hemorrhages. Each picture (C) and bar (E) represent 4 samples per group (n =4).

DISCUSSION

The present study indicates that using the transgenic Ad-MSCs of different strengths i.e. BDNF MSCs and HO-1 MSCs in combination, has more favorable effect upon healing than using the BDNF MSCs and HO-1 MSCs alone. The results indicated that anti-inflammatory functions of HO-1 positively manipulate the microenvironment of the SCI which allows the BDNF to promote nerve cells growth, more significantly, than the BDNF cells transplanted alone.

Ad-MSCs have been used in SCI dogs with improved functional recovery, due to enhanced neuroregeneration, differentiation and reduced intraparenchymal fibrosis. The treatment groups had shown higher Olby, modified Tarlov and BBB scores with an improvement in conduction velocities observed through somatosensory evoked potentials (Lim et al. 2007; McMahill et al. 2015; Park et al. 2011; Ryu et al. 2009). Similarly, in clinical cases of dogs with a chronic SCI, showed improved Olby score after an intramedullary injection of human dental pulp stem cells and physical therapy (Feitosa et al. 2017). We potentiated the usefulness of Ad-MSCs by transfecting them with BDNF and HO-1 genes, through a lentivirus that showed successful expression in HO-1 MSCs and BDNF MSCs, both in-vitro and in-vivo. As previously described, the transfection did not negatively affect the viability and proliferation of both HO-1 MSCs and BDNF MSCs (Mijung

et al. 2016; Ritfeld et al. 2015). In our previous studies, we found that both BDNF MSCs and HO-1 MSCs were more potent than MSCs in terms of healing of the spinal cord injury and improvement in functional recovery (S. H. Lee et al. 2016a, 2016b). In the present study we injected both HO-1 and BDNF MSCs called the 'combination group' and compared it with HO-1 MSCs, BDNF MSCs and GFP-MSCs group. We found improved functional ability, in terms of a highest cBBB score in the combination group compared to other groups. This improvement might be due to favorable microenvironment created by the types of cells that were transplanted.

BDNF is a neurotrophic factor which is responsible for the neuronal survival, growth and maturation (Wollen 2010). HO-1, is a kind of an anti-stress protein, which is normally expressed in spinal cord neurons and induced after a SCI, both in gray and white matter, primarily in microglial and glial cells (Y. Liu et al. 2002; Stahnke et al. 2007) that reduce inflammation, apoptosis and oxidative injury (Negi et al. 2015). Because, the pathophysiological events with an expression of proinflammatory cytokines, during SCI, create an unfavorable micro environment for the regeneration of injured neurons (Dasari et al. 2014), therefore it is desirable to control inflammation to get optimum healing effect of BDNF. BDNF MSCs have been found to induce regeneration in a contused spinal cord of mice, by upregulating GAP-43 and neuron specific enolase positive cells (NSE) (W. Ji and Qiu 2016). Similarly in SCI dogs, BDNF MSCs and HO-1 MSCs induce

neuroregeneration by upregulating the expression of Tuj-1, NF-M and nestin (S. H. Lee et al. 2016a, 2016b). In the present study, we found an increase in neuroregeneration in both HO-1 and BDNF MSCs groups, evidenced by a higher expression of Tuj-1, GAP-43 and NF-M. In BDNF group, it was due to the enhanced expression of BDNF by BDNF MSCs, which stimulated the neuroregeneration. Similar increase in neuroregeneration in HO-1 MSCs group is attributed to an upregulation of BDNF in the group. This happen because HO-1 upregulate the expression of BDNF by activating ERK and PI3K-AKT pathways (Hung et al. 2010), which subsequently activate the TrkB, PI3K/Akt signaling pathways for neuroregeneration and survival (Qi et al. 2014). However, we found more robust neuroregeneration in the combination group, which might be due to the different contribution of two different types of cells, i.e. the control of inflammation by HO-1 MSCs, and neuroregeneration by BDNF MSCs.

As previously described, the BDNF MSCs did not reduce the expression of inflammatory cytokines in chronic spinal cord injury of dogs, however it was reduced with higher expression of neural and glial markers when the dogs were intravenously (IV) injected with Ad-MSCs (S. H. Lee et al. 2016a). The MSCs injected IV cause systemic immunomodulation, which affects the inflammatory status of SCI and promote healing (Badner et al. 2016). We also found the compatible results, regarding the expression of TNFα and IL-6 in the combination group. It seems that BDNF overtake its

role of nerve growth regardless of the presence of pro-inflammatory cytokines but it is more significant when the inflammation is controlled. We got the highest expression of neural markers in the combination group with GAP43 than both BDNF and HO-1 MSCs groups which represent more favorable neuroregeneration due to local anti-inflammatory effect of HO-1 MSCs.

HO-1 mediates the anti-inflammatory effect of IL-10 in mice (T.-S. Lee and Chau 2002), and confers protection to the hepatic ischemia reperfusion injury by inhibiting TNF-α mediated activation of apoptotic pathways (S.-J. Kim et al. 2013). IL-10 inhibits the expression of pro-inflammatory cytokines and pro-apoptotic factors such as IL-6, TNF-α etc., which increases nerve tissues sparing and improves functional recovery (Thompson et al. 2013). In the present study, the expression of inflammatory markers TNF-α and IL6 was significantly reduced in both combination and HO-1 groups which is compatible with the previously reported data of canine SCI treated with HO-1 MSCs (S. H. Lee et al. 2016b). We further confirmed that anti-inflammatory effect was due to the upregulation of IL-10. As an anti-inflammatory marker, IL-10 was significantly upregulated in both the combination and HO-1 MSCs groups, compared to the BDNF and GFP-MSCs groups, which suppressed the expression of TNF-α and IL-6, increasing nerve tissue sparing by impeding further progress into the secondary SCI, and this might be the reason for more potent action of BDNF in the combination group.

COX2 expression is induced by TNF-α, which further induce

inflammation by regulating prostaglandin E2 synthesis (Miyamoto et al. 2000). HO-1 overexpression attenuates the lipopolysaccharide induced COX2 expression in the mouse brain (Shih and Yang 2010). Additionally, BDNF overexpression suppresses COX2 expression in a transgenic mice model of Alzheimer's disease (Koo et al. 2013). In the present study, COX2 was downregulated in the combination, BDNF, and HO-1 MSCs groups, which confirms the inhibitory effect upon COX2 in dogs with SCI, however this effect was not observed in a chronic spinal cord injury model of dog, treated with BDNF MSCs (S. H. Lee et al. 2016a). This may indicate that BDNF MSCs act differently in controlling the expression of COX2 when transplanted in different stages of SCI.

Previously both HO-1 MSCs and BDNF MSCs were found to reduce the degree of fibrosis and hemorrhages at the injury site (T.-S. Lee and Chau 2002; S. H. Lee et al. 2016a); however, we report a reduction in the degree of fibrosis, more significantly in the combination group than in the HO-1 and BDNF MSCs groups.

As a result of spinal cord injury a blood brain barrier is disrupted which allows the infiltration of leukocytes at the injured segment of the spinal cord, which together with glial cells, build a glial scar. The glial scar inhibits further injury to the injured spinal cord segment however it also interferes with healing process (Orlandin et al. 2017). Resident cells in the glial scar produce copious amounts of inhibitory factors, such as chondroitin sulphate

proteoglycans (CSPG) and matrix metalloproteinases, that inhibit axonal regeneration (Yuan and He 2013). Various experimental data suggests that inhibiting the glial scar ameliorate the micro-environment of the injured spinal cord for the growth of neurofilaments. Various agents such as decorin, hepatocyte growth factors, curcumin macrophage colony stimulating factors etc. have been experimentally found effective in reducing the glial scar with improved functional recovery and increase gray matter (Orlandin et al. 2017). There is also a conflicting data available in the support of astro-gliosis. Astrocyte scar formation has been found to support the regeneration of corticospinal and serotonergic axons in SCI of rats (Anderson et al. 2016). The role of astrocytes in neuroregeneration is significant because they inhibit microglial activation and control inflammation (J. h. Kim et al. 2010). We also found the favorable effect of astrocytes on neuroregeneration, as both GFAP and nestin were equally upregulated in both the BDNF MSCs and combination groups, and neuroregeneration was not affected by astrocytes, as evidenced by a higher expression of Tuj-1, GAP-43 and NF-M.

A challenge during the repair of SCI is the re-myelination of newly formed neurons, and the neurons that get demyelinated during secondary SCI. Fibroblasts overexpressing BDNF were found to promote myelination of newly formed neurons by inducing oligodendrocyte and Schwan cells proliferation (McTigue et al. 1998) and the expression of myelin basic protein (Koda et al. 2002). The marker specific for oligodendrocytes and re-

myelination, GALC, was upregulated, equally, in both the combination and BDNF MSCs groups, compared to the HO-1 and GFP-MSCs groups, which may represent a stimulatory effect of BDNF upon re-myelination. GALC was also upregulated in the HO-1 group compared to GFP-MSCs group, and this may be due to a higher expression of BDNF in the HO-1 MSCs group. These results do not comply with our previous findings which showed the down regulation of GALC in both BDNF MSCs and HO-1 MSCs used in chronic and subacute spinal cord injury model of dogs respectively (S. H. Lee et al. 2016a, 2016b). The GALC expression was reduced with systemic immunosuppression due to MSCs injected IV (S. H. Lee et al. 2016a), however the local immune suppression in the combination group of present study, did not reduce the expression of GALC, which may indicate the different behavior of BDNF MSCs in the chronic stage of spinal cord injury.

Signal transducer and activator of transcription 3 (pSTAT-3) is a transcription factor that mediate multiple pathways related to neuroregeneration and differentiation. STAT-3 activation enhance axonal regeneration and neuroprotection after a SCI by activating the JAK/STAT3 and PI3K/AKT/mTOR pathways (Mehta et al. 2016; Qi et al. 2014). STAT-3 activation mediates the activation of tyrosine kinase-A receptors by BDNF, which are required for nerve growth and survival (Ng et al. 2006). Pertaining to HO-1 expression, IL-10 induce HO-1 expression by activating STAT-3 (Ricchetti et al. 2004). In the present study, STAT3 was highly

upregulated in both the combination and HO-1 groups, which suggests the activation of IL-10/pSTAT-3/HO-1, as well as the pathways associated with neuronal regeneration, as previously described. STAT3 was also upregulated in the BDNF group, compared to the GFP-MSCs group, which may indicate the BDNF activation of STAT-3 for TrkB signaling, required for neural regeneration (Chen et al. 2016).

