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Doctoral Thesis of Philosophy

**Therapeutic effects and its mechanism of
lithium in spinal cord injury**

Department of Veterinary Medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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Feb, 2018

Therapeutic effects and its mechanism of lithium in spinal cord injury

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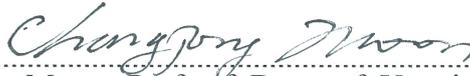
A thesis submitted in partial fulfillment of the requirement for the degree of Doctor
of Veterinary Medicine

2017. 12.

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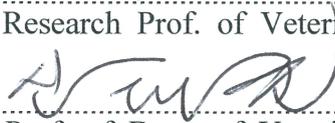
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List of Abbreviations

GSK-3 β	Glycogen synthase kinase-3 β
SCI	Spinal cord injury
Nrf-2	Nuclear factor erythroid 2-related factor 2
HO-1	Heme oxygenase-1
DPI	Day post-injury
Iba-1	Ionized calcium-binding protein-1
MASCIS	Multicenter animal spinal cord injury study
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
EAE	Experimental autoimmune encephalomyelitis
MOG	Myelin oligodendrocyte glycoprotein
NIH	National institutes of health
PBS	Phosphate-buffered saline
BBB	Basso, Beattie, and Bresnahan
SDS	Sodium dodecyl sulfate
SLS	Sodium lauryl sulfate
H&E	Hematoxylin and eosin
CNS	Central nervous system

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General Introduction

Spinal cord anatomy

The spinal cord is connected to the brain. It is the part of the central nervous system (CNS) located within the vertebral column, and it includes both sensory and motor nerves. It is also the main pathway for information between the brain and peripheral nervous system (Squire *et al.*, 2013). It carries sensory information from the body and part of the head to the CNS via afferent fibers, and performs the initial processing of this information (Figure 1).

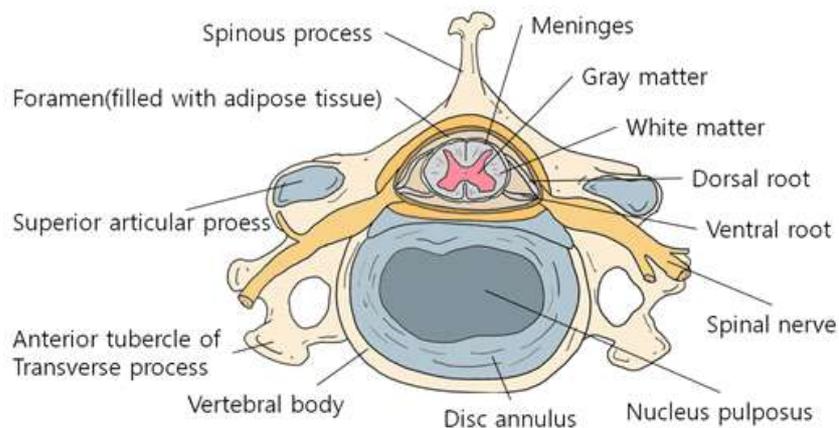


Figure 1. Schematic illustrations of cross section of spinal cord in the vertebral column (Evans. 1993).

Axons of the motor neurons in the ventral horn project to the periphery to innervate skeletal and smooth muscles and mediate voluntary and involuntary reflexes. The spinal cord contains neurons whose descending axons mediate autonomic control of most visceral functions. The spinal cord is composed of central gray matter and peripheral whiter matter (Figure 2). Two systems are responsible for the transmission of motor function: the upper and lower motor neuron systems (Olby *et al.*, 1999).