Collectively, we get enhanced neuroregeneration in the combination group, i.e., a higher expression of Tuj-1, GAP-43 and NF-M, with BDNF associated benefits of nestin and GFAP expression favored because of the non-inhibitory environment created by HO-1 MSCs, by controlling the expression of TNF-α, IL-6 and COX-2. Thus, this could be a novel strategy to selectively control inflammation, and induce neuroregeneration, by using two different types of MSCs, with good clinical outcomes. We used dog as an experimental SCI model because large animal's experimental SCI models are considered more appropriate than rodents in order to translate these studies in human beings. Because of most of the anatomical and histopathological similarity with human beings, the studies conducted on dogs and primates with experimental SCI can be applied in human beings as well (Gabel et al. 2017). The injury pattern of spinal cord in dogs, such as, naturally occurring intervertebral disk herniation (IVDH), has a close resemblance with IVDH of humans. Magnetic resonance imaging showed similar post traumatic myelopathies such as cystic cavities, spinal cord atrophy and syringomyelia in

dogs and humans due to IVDH (Alisauskaite et al. 2017). We used balloon compression SCI model in dogs which closely mimic the contusion or compression caused by IVDH in dogs and humans (Cheriyan et al. 2014). Therefore, the methodology and techniques used in present study could have favorable outcomes in the SCI of humans as well. Moreover, it will also help to investigate more combinations of suitable cell types in the future, to selectively manipulate various factors for better clinical outcomes.

From the present study, it can be concluded that HO-1 MSCs inhibit the expression of inflammatory cytokines, which establishes a favorable environment for BDNF-induced neuroregeneration in dogs with sub-acute SCI.

CHAPTER II

Therapeutic effects of intravenous injection of fresh and frozen-thawed HO-1 overexpressed Ad-MSCs in dogs with acute spinal cord injury

ABSTRACT

Owing to the anti-oxidant and anti-inflammatory functions of hemeoxygenase-1 (HO-1), HO-1 expressing stem cells could be efficacious in treating spinal cord injury (SCI). Further, frozen-thawed HO-1 MSCs could be instantly available as an emergency treatment for SCI. We compared the effects of intravenous treatment with freshly cultured HO-1 MSCs (HO-1 MSCs), only green fluorescent protein expressing MScs (GFP MSCs) and frozen-thawed HO-1 MSCs (FT-HO-1 MSCs) in dogs with acute SCI. For four weeks, dogs were evaluated for improvement in hind limb locomotion using a canine basso beattie bresnahan (cBBB) score. Upon completion of the study, injured spinal cord segments were harvested and used for western blot and histopathological analyses. All cell types had migrated to the injured spinal cord segment. The group that received HO-1 MSCs showed significant improvement in cBBB score within four weeks. This group also

showed significantly higher expression of NF-M, and reduced astrogliosis. There was reduced expression of pro-inflammatory cytokines (IL6, TNF-α, IL-1β) and increased expression of anti-inflammatory markers (IL-10, HO-1) in the HO-1 MSCs group. Histopathological assessment revealed decreased fibrosis at the epicenter of the lesion and increased myelination in the HO-1 MSCs group. Together, these data suggest that HO-1 MSCs could improve hind limb function by increasing the anti-inflammatory reaction, leading to neural sparing. Further, we found similar results between GFP MSCs and FT-HO-1 MSCs, which suggests that FT-HO-1 MSCs could be used as an emergency treatment for SCI.

INTRODUCTION

Presently, a high dose of methylprednisolone sodium succinate (MPSS) is injected intravenously (IV) as an emergency treatment to induce anti-oxidation in dogs with acute spinal cord injury (SCI). However, meta-analysis has demonstrated that there is no satisfactory improvement with the use of MPSS, and it is associated with adverse side effects such as severe gastrointestinal bleeding (Evaniew et al. 2016; Olby et al. 2016). Thus, a safer and more effective emergency treatment option is required. An alternative that has been attempted is IV administration of adipose-derived mesenchymal stromal cells (Ad-MSCs). Ad-MSCs and MPSS have different roles in controlling oxidative stress and inflammation in dogs with acute SCI (Y. Kim et al. 2015). Owing to the strong anti-oxidant and anti-inflammatory effects of hemeoxygenase 1 (HO-1), intra-parenchymal injection of HO-1-expressing Ad-MSCs reduce inflammation and improve neuronal sparing in canine SCI compared to control Ad-MSCs (S. H. Lee et al. 2016b).

HO-1 is an anti-stress enzyme which causes oxidative degradation of deleterious heme into biologically active products such as carbon monoxide, biliverdin and ferrous iron (Kozakowska et al. 2014). HO-1 causes immunomodulation by altering the function of macrophages, regulatory T cells and dendritic cells (Ryter and Choi 2016). It is normally expressed in

neurons in the spinal cord (A. Mautes et al. 1998). During SCI, the expression of HO-1 is induced in glial cells, which prevents inflammatory damage to the neurons (Stahnke et al. 2007). Due to the concerted actions of HO-1, the therapeutic effects of HO-1 have been evaluated in various inflammatory conditions, neuropathological disorders and cardiovascular diseases (Dunn et al. 2014). Adeno-associated HO-1 induction following SCI causes neuroprotection by inhibiting pro-apoptotic pathways in the neurons (Lin et al. 2017). It protects against neuronal damage due to ischemia-reperfusion injury by inducing the expression of anti-oxidant enzymes such as Cu and Zn-superoxide dismutase and neurotrophic factors such as BDNF, and inhibiting lactic acidosis (Jung et al. 2016). In SCI and cardiovascular disease, transplantation of mesenchymal stem cells (MSCs) modified by viral-mediated HO-1 gene insertion shows improved healing due to the stimulated expression of anti-inflammatory cytokines and vascular endothelial growth factor (Jiang et al. 2011; S. H. Lee et al. 2017).

MSCs have been administered through various routes such as IV (Y. Kim et al. 2015; Morita et al. 2016), intra-parenchymal (S. H. Lee et al. 2017), and into the subarachnoid (Nategh et al. 2016) and epidural spaces (Krueger et al. 2017). The IV route is simple and minimally invasive, with favorable clinical outcomes, and avoids complications associated with the other relatively invasive procedures used for cell transplantation (Taylor-Brown et al. 2015).

Cryopreservation of cells could lead to more instantaneous availability for use after SCI, and frozen-thawed HO-1 overexpressed stem cells could be the best option for critical care. In the present study, I evaluated whether IV injection of freshly cultured and frozen-thawed HO-1 overexpressed Ad-MSCs is effective as an emergency treatment in dogs with acute SCI.

MATERIALS AND METHODS

1. In vitro procedures

1.1.Isolation and culture of Ad-MSCs

Adipose tissue were collected aseptically from the sub-gluteal region of a healthy beagle dog under general anesthesia. The tissue was homogenized and incubated with collagenase type-1 enzyme (1 mg/mL, Sigma, St. Louis, USA) for 2 h at room temperature (16-20°C). The suspension was filtered through a 100 µm nylon mesh then centrifuged at 4°C for 10 min. The supernatant was discarded and the stromal vascular fraction (SVF) was carefully isolated. The SVF was resuspended with phosphate buffer saline (PBS) and cultured in a 150 mm Petri dish containing low-glucose Dulbecco's modified eagle medium (DMEM; GenDEPOT, USA) with 10 % fetal bovine serum (FBS; Gibco BRL, Grand Island, USA) and 1% penicillin and streptomycin (PS). Cells were washed with PBS after 24h of incubation to remove the dead cells and tissue debris, and media was changed. The cells were subcultured when 90% confluence was reached. The third passage (P3) of Ad-MSCs were used in subsequent experiments (Neupane et al. 2008).

1.2.Lenti-Virus mediated HO-1 gene insertion into Ad-MSCs

A Dharmacon trans lentivirus packaging system (GE Healthcare, Bio-Sciences U.S.A.) was used to produce HO-1-overexpressing Ad-MSCs. The transgenic lentiviruses express green fluorescent protein (GFP), and GFP with HO-1 were produced, which were used to transduce Ad-MSCs. Two types of cells were produced: GFP expressing Ad-MSCs (GFP-MSCs) and GFP-HO-1-expressing Ad-MSCs (HO-1 MSCs).

According to manufacturer's guidelines, we first amplified the flag-tagged HO-1 gene by using Phusion DNA Polymerases (Thermo Scientific, Pittsburgh, U.S.A.) from the cDNA of canine peripheral blood. The HO-1 primer (Forward GACAGCATGCCCCAGGAT, Reverse CACAGCCTAAGGAGCCAGT) specific for canines was inserted into the pCDH-EF1-MCS-pA-PGK-copGFP-T2A-Puro vector using the restriction enzymes EcoRI and BamHI (System BioSciences) (Mijung et al. 2016). HEK293T cells were cultured in 100 mm culture dish. Culture medium DMEM containing high glucose, 10% FBS and 1% PS was used to grow the cells at 37°C and 5% CO_2. When cells reached 90% confluence, the vector encoding GFP and HO-1 genes was mixed with lentiviral packaging mix (Fisher Scientific Cat#14-432-23) and added dropwise to the cultured HEK293T cells. The cells were cultured for 48 hr at 37°C and 5% CO_2. The

medium was changed once after 16 hr of incubation, and viral particles were collected from culture media after 48 hr of incubation. Hence, two types of viruses were produced: Lenti-GFP and Lenti HO1-GFP. Using the manufacturer guidelines, the concentration of viral particles was determined. The second passage Ad-MSCs were infected with lentiviruses at MOI 100(McGinley et al. 2011) at 50-60% confluence. At 90% confluence, the cells were subcultured. Successful transduction was confirmed by the expression of GFP under a fluorescent microscope (EVOS FL imaging system, USA), quantitative polymerase chain reaction (qPCR) and western blot analysis. The cells were treated with 3 µg/ml puromycine to obtain a higher percentage of transduced stem cells. Finally, we got two types of cells: Ad-MSCs expressing GFP (GFP-MSCs) and Ad-MSCs expressing GFP and HO-1 (HO1-MSCs). The transduced Ad-MSCs were cultured to P3 and used in the subsequent experiments.

1.3.Analysis of the expression of HO-1 gene in Ad-MSCs

The transduced Ad-MSCs were analyzed for the expression of transfected genes by qPCR and western blot analysis. The cells were cultured in four 150 mm culture dishes until they reached 90% confluence. One culture dish was utilized for qPCR, and 3 culture dishes were utilized for western blot

analysis. For qPCR, mRNA was harvested using Hybrid-R RNA extraction kit (geneAll, Seoul, Republic of Korea) according to manufacturer guidelines. Total mRNA was determined by Gen 5.2 reader type, Epoch (BioTek, Winooski, Vermont, USA) by measuring optical density at a wavelength of 260 nm. cDNA was synthesized by using prime script II First-Strand cDNA Synthesis Kit (Takara, Otsu, Japan). cDNA was amplified by ABI Step one plus Real-Time PCR system (Applied Biosystems, California, U.S.A.) after mixing it with SYBR premix EX Taq (Takara, Otsu, Japan) and HO-1 primers (Forward GACAGCATGCCCCAGGAT, Reverse CACAGCCTAAGGAGCCAGT). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (Forward CATTGCCCTCAATGACCACT, Reverse TCCTTGGAGGCCATGTAGAC) was used as a reference gene. The expression of HO-1 gene was quantified by the $2^{-\Delta\Delta CT}$ Method(Livak and Schmittgen 2001).

For western blot analysis, cells from three culture dishes were scraped and centrifuged. The cell pellet was resuspended and incubated with 300 μL Ripa Lysis Buffer (Gen Depot, USA) for 30 min on ice. The suspension was centrifuged at 4°C for 10 min with 13,000 rpm. The supernatant containing the proteins were separated and mixed with proteinase inhibitor solution (Gen depot, USA) at the rate of 1 μL/100 μL of cell's lysate. Protein concentration was measured by Bradford assay(Bradford 1976). Approximately 20 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and incubated with primary antibodies for GFP (GF28R), β-actin (sc-47778) and HO-1 (ab-13243), followed by incubation with secondary antibodies anti-mouse secondary antibody (ab6728) and anti-rabbit secondary antibody (ab6721). The protein bands were visualized with enhanced chemiluminescent substrates (ECL) (Bio-Rad, USA), by using image quant LAS 4000 mini system (GE Healthcare Biosciences, Sweden).

1.4.Cryopreservation of HO-1 MSCs

P3 HO1-MSCs cultured in 150 mm culture plate were harvested at 90% confluence. The cells were washed twice with PBS and incubated with 0.05% trypsin-EDTA for 15 min at 37 ˚C and 5% CO_2 (Sigma-Aldrich, Saint Louis, USA). The cells were then centrifuged at 2,500 rpm for 5 min at 4°C and the supernatant was discarded. Approximately 5 $\times 10^6$ cells were resuspended in the cryogenic medium (50% DMEM, 40% FBS, 10% Dimethyl sulfoxide) and transferred into a Cryovial (1.2 ml, Sigma-Aldrich, Saint Louis, USA). The cells were kept at 4 C° for 1 hr, -20 C° for 2 hrs, -80 C° for 24 hrs and -150 C° for 2-3 weeks(Mijung et al. 2016).

2. In vivo procedures

2.1.Selection and identification of animals

The study was performed on 12 healthy beagle dogs aged 1.2 ± 0.2 years and weighing 12 ± 3.0 kg. This study was approved by the Institute of Animal Care and Use Committee (ICAUC) of Seoul National University (SNU-170417-12). The dogs were randomly divided into four groups comprising four dogs each. The groups were named according to the type of cells transplanted IV: GFP-MSCs (fresh Ad-MSCs expressing GFP), HO-1 MSCs (fresh Ad-MSCs expressing GFP and HO1), FT-HO-1 MSCs (frozen thawed HO-1 Ad-MSCs).

2.2.Induction of spinal cord injury

SCI was induced by hemilaminectomy as previously described(Park et al. 2012). Dogs were restrained in ventral recumbence position under general anesthesia. A small opening was made using a pneumatic burr between the third and fourth lumbar vertebra. Under fluoroscopic guidance, a 6-french embolectomy catheter (Sorin, Biomedica, Italy) was inserted into the

spinal canal and dragged up to the 1st lumbar vertebra (L1), until its tip reached the cranial border of L1. The catheter balloon was inflated with contrast media at a rate of 50 µl/kg. The catheter was deflated and removed after 6 hrs. The dogs were closely evaluated for the presence of sensory and motor reflexes. Only the dogs which showed complete paraplegia, with no sensory and motor reflexes, were included in the study. Twelve properly induced dogs were kept in an intensive care unit for 3 days. Urination was assisted by manual bladder compression three times per day. Soft bedding was provided to prevent bed sores, and food and water were provided ad-libitum.

2.3.Transplantation of Ad-MSCs

Stem cells were transplanted immediately after the removal of the embolectomy catheter. In the case of transplantation of fresh MSCs, the cells were cultured in a 150-mm culture dish until they had reached 90% confluence. The cells were harvested, centrifuged, and pelleted as described above. In the case of transplantation of frozen-thawed MSCs, the cryopreserved cells were thawed by warming the cryovial at 37°C for 5 min. The cell suspension was mixed with an equal volume of DMEM and centrifuged at 2,500 rpm for 5 min at 4°C. The medium

was discarded and the cell pellet was resuspended in PBS and centrifuged. The cell pellets obtained from both fresh and frozen-thawed preparations were resuspended in 1 ml of Hartmann's solution. The cell number required for transplantation was counted using a Countess FL Automated Cell Counter (ThermoFisher Scientific, MO, USA) after staining with Trypan blue. The %age of GFP expression for HO-1 MSCs, GFP MSCs and FT-HO1 MSCs was 91.03 ± 0.8, 91.30 ± 0.7 and 90.56 ± 0.95 respectively. About 1×10^7 fresh GFP MSCs and HO-1 MSCs, and 1.5×10^7 frozen-thawed HO-1 MSCs were diluted in 20 ml of Hartmann's solution and injected by slow IV infusion in 10 min. The cells were injected IV for three consecutive days with an interval of 24 hrs between each injection. The dogs were kept for four weeks after cell transplantation.

2.4.Behavioral assessment

All the dogs were observed for the improvement in hind limb movements every week for four weeks. The assessment was made by three people blinded to the study, and the score for each week was presented as an average. The motor activity of hind limbs was assessed by using the canine basso beattie

bresnahan (cBBB) scale(Song et al. 2016). Each week, the dogs were released into a confined area for 5 min and observed from each side to note their ability to use the hind limbs. Hind limb activity was observed when the dogs were at rest, walking and forced walking. The hind limb joints were flexed while the dogs were sitting and when lifted up, to observe the reflex motor response and movements of the joints. Deep pain perception was evaluated by pinching the phalangeal joints with artery forceps.

2.5.Harvesting of the spinal cord and sample preparation

At the end of the four-week experimental period, the twelve dogs were anesthetized and the spinal cords were harvested from T13 to L2. The spinal cord segments containing the entire injured segment were put into 10% sucrose for 24 hrs and then into 20% sucrose for the subsequent 24 hrs. The dura mater was carefully removed and the injured spinal cord segments were frozen in OCT compound using liquid nitrogen. Injured spinal cord segments were carefully sliced from the center into two equal longitudinal halves. One half was used for western blotting while the other half was used for histopathology.

2.6.Western blot analysis for the expression of proteins

The injured spinal cord segments were crushed and minced by hammering after freezing with liquid nitrogen. Crushed tissue was then incubated with Ripa lysis buffer and proteinase inhibitor solution for 30 min according to manufacturer's guidelines. Samples were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was collected into a separate Eppendorf tube and the protein concentration was determined by a Bradford assay. Approximately 20 μg of protein was separated through 10% acrylamide gel by a water tank gel electrophoresis. The proteins were electrically transferred onto a PVDF membrane. The membrane was blocked with 20% skim milk for 1 hr and washed. Membranes were then incubated with primary antibodies for 16-24 hr. The primary antibodies specific for neural markers Tuj-1 (sc69966, β-III tubulin), NF-M (sc-398532, neurofilament,), and nestin (ab7695, neural progenitor stem cells), and glial markers GFAP (sc65343, astrocytes), GALC (sc67352, Galactocylceramidase) and CD11b (ab62817 microglia) were used. To stain for inflammatory cytokines, the following primary antibodies were used: TNF-α (sc1350, tumor necrosis factor alpha), IL6 (ab6672, interleukin-6) and IL1-β (A13268, interleukin 1β). To stain for anti-inflammatory markers, the following primary antibodies were used: IL-10 (R&D, MAB7352) and HO-1 (ab13243). The primary antibody for GFP (GF28R) was used to assess the migration of transplanted cells into the

injured segment of the spinal cord. The membrane was then incubated for 2 hr at 4°C with the anti-mouse secondary antibody (ab6728), anti-rabbit secondary antibody (ab6721) and anti-goat secondary antibody (sc2354). β-actin was used as a reference antibody (sc47778). Enhanced chemiluminescent substrates (ECL) (Bio-Rad, USA) were used to visualize the protein bands and images were quantified using the LAS 4000 mini system (GE Healthcare Biosciences, Sweden).

2.7.Immunocytochemistry

The remaining half of the spinal cord was cryosectioned at -20°C. The segments containing the injury were sliced at 10 μm and mounted on silane-coated slides. OCT compound was carefully removed by pouring phosphate buffer saline (PBS) on the slides, followed by fixing with 4% paraformaldehyde for 10 min, then permeating with 0.1% v/v Triton X-100 for 3 min. Slides were blocked with 10% FBS (FBS, Gibco BRL, Grand Island, USA) in PBS for 1 hr, washed, and incubated overnight with primary antibodies for GFP, Tuj-1, NF-M, nestin, GFAP, and GALC at 4°C. Slides were washed and incubated with fluorescein iso-thio-cyanate conjugated anti-rabbit (Flamma 648) and anti-mouse (Alexa flour, ab-150111) secondary antibodies. One or two drops of DAPI were added to sections to stain the nuclei, and slides were coverslipped. The sections were examined for the expression of relevant markers with a fluorescent microscope (EVOS FL

imaging system, USA). The injured segments of the spinal cord were examined at low magnification 100 X (scale bar 400 μm), and the positively stained areas for the specific markers were measured as percent values through an integrated cell counting system in the microscope software.

2.8.Histopathological assessment

Histopathology was performed to assess the degree of fibrosis, demyelination, hemorrhage and vacuoles formation. Following the manufacturer guidelines, slides were stained with hematoxylin and eosin (ThermoFisher scientific, 7212, USA) to assess the degree of fibrosis, and with luxol fast blue (American Master Tech, STLFB100) to assess the degree of demyelination. Slides were observed at 40 X (scale bar 1000 μm) (Ninomiya et al. 2015). About 2-3 images that comprised the entire injured segment were obtained, and the lesion area was measured using Image J software (Version 1.37; National Institute of Health, USA). Values are presented as the average for each slide of a corresponding group.

3. Statistical analysis

Data are expressed as mean ± standard error (SE), and analyzed for the presence of significance by non-parametric Kruskal Wallis test. A Man-Whitney U test was used to determine significance between two specific groups. SPSS software (version 23 IBM, USA) was used to analyze the data, and a p-value ≤ 0.05 was considered significant.

RESULTS

1. HO-1 overexpression in Ad-MSCs

HO-1 gene was successfully transfected into Ad-MSCs. The mRNA gene expression of HO-1 was significantly higher (Fig 2.1C, $^{*}p \leq 0.05$) than the GFP MSCs. Similarly, at a translation level, the cell lysate obtained from HO-1 MSCs contained higher levels of HO-1 protein (Fig 2.1A, B., $^{*}p \leq 0.05$) than the GFP MSCs. The expression of GFP was not significantly different between HO-1 MSCs and GFP MSCs (Fig 2.1B, D, E). GFP MSCs showed thin elliptical fibroblast-like morphology, while the HO-1 MSCs were a slightly broader elliptical shape (Fig 2.1 D, E). The GFP MSCs showed slightly high proliferation, which was lower in HO-1 MSCs.