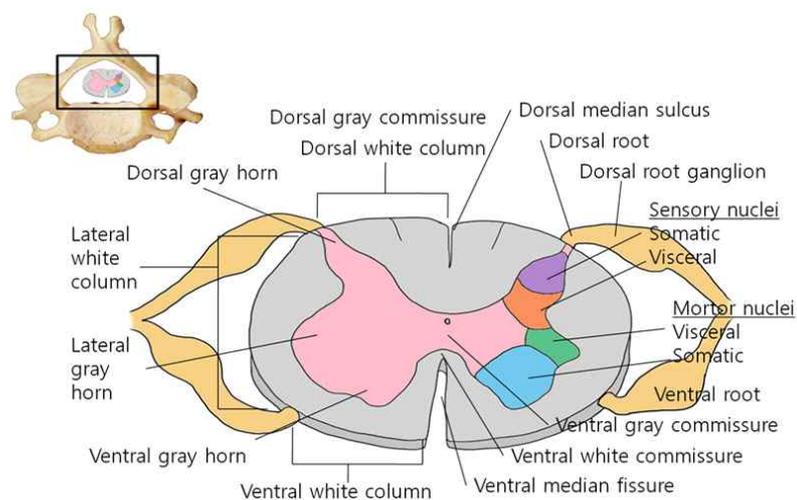


Figure 2. Schematic illustrations of functional lesions of spinal cord (Evans, 1993).

Classification of spinal cord injury

The spinal cord can be damaged by various primary injuries, including concussion (Vitale *et al.*, 2007), shear (Griffin *et al.*, 2009), laceration (Olby, 1999), intervertebral disk herniation (Griffin *et al.*, 2009), vertebral injuries such as fractures, dislocations, and subluxations (Jeffery *et al.*, 2010), penetrating injuries including gunshot and stab wounds (Dumont *et al.*, 2001), and non-traumatic injuries including embolism (Abrhamson *et al.*, 2002). Intervertebral disk herniation is classified into Hansen types I (disk extrusion), II (disk protrusion), and III (nucleus pulposus extrusion) (Griffin *et al.*, 2009, de Lahunta *et al.*, 2009). Table 1 groups the various types of SCI.

Table 1. Classification of spinal cord injury.

Classification	
Primary Injury	Concussion, Compression (Vitale <i>et al.</i> 2007)
	Shear (Griffin <i>et al.</i> , 2009), Laceration (Olby. 1999)
Intervertebral Disk Herniation	Contusion (Edward <i>et al.</i> , 2012)
	Hansen Type I -disk extrusion, Hansen Type II -disk protrusion (Griffin <i>et al.</i> , 2009)
	Hansen Type III -nucleus pulposus extrusion (De Lahunta <i>et al.</i> , 2009)
Vertebral Injuries	Fractures, Dislocations, Subluxations (Jeffery <i>et al.</i> , 2010)
Penetrating Injuries	Gunshot, Stab wounds (Dumont <i>et al.</i> , 2001)
Non-traumatic Injuries	Fibrocartilage emoli (Abrhamson <i>et al.</i> , 2002)

Irrespective of the primary injury type, secondary degeneration follows, leading to severe damage (Figure 3) (Tator *et al.*, 1991; Olby, 1999, 2010; Dumont *et al.*, 2001). The neuropathological outcome of SCI is characterized by edema, axonal degeneration, inflammatory cell infiltration, fibronectin exudation through the damaged blood–brain barrier, microglia activation, and reactive astrogliosis (Prewitt *et al.*, 1997; Jung *et al.*, 2003; Kim *et al.*, 2003; Shin, 2007; Song *et al.*, 2009).

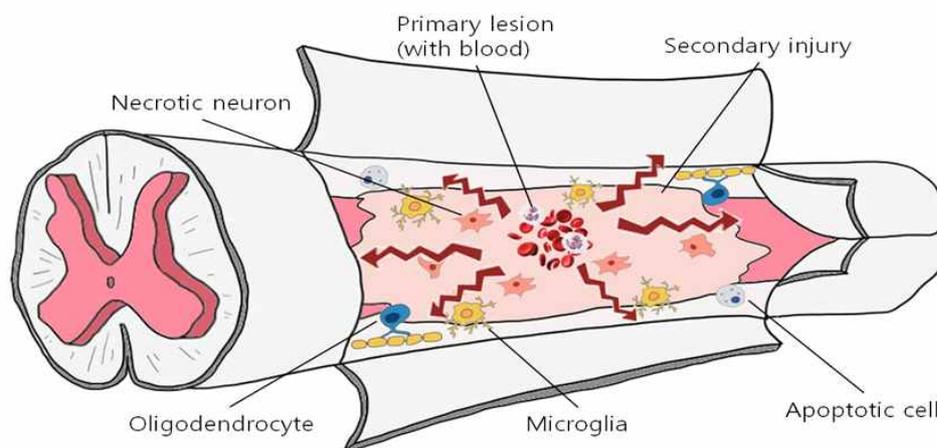


Figure 3. Schematic illustrations of both mechanical and inflammatory response-induced damage of spinal cord (Beattie, 2004).

Animal models of spinal cord injury

Many animal models have been developed to study the pathogenesis of SCI. Models based on mechanical impact include the New York University (NYU) impact device injury, Ohio State University (OSU) impact device injury, and clip-compression injury models (Kwon *et al.*, 2002; Shin *et al.*, 2013). Compression models include balloon compression (Tarlov, 1953) and lamina compression (Sun *et al.*, 2017) models. Transection and partial transection have also been used to study SCI (Basso *et al.*, 1996; Zeman *et al.*, 1997). Basso *et al.* (1996) used a rat model of SCI in a preliminary study for the application of candidate therapeutic agents in dogs with SCI to study their therapeutic effects and mechanisms. Table 2 summarizes the animal models of spinal cord injury.

Table 2. Animal models of spinal cord injury.

Models	Methods
Contusion models	Weight drop model (Basso <i>et al.</i> , 1995) Weight drop impact sys.(Kim <i>et al.</i> , 2017)
Compression models	Clip compression (Rivin <i>et al.</i> , 1978) Balloon compression (Tarlov. 1953) Lamina to compress (Sun <i>et al.</i> , 2017)
Section models	Transection (Basso <i>et al.</i> , 1996) Partial transection (Zeman <i>et al.</i> , 1997)

Occurrence of spinal cord injury in dogs and cats

In veterinary medicine, the incidence of spinal cord injury in dogs and cats is increasing, and emergency care is necessary for such injured animals. Because patients suffer from secondary insults after SCI, drugs are used to reduce neuronal cell death and glial activation. Opiate receptor antagonists and thyrotrophin-releasing hormones have been used to improve spinal cord blood flow (Faden *et al.*, 1981, Pitts *et al.*, 1989). The neuroprotective effects of minocycline (Cho *et al.*, 2006) and anti-oxidant therapies (Hall, 1987; Gul *et al.*, 2005; Kaplan, 2005; Baltzer *et al.*, 2008) are being studied. Olfactory glial cells have been transplanted into the spinal cord (Jeffery *et al.*, 2005). Table 3 summarizes SCI cases in dogs and cats and shows the clinical care and therapeutic agents used. In general, the therapeutic strategy aims to minimize secondary degeneration following SCI.

With the increase in SCI in dogs, case studies and therapeutic trials in rodent models are needed.

Table 3. Classification of spinal cord injury and therapeutic substance in dog and cat.

Type	Distinction
Initial Stabilization (Muir.1998)	ABCs (Airway, Breathing, Circulation)
Prevention of Secondary Injury (Platt <i>et al.</i> , 2005)	Controversial use of methylprednisolone
Calcium-Channel Antagonists (Guha <i>et al.</i> , 1985)	Improve spinal cord blood flow
21-Aminosteroids (Hall. 1998)	Alternative to methylprednisolone
Opiate Receptor Antagonists (Faden <i>et al.</i> , 1981)	Decrease spinal cord blood flow
Thyrotrophin-Releasing Hormone (Pitts <i>et al.</i> , 1989)	Improve spinal cord blood flow
Minocycline (Cho <i>et al.</i> , 2006)	Anti-inflammatory and neuroprotective effects
Intraspinal Olfactory Glial Transplantation (Jeffery <i>et al.</i> , 2005)	Need further research
Anti-Oxidant Therapy	Vitamin E and selenium (Hall. 1987) Melatonin (Gul <i>et al.</i> , 2005) Resveratrol (Kaplan. 2005) N-actylcystenine (Baltzer <i>et al.</i> , 2008)