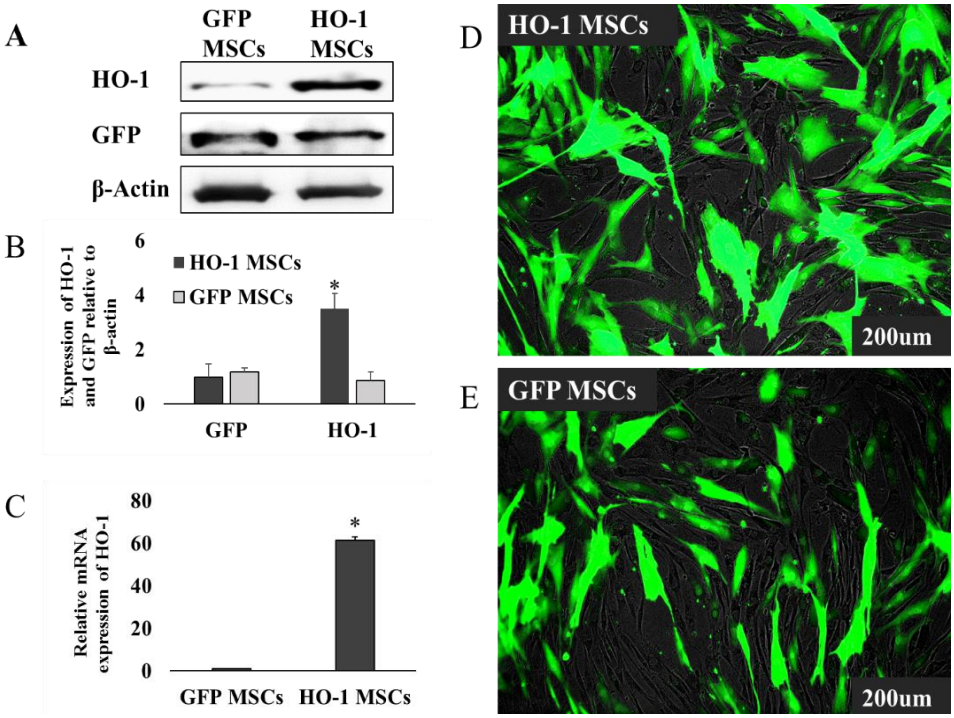


Figure 2.1. HO-1 and GFP gene transfection in Ad-MSCs. A) Representation of the densities of HO-1 and GFP proteins obtained from a cell lysate of HO-1 and GFP MSCs. B) Quantitative expression of densities obtained by western blot relative to β-Actin. Each bar represents the average of six samples and error bars represent standard error. C) mRNA expression of HO-1 in HO-1 MSCs and GFP MSCs. Each bar represents the average of six samples and error bars represent standard error. * indicates significance at $p \leq 0.05$ (Student t-test). D) Positive GFP expression by GFP and HO-1 MSCs.

2. Migration of cells after IV injection

Based upon western blot analysis and immunocytochemistry, cells from all groups had successfully migrated into the injured spinal cord segment after IV injection (Fig 2.2). Regarding the expression of GFP in vivo, no significant difference was observed among the groups (Fig 2.2 A, B, C). As indicated by the positive expression of GFP detected using immunocytochemistry (Fig 2.2C) the GFP positive cells were found in abundance at the rostral and caudal margins of the injured spinal cord segment; however, their expression was comparatively lower in the medial area of the injured spinal cord segment. More cells were present in gray matter at the rostral, medial, and caudal site of the injured spinal cord segment compared to white matter (Fig 2.2C). The percentages of GFP expression quantified were 12.18% ± 2.84, 10.85% ± 3.03 and 8.34% ± 4.04 for HO-1 MSCs, GFP MSCs and FT-HO-1 MSCs, respectively.

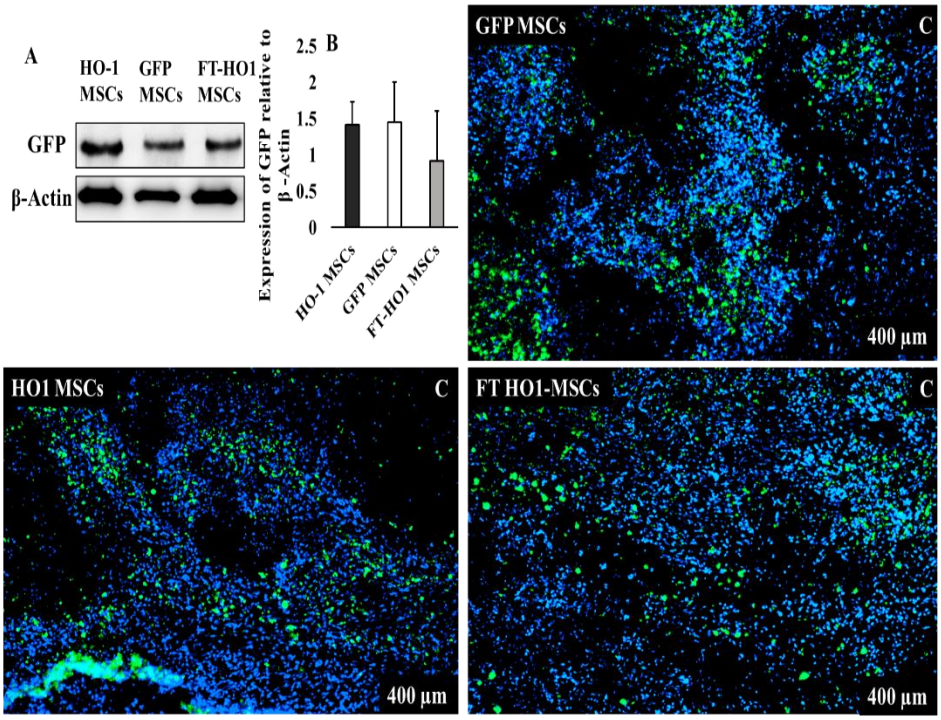


Figure 2.2. Migration of injected cells at the injured spinal cord segment following 4 weeks of experimental period. A) Representative densities of GFP protein in groups obtained through western blot analysis of tissue lysate. B) Depiction of the quantitative expression of GFP protein relative to β-Actin. Each bar represents the average of 4 samples in a group and error bars represent standard error. C) Immunostaining for the location of transplanted cells at the injured spinal cord segment. The nuclei are stained blue and GFP protein is stained green, demonstrating the migration of transplanted cells. Each figure is obtained with a scale bar of 400 µm.

3. Functional recovery

Recovery in hind limb locomotion was assessed using the 19-point cBBB scoring system. After SCI induction, dogs were closely evaluated for the presence of deep pain and joint movement. Before cells transplantation, the cBBB score of all dogs was zero. The hind quarter was completely paralyzed with no pain perception or movement in the joints. Defecation was normal in all the dogs but they failed to urinate voluntarily.

After cells transplantation, the dogs showed gradual improvement in the motor recovery. The HO-1 MSCs group showed rapid locomotor recovery in 4 weeks, demonstrated by a significantly higher cBBB score compared to the GFP MSCs and FT-HO-1 MSCs groups (Fig 2.3, $^{*}p \leq 0.05$). The improvement in cBBB score observed after 4 weeks was 7.5 ± 1.70, 4.25 ± 1.50, and 5.5 ± 1.29 for HO-1 MSCs, GFP MSCs, and FT-HO-1 MSCs groups, respectively. The HO-1 MSCs group showed extensive movements of all the joints. Two of the dogs could support their body weight, and attempted to stand when feeding or undergoing physical assessment. When they were forced to walk, one dog failed to support his body weight while the other dog could weakly lift his hind limb, with dorsal stepping and incoordination between hind limbs. No dog in either of the other groups could lift their body weight either at rest or during assisted walking. No dog showed recovery of

pain perception. The dogs did not vocalize or react while their phalanges were pinched with artery forceps. Voluntary urination was recovered only in 2 dogs of HO-1 MSCs group, 1 dog of GFP MSCs group and none in the FT-HO-1 MSCs group.

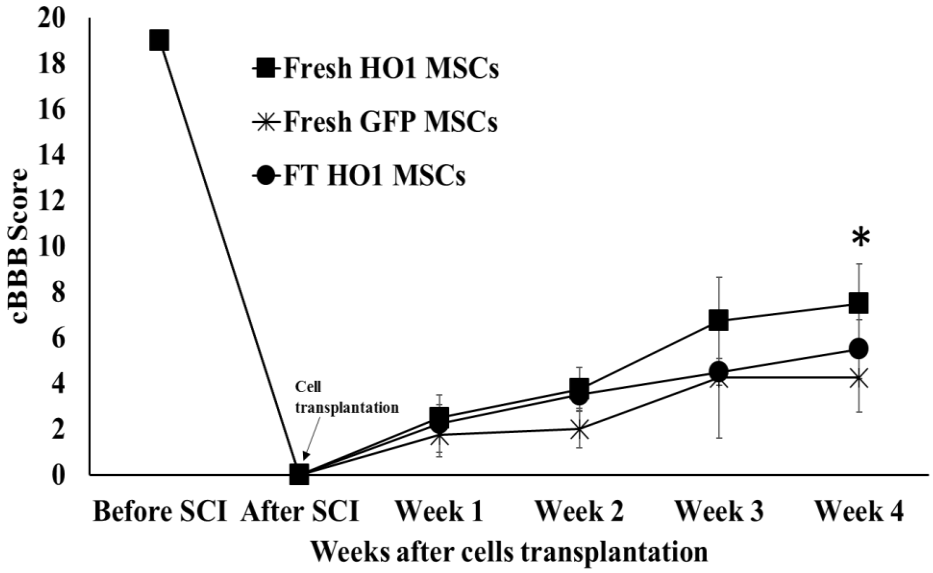


Figure 2.3. Improvement in cBBB score during the 4 weeks following cell transplantation. *Indicates significance at $p \leq 0.05$ among the groups. Each point represents the average of 4 samples and error bars represent standard error.

4. Neural sparing and astrogliosis

The expression of NF-M was significantly higher in HO-1 MSCs group than the GFP MSCs and FT-HO-1 MSCs groups (Fig 2.4, $^{*}p \leq 0.05$). Similarly, the expression of GFAP was significantly reduced in HO-1 MSCs group compared with GFP MSCs and FT-HO-1 MSCs groups (Fig 2.4, $^{*}p \leq 0.05$). The expression of NF-M was similar between the GFP MSCs and FT-HO-1 MSCs groups. However, the expression of GFAP was significantly higher in FT-HO-1 MSCs than the GFP MSCs and HO-1 MSCs groups (Fig 2.4, #$p \leq 0.05$). No statistical significance was detected when comparing the expression of Tuj-1, nestin, GALC and CD11 among the groups (Fig 2.4).

The expression of various neural and glial markers was further confirmed by immunocytochemistry. The expression of these markers followed a similar pattern as what was found with western blot technique. The percent expression of Tuj-1 was similar between all groups, at 16.8% ± 2.14, 10.7% ± 6.14, 13.23% ± 5.40 in the HO-1 MSCs, GFP MSCs and FT-HO-1 MSCs groups, respectively (Fig 2.6). Similarly, the expression of nestin was similar in all groups, at 12.07% ± 0.46, 9.33% ± 2.23, 10.03% ± 2.33, in the HO-1 MSCs, GFP MSCs, and FT-HO-1 MSCs groups, respectively (Fig 2.6). The percent expression of NF-M was higher in the HO-1 MSCs group at 14.50% ± 2.30, compared to the GFP MSCs and FT-HO-1 MSCs groups,

which were 9.33% ± 2.03 and 10.03% ± 2.33, respectively (Fig 2.6, $p \leq 0.05$). Regarding the expression of glial markers, the expression of GFAP was significantly reduced in HO-1 MSCs (10.87% ± 3.30) compared to the GFP MSCs group (14.92% ± 4.14) (Fig 2.6, $p \leq 0.05$). However, the expression was significantly higher in FT-HO-1 MSCs (16.82% ± 2.14) compared to the GFP MSCs group (Fig 2.6, $p \leq 0.05$). The GALC expression was not significantly affected, at 8.67% ± 2.30, 6.24% ± 3.09, 9.29% ± 1.34 for the HO-1 MSCs, GFP MSCs and FT-HO-1 MSCs, respectively (Fig 2.6).