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CHAPTER I

A case of spinal cord injury in a dog

1. Abstract

A 4-year-old male miniature poodle developed chronic progressive paraplegia of the hind limbs following an accident 2 years prior to presentation for treatment. All hematological parameters, including the complete blood count, were normal.

Neurological examination revealed that the dog was ambulatory, with severe ataxia of the hindlimbs. Radiological examination showed a fracture of the 10th thoracic vertebra in the lateral view. The bladder was expanded, and the gas shadow in the stomach was elevated. Computed tomography revealed a fracture in the 10th thoracic vertebra in the sagittal and coronal views, and lack of continuity of the thoracic vertebrae.

We diagnosed complete transection of the spinal cord, and postulated that connective tissue injury in the damaged spinal cord was followed by permanent paralysis. The hematological parameters were in the normal range, although there might have been transient changes immediately after the spinal cord injury (SCI). A therapeutic trial is recommended only within a few days after SCI in cases with potential for improvement.

Key words: paraplegia, Computed tomography, Fracture, Spinal cord injury.

2. Introduction

Spinal cord injury (SCI) can result in complete or incomplete damage and can compromise the major functions of the spinal cord (*e.g.*, proprioception, motor, nociception, and reflexes) (Edward *et al.*, 2012). The principle mechanical forces involved in acute traumatic injury are concussion and compression (Vitale and Coates, 2007), shear (Griffin *et al.*, 2009), laceration (Olby, 1999), and distraction (Dumont *et al.*, 2001). Acute intervertebral disk herniation is a common acute SCI seen primarily in canine patients. The types of hernia are disk extrusion, disk protrusion (Griffin *et al.*, 2009), and nucleus pulposus extrusion (De Lahunta *et al.*, 2009). However, the most common cause of paraplegia in clinical practice is traumatic SCI, such as with fractures.

Spinal cord injury is characterized by both mechanical and inflammatory response-induced damage (Jung *et al.*, 2003; Ahn *et al.*, 2015) (Figure 3). The mechanical forces that affect the spinal cord at the time of injury may cause immediate tissue bursting. The neuropathological features of SCI include edema, axonal degeneration, inflammatory cell infiltration, and fibronectin exudation through the damaged blood–brain barrier (Jung *et al.*, 2003; Shin *et al.*, 2013). This report describes a case of SCI in a dog.

3. Case description of spinal cord injury in a dog

Case history

A 4-year-old male miniature poodle was evaluated because of chronic progressive paraplegia of the hind limbs (Figure 4). The dog had been in an accident 2 years earlier. Its body temperature was 38.7°C, and heart rate was 130 beats/minutes. This case was diagnosed based on the clinical signs and computed tomography (CT) obtained at Jeju National University Animal Hospital.



Figure 4. A case of spinal cord injured dog.

Hematological analysis and urinalysis

The complete blood count was normal except for leukocytosis: white

blood cell count (WBC) $17.5 \times 10^9/L$ and monocytes $0.5 \times 10^9/L$ (Table4). Biochemistry included normal blood urea nitrogen (BUN)16mg/dL, phosphorus 3.8mg/dL, and creatinine 0.8mg/dL. The specific gravity of the urine was 1.035, which was lower than normal. The urine sediments after centrifugation were normal.