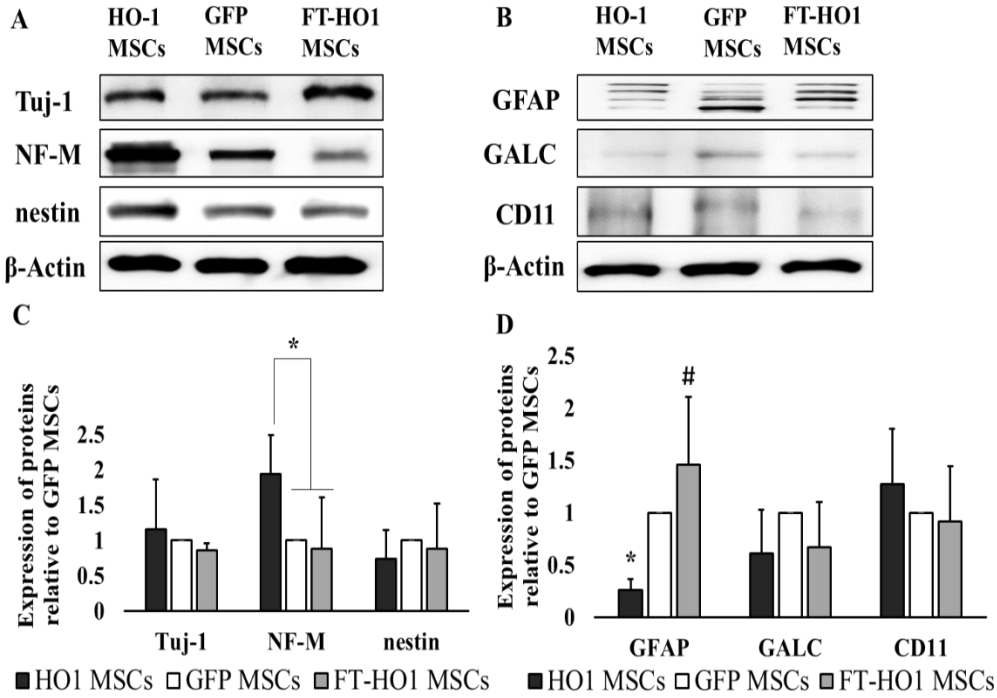
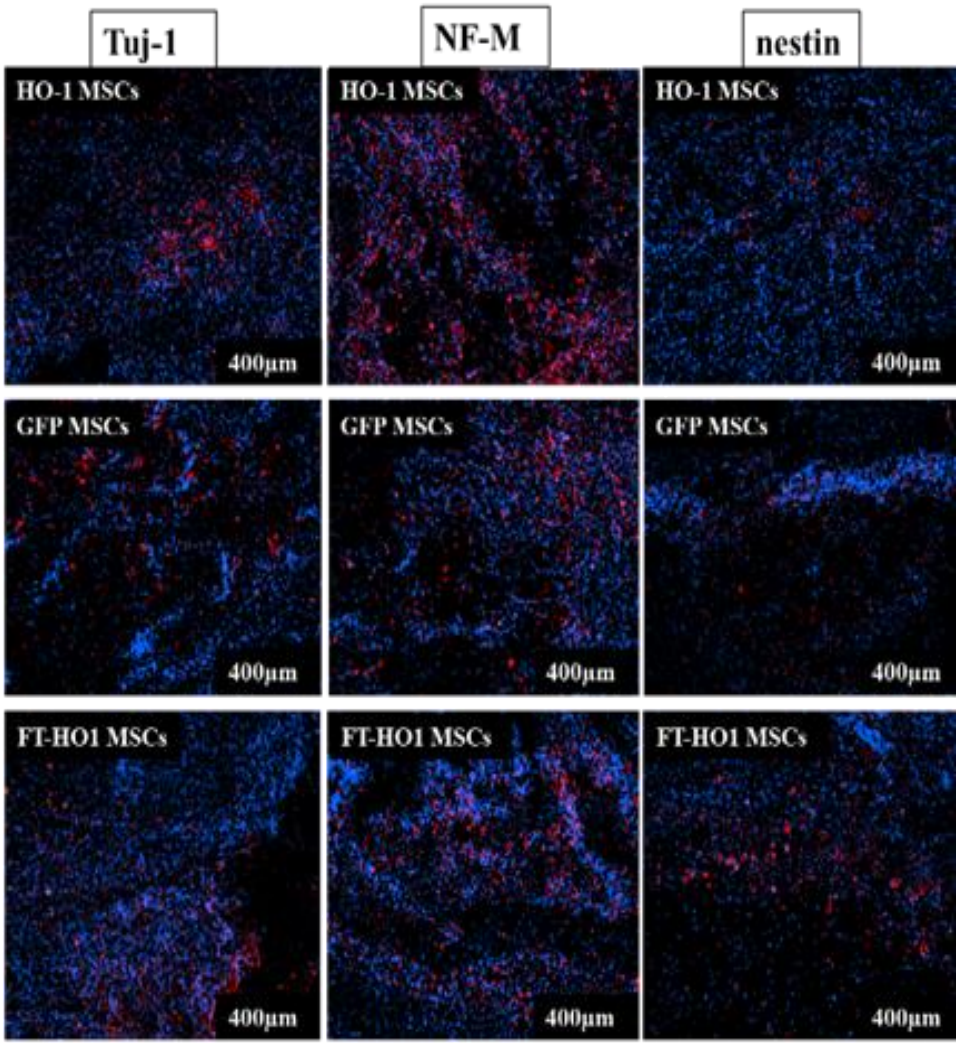


Figure 2.4. Expression of neural and glial cell markers. A) Representative densities of neural markers. B) Representative densities of astroglial cell markers. C, D) Quantitative analysis of densities obtained for neural and astroglial cell markers relative to GFP MSCs. Each bar represents the average of 4 samples in a group and error bars represent standard error. * and # indicate significance at $p \leq 0.05$.


Tuj-1
NF-M
nestin
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400μm

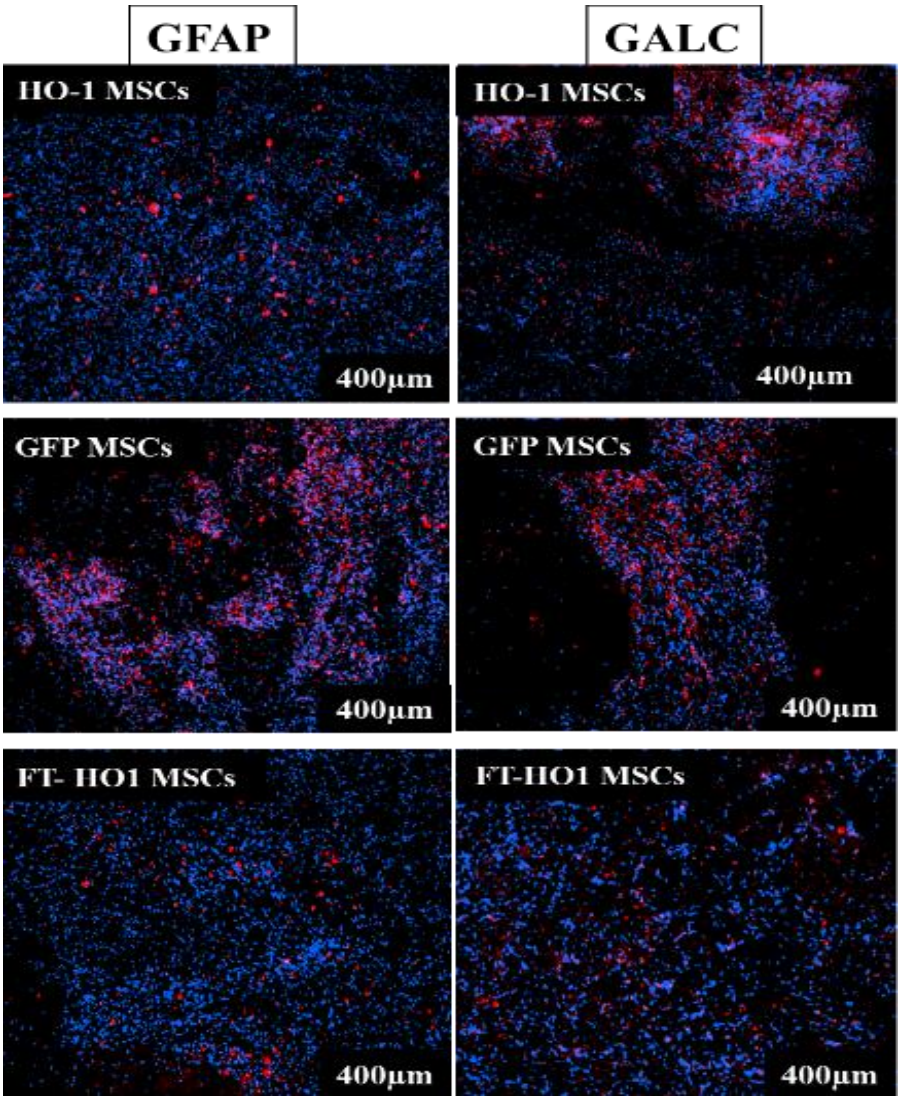


Figure 2.6. Immunofluorescent staining for the expression of neural (Tuj-1, NF-M, nestin) and glial (GFAP, GALC) cells. Sections were stained with Tuj-1, NF-M, nestin, GFAP, and GALC in red, and nuclei were stained with DAPI in blue. Each image represents 4 samples per group with a scale bar of 400 μm

5. Immune modulation

The fresh HO-1 MSCs group showed a robust immune suppression at the injured segment of the spinal cord after migration. The expression of TNF-α was significantly lower in the HO-1 MSCs and FT-HO-1 MSCs groups (Fig 2.5, $^{*}p \leq 0.05$) than the GFP MSCs group. The expressions of IL-6 and IL-1β were significantly lower in the HO-1 MSCs group compared to the GFP MSCs and FT-HO-1 MSCs groups (Fig 2.5, $^{*}p \leq 0.05$); however, expression was not significantly different between the GFP MSCs and FT-HO-1 MSCs groups.