Neurological examination

Neurological examination revealed that the dog was ambulatory, with severe ataxia of the hindlimbs. Proprioception was decreased in both hindlimbs, although the bilateral spinal reflexes were unremarkable. In hopping and extensor postural thrust, the forelimbs showed a normal response (2+), while the hindlimbs did not respond (0). Deep pain sensation was detected during palpation of the lumbar portion of the vertebral column (Table 6).

Radiological examination

The lateral view showed a fracture of the 10th thoracic vertebra, with signs of ossification. The bladder was expanded, and the gas shadow in the stomach was elevated (Figure 5, arrow). CT revealed a fracture of the 10th thoracic vertebra in the sagittal and coronal views (Figure 6A, arrow), lack of continuity of the thoracic vertebrae, and a spinal cord shadow in the thoracic vertebral cavity (Figure 6B, arrow). There was severe torsion around the 10th thoracic vertebra (Figure 6C, circle).

Table 4. Hematological changes of spinal cord injured dog.

Item	Result	Reference value (Latimer et al., 2009)	Unit
WBC	17.5	6-17	$\times 10^9/L$
WBC(Diff)-Lymphocyte	4.9	0.8-5.1	$\times 10^9/L$
WBC(Diff)-Monocyte	0.5	0-1.8	$\times 10^9/L$
WBC(Diff)-Granulocyte	5.6	4-12.6	$\times 10^9/L$
RBC	6.86	5.5-8.5	$\times 10^6/ul$
Hb	15.8	11-19	g/dL
HCT	52.5	39-56	%
MCV	66.6	62-72	fl
MCH	23	20-25	Pg
MCHC	30	30-38	g/dL
Platelets	411	117-460	$\times 10^3/ul$

WBC: white blood cells, Diff: differential count, RBC: red blood cell, Hb: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration

Table 5. Serum chemistry profile of spinal cord injured dog.

Item	Result	Reference value (Latimer et al., 2009)	Unit
Albumin	3.2	2.3-4	g/dL
ALKP (Alkine phosphatase)	56	23-212	U/L
ALT (Alanine transaminase)	64	10-100	U/L
Amylase	891	500-1500	U/L
BUN (Blood urea nitrogen)	16	7-27	mg/dL
Calcium-Total	10.4	7.9-12.0	mg/dL
Cholesterol	162	110-320	mg/dL
Glucose	109	74-143	g/dL
Phophorus	3.8	2.5-6.8	mg/dL
Bilirubin-Total	0.1	0-0.9	mg/dL
Protein-Total	6.3	5.2-8.2	g/dL
Creatinine	0.8	0.5-1.8	mg/dL
Globulin	3.1	2.5-4.5	g/dL

Table 6. Neurological evaluation of spinal cord injured dog.

	Rt.FL	Lt.FL	Rt.HL	Lt.HL
Proprioceptive placing	2+ ^a	2+	0	0
Hopping	2+	2+	0	0
Extensor postural thrust	2+	2+	0	0
Deep pain sensation	2+	2+	0	0

Rt: Right, Lt: left, FL: forelimb, HL: hind limb

^aAbsent 0, No reflex movement; Reduced 1+, Slow, decreased, reduced movement; Normal 2+, Brisk reflex action followed by relaxation; Increased 3+, Exaggerated, larger, brisker, reflex action; Clonus 4+, Increased, repetitive, sustained reflex action (Susan, 2010).