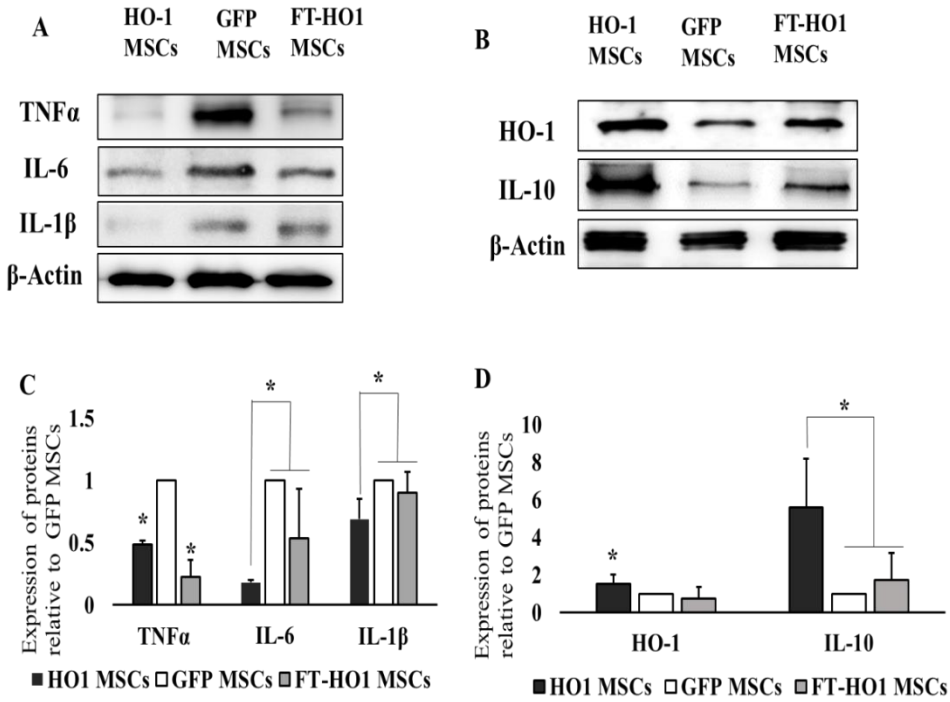


Figure 2.5. Expression of inflammatory and anti-inflammatory cytokines. A) Representative densities of markers for inflammatory cytokines. B) Representative densities of markers for anti-inflammatory cytokines. C, D) Quantitative analysis of densities obtained for markers related to inflammatory and anti-inflammatory cytokines. Each bar represents the average of 4 samples per group and error bars represent standard error. * indicates significance at $p \leq 0.05$.

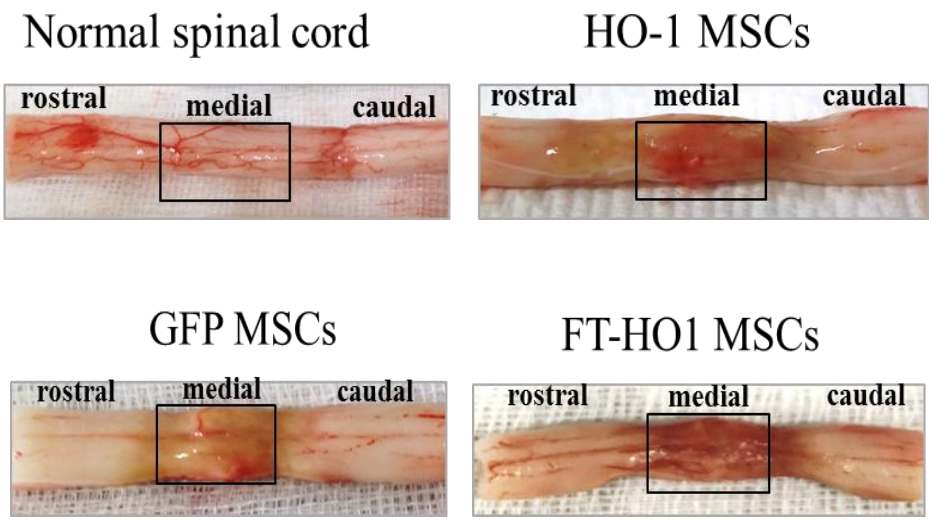
6. Fibrosis and demyelination

Upon gross evaluation, the injured area of the spinal cord showed hemorrhages, fibrosis, and atrophy (Fig 2.7A). The extent of the gross lesion area of the injured spinal cord was not significantly different among the groups (Fig 2.7D1). The gross lesion areas quantified were 37.29% ± 6.84, 42.36% ± 10.80 and 44.50% ± 10.70 for HO-1 MSCs, GFP MSCs and FT-HO-1 MSCs, respectively.

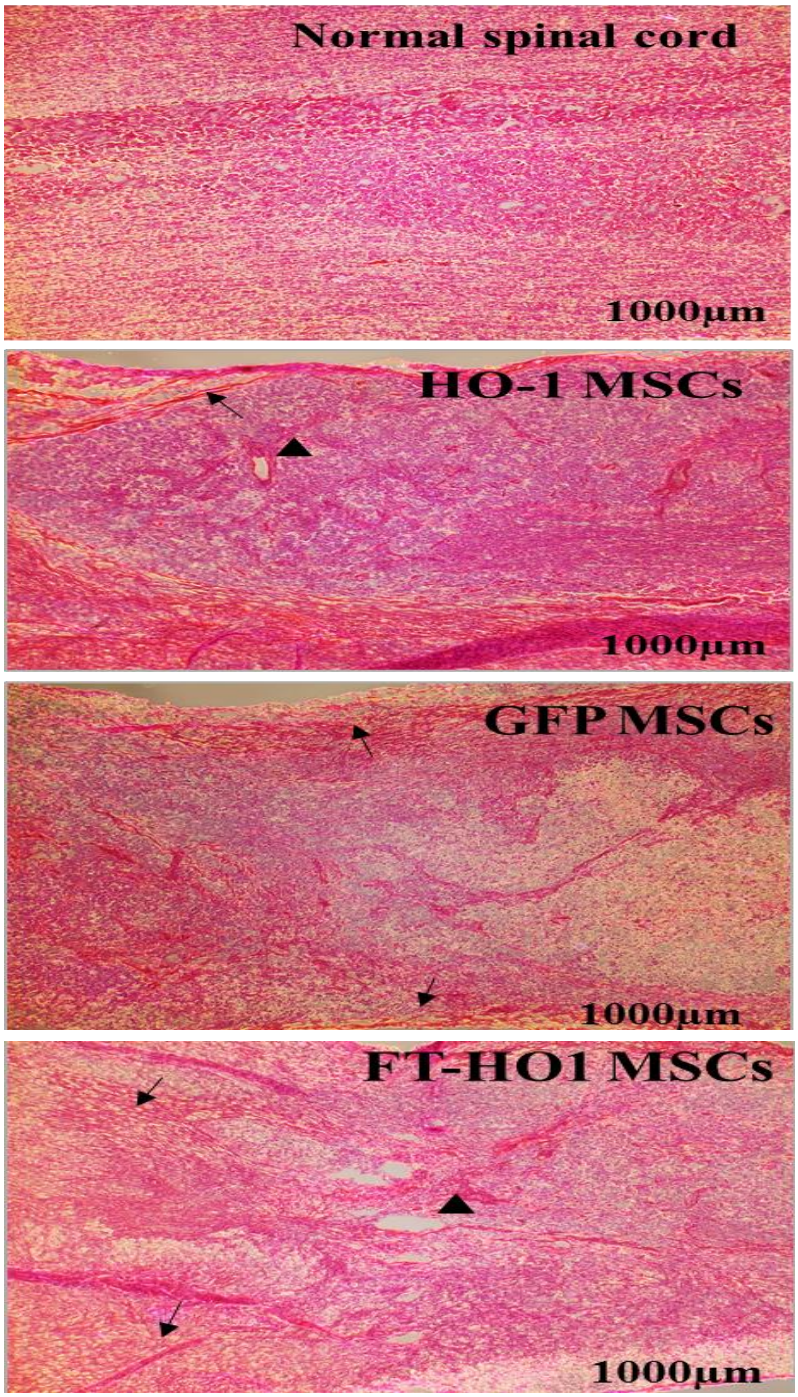
As detected by H & E staining, fibrosis was significantly reduced in the HO-1 MSCs group compared with GFP MSCs and FT-HO-1 MSCs groups (Fig 2.7B, D2) ($^{*}p \leq 0.05$). The degree of fibrosis was 20.20% ± 5.0, 41.80% ± 10.04 and 46.21% ± 5.51 for HO-1 MSCs, GFP MSCs and FT-HO-1 MSCs groups, respectively. Fibrosis was mainly confined at the epicenter of the injured area (Fig 2.7B arrow). The gray matter was well preserved in the HO-1 MSCs group. Vacuoles were observed in all the groups; however, they were more numerous in the FT-HO-1 MSCs group (Fig 2.7 arrowheads).

As detected by luxol fast blue staining, the injured areas of the spinal cords of the HO-1 MSCs group were more deeply stained compared to the GFP MSCs and FT-HO-1 MSCs groups (Fig 2.7C, D3) ($^{*}p \leq 0.05$).

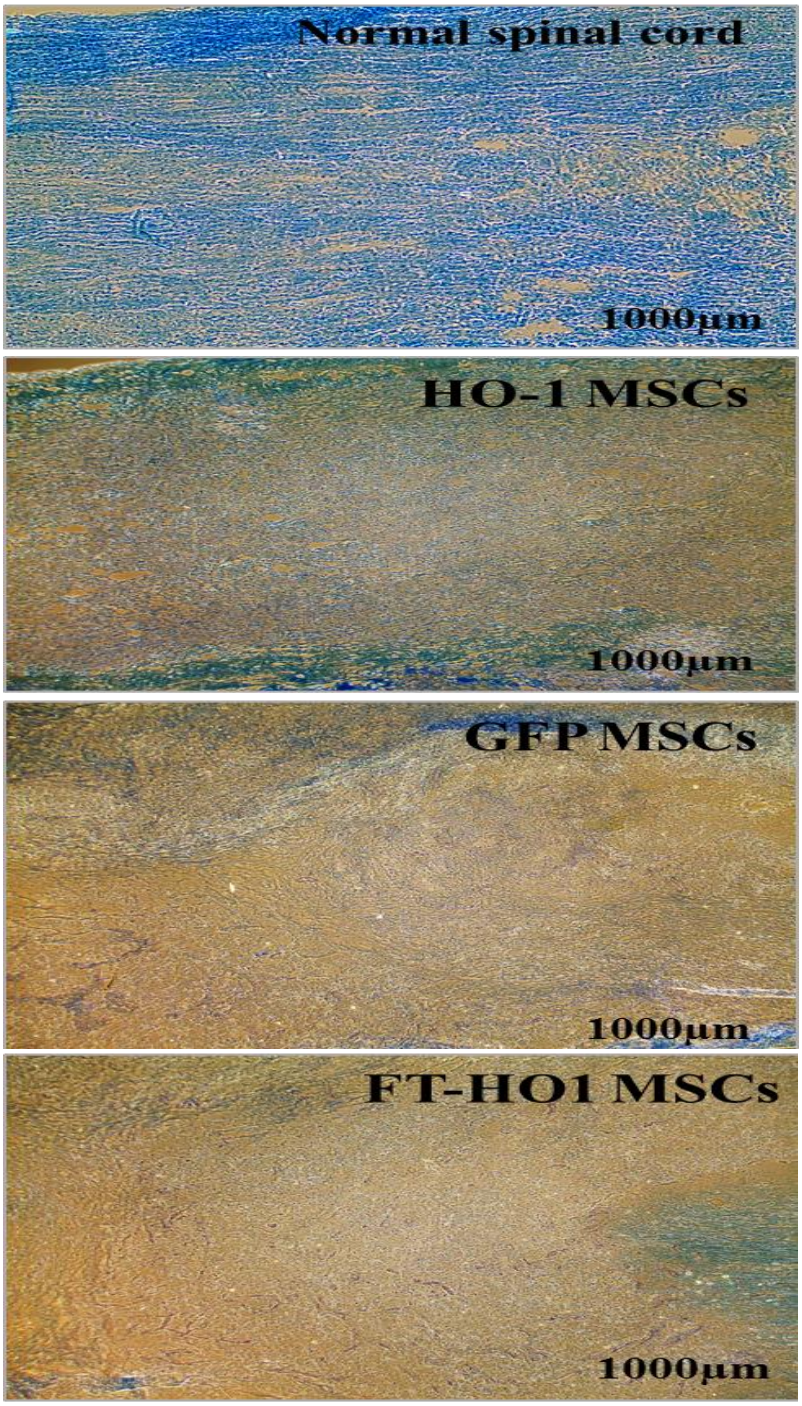
A


Normal spinal cord
rostral
medial
caudal
HO-1 MSCs
rostral
medial
caudal
GFP MSCs
rostral
medial
caudal
FT-HO1 MSCs
rostral
medial
caudal

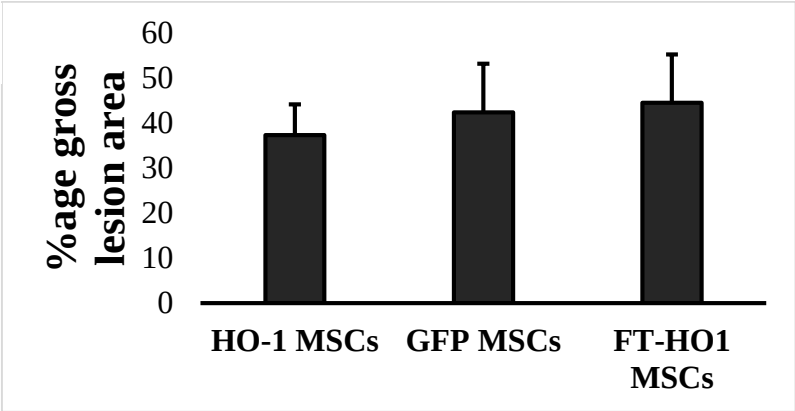
B


Normal spinal cord
1000μm
HO-1 MSCs
1000μm
GFP MSCs
1000μm
FT-HO1 MSCs
1000μm

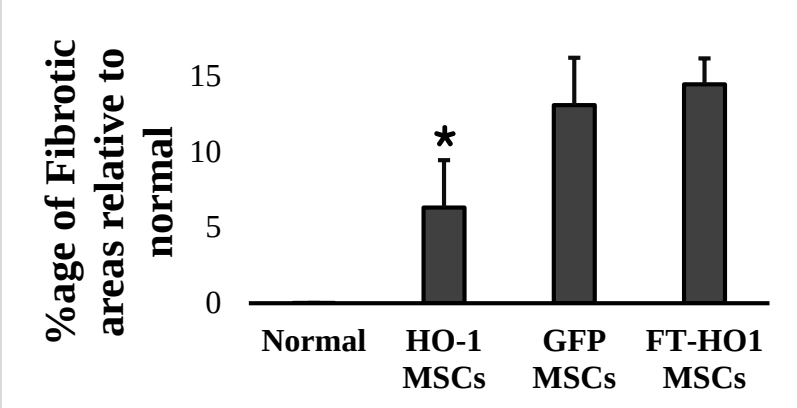
C


Normal spinal cord
1000μm
HO-1 MSCs
1000μm
GFP MSCs
1000μm
FT-HO1 MSCs
1000μm

D1


%age gross lesion area
60
50
40
30
20
10
0
HO-1 MSCs
GFP MSCs
FT-HO1 MSCs

D2


%age of Fibrotic areas relative to normal
15
10
5
0
*
Normal
HO-1 MSCs
GFP MSCs
FT-HO1 MSCs

D3

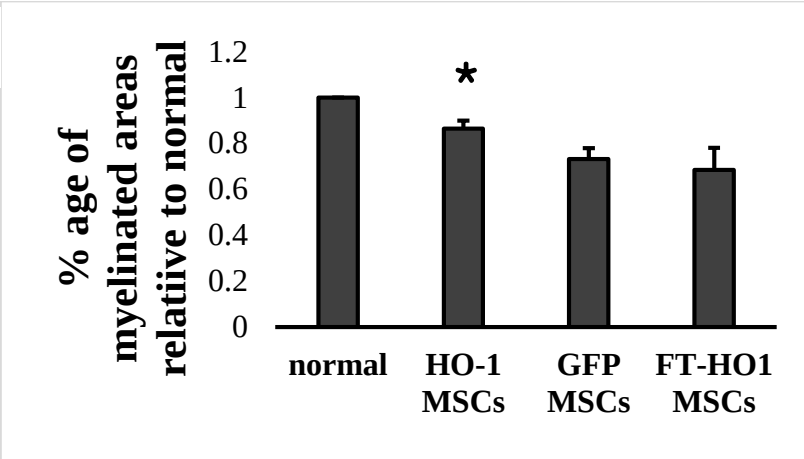

% age of myelinated areas relatiive to normal
1.2
1
0.8
0.6
0.4
0.2
0
*
normal
HO-1 MSCs
GFP MSCs
FT-HO1 MSCs

Figure 2.7. Histopathology of injured spinal cords stained with H & E and luxol fast blue. A) Injured spinal cord segments with fibrosis, hypotrophy, and hemorrhages. B) Representative H & E staining of injured spinal cord segments. The fibrotic area is observed as a red & pink color, while the healthy area is observed as purple. C) Representative staining of myelin with luxol fast blue. Positive staining is depicted by the intensity of blue color. The images were obtained at low magnification 40X with a scale bar of 1000 μm. D1) % expression of gross lesion area. D2) % expression of fibrosis relative to normal. D3) % expression of the myelinated area relative to normal. Each bar represents the average of 4 samples per group. * represents the significance of HO-1 MSCs with the GFP MSCs and FT-HO-1 MSCs groups at $p \leq 0.05$. Arrows denote fibrotic areas; arrowheads denote vacuoles.

DISCUSSION

It has been previously found that IV delivered Ad-MSCs improved healing in dogs with acute SCI, compared with non-treated controls (Y. Kim et al. 2015). Similarly, dogs with chronic SCI (S. H. Lee et al. 2015) and subacute SCI (Lim et al. 2007; Ryu et al. 2009) showed significant improvement in healing after Ad-MSCs transplantation compared to non-treated SCI dogs. There are multiple studies in rabbits as well as rats which demonstrate the efficacy of stem cells in the healing of spinal cord injury, through immune modulation, differentiation, and regeneration (Morita et al. 2016; Shi et al. 2007; Urdzíková et al. 2006). As the efficacy of MSCs has been established in different animals, we did not include a negative control (non-treated) group in our study for ethical reasons, and considered the GFP MSCs group as our control.

In the present study, spinal cord injury was induced through a balloon compression method, chosen based on the simplicity, severity (covering approximately 80% of the space in the spinal canal), and resulting severe compression spinal cord injury (Lim et al. 2007; Park et al. 2012; Ryu et al. 2009). This technique is clinically significant as it closely mimics SCI caused by naturally occurring intervertebral disc herniation (IVDH) in dogs (Alisauskaite et al. 2017).

HO-1 is a stress-induced enzyme which mainly functions to control homeostasis during disease. The anti-oxidant and anti-inflammatory effects of HO-1 in the treatment of various disease processes have been well documented (Konrad et al. 2016; Ryter and Choi 2016). Therefore, HO-1 expressing stem cells were produced to deliver increased levels of HO-1 at the injury site. We previously found that HO-1 MSCs have higher anti-oxidant capacity; however, their proliferation rate was lower than that of Ad-MSCs. They exhibit a broader spindle shape morphology than Ad-MSCs (Mijung et al. 2016). We found similar physiological characteristics in HO-1 MSCs as those described previously.

It has been previously reported that in dogs with acute SCI, Ad-MSCs migrate to the injured spinal cord segment, lungs, and spleen following IV administration (Y. Kim et al. 2015). Similarly, in mice, they migrated into injured spinal cord, lungs, spleen, and liver after early IV administration (Badner et al. 2016). In the present study, we found that HO-1 MSCS also migrated to injured spinal cord segments and in vivo produced increased levels of HO-1. The localization and degree of migration of cells were not significantly different among the HO-1 MSCs, GFP MSCs, and FT-HO-1 MSCs groups. The GFP expression was 4-12% 4 weeks after cells transplantation, which is higher than previously reported expressions of 0.002% or 0.029% in mice after 1 week of cell transplantation (Badner et al. 2016; Ohta et al. 2017). In various reports using murine models, no cells were

observed 6 weeks after IV transplantation (Takahashi et al. 2011) and 4 weeks after transplantation through subarachnoid space (Satake et al. 2004). This is likely because we transplanted a higher number of cells repeatedly for 3 consecutive days. Another reason may be species differences. It can be assumed that repeated transplantation of cells would increase migration of cells at the injured site, and would promote anti-oxidant and anti-inflammatory processes.

It has been previously shown that acute SCI dogs injected IV with Ad-MSCs showed more improvement in hindlimb locomotion after one week than controls (Y. Kim et al. 2015). Similarly, in dogs with subacute SCI, intra-parenchymal transplantation of HO-1 MSCs significantly improved hindlimb function 7 weeks after transplantation compared to Ad-MSCs injected dogs (S. H. Lee et al. 2016a). In the present study, we found more rapid recovery in 4 weeks than the previously reported 7 weeks of cell transplantation. This suggests that IV transplantation of cells in the acute stage (i.e.; immediately after SCI) could be more effective in controlling the aggression of secondary SCI, and that HO-1 MSCs perform better than MSCs.

Vascular disruption due to SCI causes neuronal ischemia which causes mitochondria to produce an excessive amount of reactive oxygen species (ROS). Increased leukocyte infiltration and intracellular accumulation of calcium further increases the production of ROS. ROS have a significant

deleterious role in secondary SCI (Jia et al. 2012). They cause lipid peroxidation of the cell membrane, which further produces neurotoxic compounds such as 4-hydroxynenonal (4HNE). Meanwhile, they cause degradation of proteins (lysine, arginine, histidine) to form protein carbonyl moieties and nitrotyrosine compounds (3NT)(Hall 2011). A concomitant infiltration of leukocytes causes increased production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, etc., which induce neuronal apoptosis by activating caspase pathways(Rust and Kaiser 2017). Hence, it is crucial to measure oxidation and inflammation during the early stages of the disease in order to promote neuronal sparing. Ad-MSCs have both anti-oxidant and anti-inflammatory functions, with improved neural sparing after early IV administration in dogs with acute SCI(Y. Kim et al. 2015). In various clinical disorders, transplantation of HO-1 MSCs showed more anti-inflammatory(N. Liu et al. 2015a; Zeng et al. 2008) and anti-oxidant effects (Hamedi-Asl et al. 2012) than MSCs. I found that the HO-1 MSCs group showed more immunosuppression (i.e.; reduced expression of TNF-α, IL6 and IL-1β), and more neuronal sparing. Considering the anti-oxidant effects of HO-1, an early anti-oxidant effect in the HO-1 MSCs group can also be assumed. MY results corroborate previous studies of dogs with sub-acute SCI, in which HO-1 MSCs were injected directly into the spinal cord parenchyma(S. H. Lee et al. 2017). Thus, HO-1 MSCs have a similar function after IV transplantation in acute SCI dogs as that observed following intra-parenchymal injection;

however, the IV route has additional advantages over an intra-thecal injection, as it is simple, rapid and avoids intra-spinal hemorrhages.