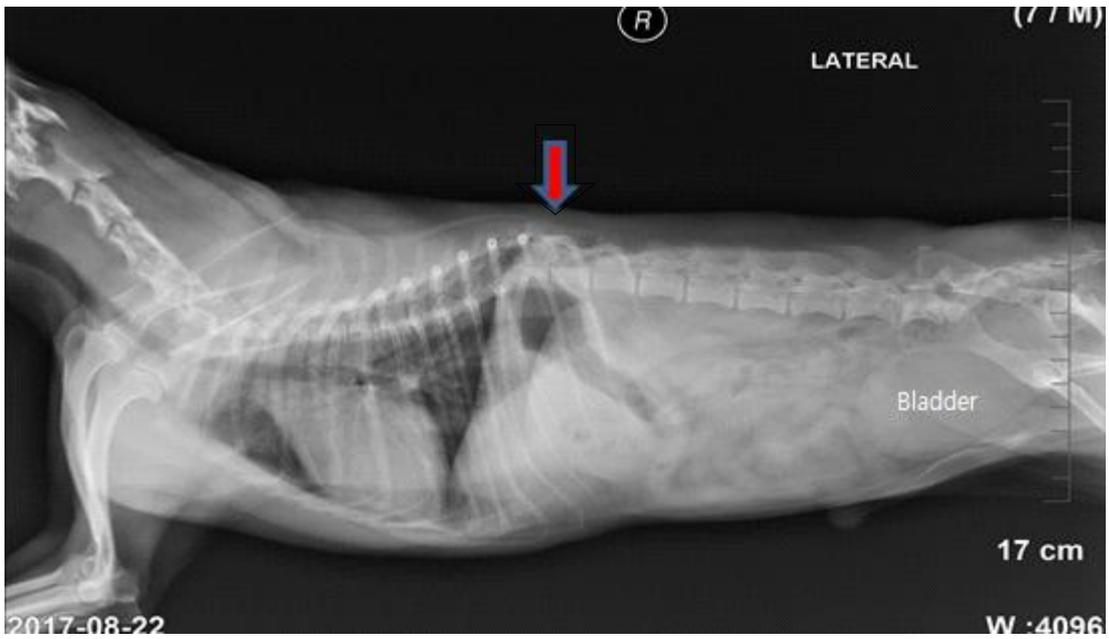


Figure 5. X-ray image of spinal cord injured dog. lateral view.

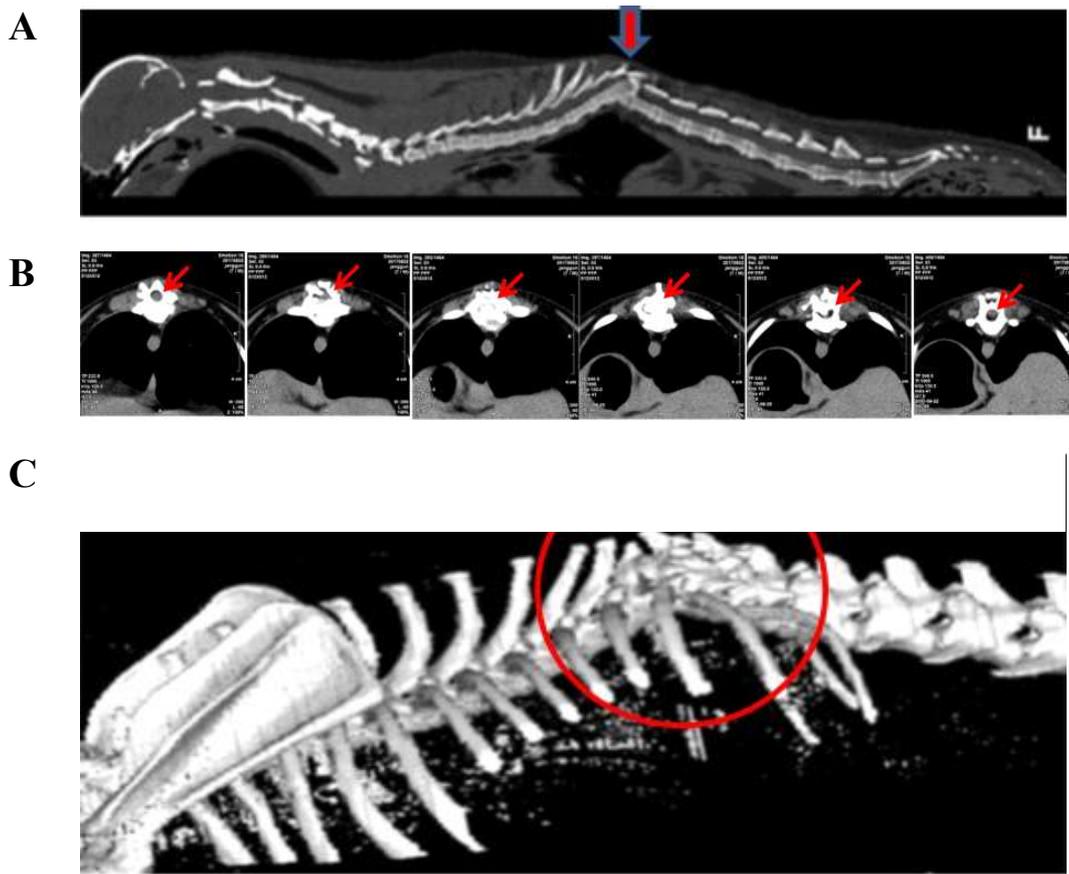


Figure 6. Computed tomography (CT) image of spinal cord injured dog.

4. Treatment

Because the caregiver's intention was symptomatic treatment rather than active treatment, eczema dermatitis treatment was performed, and lactulose 0.5 mL/kg (twice a day orally) was prescribed for smooth defecation. The caregiver was taught to perform bladder compression to prevent uremia.

5. Discussion

In most animals with spinal disease, the hematological analysis is unremarkable, although leukocytosis is a common finding (Nicholas and Simon, 2005), as seen in this case.

Paraplegia due to a vertebral fracture has been reported in dogs (Fitzmaurice, 2010). In this case, proprioceptive placing, hopping, extensor postural thrust, and deep pain sensation were normal in the forelimbs, while the hindlimbs were unresponsive. The spinal cord injury was caused by a thoracic fracture, which caused paraplegia. The radiological examination confirmed that the 10th thoracic vertebra was fractured, and we speculated that the fracture caused the paraplegia. Furthermore, the fracture caused bladder expansion due to the loss of reflexes for defecation and urination.

Following a vertebral fracture or chronic spinal cord injury, time is required for the growth of new tissues to protect the nerves in the spinal cord. In this case, there was no emergency care, diagnosis, or appropriate treatment at the time of the SCI.

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CHAPTER II

Therapeutic trials of lithium, an inhibitor of glycogen synthase-3 β in rat spinal cord injury

1. Abstract

Glycogen synthase kinase (GSK)-3 β and related enzymes are associated with various forms of neuroinflammation, including spinal cord injury (SCI). Our aim was to evaluate whether lithium, a non-selective inhibitor of GSK-3 β , ameliorated SCI progression, and also to analyze whether lithium affected the expression levels of two representative GSK-3 β -associated molecules, nuclear factor erythroid 2-related factor 2 (Nrf-2) and heme oxygenase 1 (HO-1) (a target gene of Nrf-2). Intraperitoneal lithium chloride (80 mg/kg/day for 3 d) significantly improved locomotor function at 8 d post-injury (DPI); this was maintained until 14 DPI ($p < 0.05$). Western blotting showed significantly increased phosphorylation of GSK-3 β (Ser9), Nrf-2, and the Nrf-2 target HO-1 in the spinal cords of lithium-treated animals. Fewer neuropathological changes (e.g., hemorrhage, inflammatory cell infiltration, and tissue loss) were observed in the spinal cords of the lithium-treated group compared with the vehicle-treated group. Microglial activation (evaluated by measuring the immunoreactivity of ionized calcium-binding protein 1 [Iba-1]) was also significantly reduced in the lithium-treated group. These findings suggest that GSK-3 β becomes activated after SCI, and that a non-specific enzyme inhibitor, lithium, ameliorates rat SCI by increasing phosphorylation of GSK-3 β and the associated molecules Nrf-2 and HO-1.

Key words: spinal cord injury, lithium, glycogen synthase kinase 3 β , nuclear factor erythroid 2-related factor 2, heme oxygenase 1.