In rats with SCI, human umbilical cord MSCs reside in the spleen and upregulate the expression of IL-10 in blood plasma. This systemic immunosuppression has neuroprotective and vasoprotective effects(Badner et al. 2016). We found that IV administration of HO-1 MSCs in dogs with SCI upregulated the local expression of IL-10 and HO-1 more than GFP MSCs. However, the impact of HO-1 MSCs upon systemic immune modulation requires further detailed investigation.

In one group in I injected frozen thawed HO-1 MSCs, because the cryopreservation of stem cells allows instant use when required, controlling the progress of the disease during secondary spinal cord injury. HO-1 MSCs have a higher anti-oxidant capacity; however, the viability of FT-HO-1 MSCs was lower than that of fresh HO-1 MSCs(Mijung et al. 2016). In the present study, we found that the FT-HO-1 MSCs successfully migrated to injured spinal cord segments, although they were not as physiologically active as HO-1 MSCs despite a higher number being transplanted. This indicates that freeze-thawing procedures negatively influenced the biodistribution and homing properties of transplanted cells in vivo (Chinnadurai et al. 2014). Moreover, FT-HO-1 MSCs did not produce sufficient amounts of anti-inflammatory cytokines, and did not efficiently suppress the expression of

proinflammatory cytokines as was observed with fresh HO-1 MSCs. This may indicate deleterious effects of freeze-thawing procedures upon the expression of certain genes (Hoogduijn et al. 2016); however, we previously found in vitro that gene expression of HO-1 was not significantly affected, and that FT-HO-1 MSCs produced higher amounts of HO-1 as did by fresh HO-1 MSCs, although their anti-oxidant capacity was reduced (Mijung et al. 2016). In the present study, the poor expression of HO-1 by FT-HO-1 MSCs in vivo might be due to a higher fragility of FT-HO-1 MSCs, reducing their ability to accommodate to the microenvironment where they migrated and further reducing their viability and gene expression (Hoogduijn et al. 2016). Plenty of data describes how cryopreservation and thawing negatively affects the physiological properties of MSCs. With respect to genetically modified MSCs, a detailed comparative study comparing wild-type and transgenic stem cells in vitro, as well as in vivo, is needed to establish methodologies to produce stem cells resistant to freeze-thawing procedures and unfavorable microenvironments of the target organs.

The present study evaluated the IV use of HO-1 MSCs as an emergency treatment for acute SCI in dogs. I found that fresh HO-1 MSCs were more efficacious in promoting functional recovery in dogs with acute SCI by reducing astrocyte infiltration and pro-inflammatory cytokines, and hence can be used as a viable alternative to Ad-MSCs and MPSS. The frozen-thawed HO-1 MSCs functioned in the same manner as did fresh MSCs and

could act as a readily available tool for the emergency treatment of acute SCI, because the freshly cultured cells are not instantly available to deliver immediately to dogs after SCI.

CONCLUSION

This study was performed to investigate the healing effects of genetically modified MSCs such as HO-1 MSC and BDNF MSCs after their co-transplantation and transplantation through minimal invasive procedure in dogs with subacute and acute SCI respectively. The conclusions are as follows:

1. Lentiviral-mediated transfection of HO-1 and BDNF genes into Ad-MSCs allows stable production of HO-1 and BDNF by Ad-MSCs *in vivo* as well as *in vitro*.

2. The cotransplantation of HO-1 and BDNF MSCs selectively inhibits the expression of pro-inflammatory cytokines which favors BDNF induced neuroregeneration and neural sparing more profoundly in dogs with sub-acute SCI than using HO-1 MSCs and BDNF MSCs alone.

3. HO-1 MSCs and FT-HO1 MSCs can migrate to an injured spinal cord after their IV transplantation in dogs with acute SCI.
4. There is an increase viability and migration of cells at the injured spinal cord segment if administered in a higher dose and multiple time through IV route.

5. Early IV transplantation of HO-1 MSCs is more helpful in controlling the expression of inflammatory cytokines and promoting the neural sparing and functional recovery in dogs with acute SCI.

6. FT-HO1 MSCs bear the same function in vivo as performed by freshly cultured MSCs and hence could act as a good alternative to freshly cultured MSCs during the critical care of SCI patient.

Overall, cotransplantation of HO-1 MSCs and BDNF MSCs, and early IV transplantation of HO-1 MSCs have more profound healing effects in dogs with SCI. FT-HO1 MSCs could be used in the emergency treatment of SCI with favorable outcomes as an alternative to freshly cultured MSCs.

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국문초록

개 척수 손상에 대한 형질 전환 개 지방유래 중간엽줄기세포의 치료 효과

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일차적 척수손상 (SCI)은 신경과 혈관에 물리적 손상을 일으킨다. 이것은 다수의 병적인 기전을 활성화시켜 2차적 SCI로 이어지는데, 이 과정에서 산화와 염증은 신경을 괴사시키는 중요한 요인들이다.

척수 손상의 초기 단계에 발생하는 미토콘드리아 대사 결핍과 세포막의 지질 과산화를 유발하는 다량의 식세포 (대식세포, 호중구) 이동은 결국 세포 사멸을 일으키고 반응성 산소 종 (ROS)을 과다하게 발현시킨다.

다량의 호중구, 대식세포 및 림프구의 이동은 신경 세포 사멸을 활성화시키는 TNF-α, IL-6, IL-1β, MMPs, SOD 및 myeloperoxidases와 같은 전염증성 사이토카인을 생성하므로, 상주세포는 염증과 산화 손상을 조절하기 위하여 heme oxygenase-1 (HO-1)과 뇌유래신경촉진인자 (BDNF)를 유도하여 결과적으로 신경재생을 촉진시키게 된다. 따라서 많은 양의 외인성 HO-1 및 BDNF를 공급한다면 최적의 치유 효과를 기대할 수 있을 것이라 추측할 수 있다.

첫 번째 장에서는 SCI 시 발생하는 염증 및 산화적 손상의 억제와 신경 재생을 척수손상의 치료 목표로 삼았다. 실험 견으로 16 마리의 비글견을 사용했으며, 각 군당 4 마리씩, 총 4 군으로 나누었다. 모든 실험 견에 아급성 척수 손상을 유발했으며, 유발한 지 1 주일 후에 첫 번째 군은 HO-1 MSC, 두 번째 군은 BDNF MSC, 세 번째 군은 HO-1 MSC + BDNF MSC (병용 투여 군), 네 번째 군은 GFP MSC 를 각각 척수 실질에 직접 주입하였다. 8 주에 걸친 후지기능 회복을 조사하였으며 8 주차에 척수 실질을 채취하여 면역세포 및 병리조직학적 검사를 하였다. 병용 투여 군이 기능 회복에 있어 유의미한 개선을 보였다. 병용 투여 군에서는 TNF-α, IL-6 및 COX-2 와 같은 전염증성 사이토카인의 발현 감소 및 IL-10, HO-1 과 같은 항염증성 사이토카인의 발현이 증가하였으며 이로 인한

신경 재생 / 신경 보존의 향상을 나타내는 신경 인자 및 성장 관련 단백질 (GAP-43)의 발현이 다른 군에 비해 증가하였다. 조직병리학적 검사에서 병용 투여 군의 손상된 척수 분절에서는 척수내 섬유증이 다른 군보다 적게 나타났다. 면역세포화학검사에서 병용 투여 군의 신경표지자 (NFM, Tuj-1)는 다른 군들에 비해 유의적으로 높음이 확인되었고, 신경교표지자 (GFAP, GALC)는 BDNF MSC 군과 유사하게 나타났으며 다른 군들에 비해 유의적으로 높게 확인되었다. 따라서 항산화, 항염증 및 신경 재생을 목표로 한 HO-1 MSC 와 BDNF MSC 의 병용투여는 척수 손상의 회복을 촉진하는 가장 좋은 방법으로 사료된다.

두 번째 장에서는 급성 SCI 에 대한 HO-1 MSC 의 정맥주사 효과를 검토하였다. 신선세포와 동결해동세포 간 기능회복능력을 비교하였다. 12 마리의 비글 견을 총 3 군으로 나누었으며, 각 군마다 4 마리씩 할당하였다. 모든 실험 견은 SCI 를 유발하였으며, 유발 직후 첫 번째 군은 HO-1 MSC 신선세포 1 x 10^7, 두 번째 군은 GFP MSC 신선세포 1 x 10^7, 세 번째 군은 동결해동 HO-1 MSC (FT-HO-1 MSC) 1.5 x 10^7 개를 3 일 연속 1 일 1 회 정맥주사 하였다. 척수손상 유발 4 주차에 안락사하여 척수를 채취하였다. 주입세포들은 모든 군의 손상된 척수 분절에서 확인할 수 있었지만, 군 간에는 차이가

없었다. HO-1 MSC 신선세포는 HO-1 과 IL-10 의 발현이 증가하였으며, 염증성 사이토 카인 (TNF-α, IL-6, IL-1b)의 발현을 억제하였다. HO-1 MSC 군에서 NF-M 의 발현은 유의적으로 증가하였으며 GFAP 의 발현은 현저하게 감소하였다. HO-1 MSC 군은 GFP MSC 및 FT-HO-1 MSC 군과 비교하여 척수 내 섬유화 및 신경수초의 분해가 적게 나타났다. NF-M, IL-6, IL-1β 의 발현은 FT-HO-1 MSC 및 GFP MSC 군에서 유사하였다. 섬유화 및 탈수초의 정도 또한 비슷하였다. 면역세포화학 검사에서 HO-1 MSC 군에서 NF-M 이 유의적으로 증가하였다. FT-HO-1 군의 GFAP 는 GFP MSC 신선 세포군 보다 유의적으로 증가하였다.

손상부로 이동된 MSC 는 HO-1 MSC 와 GFP MSC 군사이에 차이가 없었지만 HO-1 MSC 군에서 항산화 및 항염증 인자인 HO-1 을 더 많이 생산하였다. 이로 인해 HO-1 MSCs 군에서 신경 세포의 사멸이 감소되고 신경세포가 더 많이 보존된 것으로 추측된다. GFP MSC 신선세포 군과 더 많은 세포를 주입한 FT-HO-1 MSC 군 사이에 HO-1 농도의 차이는 없었다. 동결 - 해동 절차가 HO-1 MSC 의 생존성 및 유전자 발현에 나쁜 영향을 주었지만 후지기능 회복에 대해 GFP MSC 신선세포 군과 FT-HO-1 MSC 군이 유사한 결과를

보였으므로, FT-HO-1 MSC 는 MSC 신선세포 대신 척수손상의 응급 처치로 이용할 가능성을 제시할 수 있다고 사료된다.

주요어: 중간엽 줄기세포, 염증, 신경 재생, 개, 척수손상
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