2. Introduction

Spinal cord injury (SCI) is characterized by both mechanical and inflammatory response-induced damage (Jung *et al.*, 2003; Ahn *et al.*, 2015) (Figure 3). The mechanical forces that impact the spinal cord at the time of injury may cause immediate tissue bursting (Kwon *et al.*, 2004). There are a variety of animal models of spinal cord injury, including those using weight-drop impact devices (Shin *et al.*, 2013), the transection method (Donnelly and Popovich, 2008), and clip-compression injuries (Grimes and Jope, 2001; De *et al.*, 2002; Ahn *et al.*, 2017).

Clip compression, which results in the progressive recovery of hindlimb paralysis in rats (Fang *et al.*, 2016), is regarded as a manual injury technique for spinal cords in the absence of digital analysis systems, such as the Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor (Young, 2009). The neuropathological features of SCI include edema, axonal degeneration, inflammatory cell infiltration, and exudation of fibronectin through the damaged blood-brain barrier (Jung *et al.*, 2003; Shin *et al.*, 2013). Activated neutrophils, microglia, and macrophages synthesize free radicals that trigger apoptosis of neurons and glia by irreversibly oxidizing polyunsaturated fatty acids, proteins, and DNA (Donnelly and Popovich, 2008). An increase in the cellular antioxidant capacity or purging of reactive oxygen species (ROS) can protect against SCI.

Lithium, a non-selective inhibitor of glycogen synthase kinase (GSK)-3 β , has been used as a mood stabilizer in patients with bipolar disorder, and possibly acts as a neuroprotectant (Grimes and Jope, 2001). Lithium has also been used to ameliorate chronic experimental autoimmune encephalomyelitis (EAE) in mice expressing anti-myelin oligodendrocyte glycoprotein (MOG) antibodies (De *et al.*, 2002) and acute monophasic EAE in the rat (Ahn *et al.*, 2017). In addition, lithium is used to protect neurons after SCI, reducing post-injury inflammation (Fang *et al.*, 2016). Lithium promotes the production and release of neurotrophins, stimulates neurogenesis, enhances autophagy, and inhibits apoptosis (Young, 2009). However, any neuroprotective effect of lithium in terms of activating antioxidative systems, such as the nuclear factor erythroid 2-related factor (Nrf)-2/heme oxygenase (HO)-1 mechanism requires further study in models of SCI.

Oxidative stress-induced cell damage is attributable to an imbalance between reactive oxygen free radical production and the efficacy of the anti-oxidant system (Pratheeshkumar *et al.*, 2014). An increase in cellular antioxidant capacity or ROS removal can ameliorate various diseases and injuries, including SCI (Jiang *et al.*, 2016). Nrf-2 and its downstream target (HO-1), along with other antioxidant enzymes including superoxide dismutase and glutathione peroxidase play important roles in protecting various tissues and cells against oxidative stress both by regulating the expression levels of cytoprotective and antioxidant genes (Jiang *et al.*, 2016) and reducing inflammation (Ahn *et al.*, 2016). Normally, Nrf-2 is associated with the Kelch like-ECH-associated protein 1 (Keap-1) in the cytoplasm; upon stimulation, Nrf-2 is translocated to the nucleus where it plays essential roles in the

transcription of various phase II and/or antioxidant enzyme genes (Chen *et al.*, 2006). Targeting of Nrf-2/HO-1 after SCI suppresses oxidative stress and exerts a neuroprotective effect (Lv *et al.*, 2015). However, any relationship between GSK-3 β and Nrf-2 expression in the SCI rat model remains unclear.

In the present study, we investigated the neuroprotective effects of lithium, a non-selective inhibitor of GSK-3 β , in rats where SCI was induced by clip compression. We explored the underlying protective molecular mechanisms via semi-quantitative analysis of Nrf-2 and HO-1 levels (Figure 4).

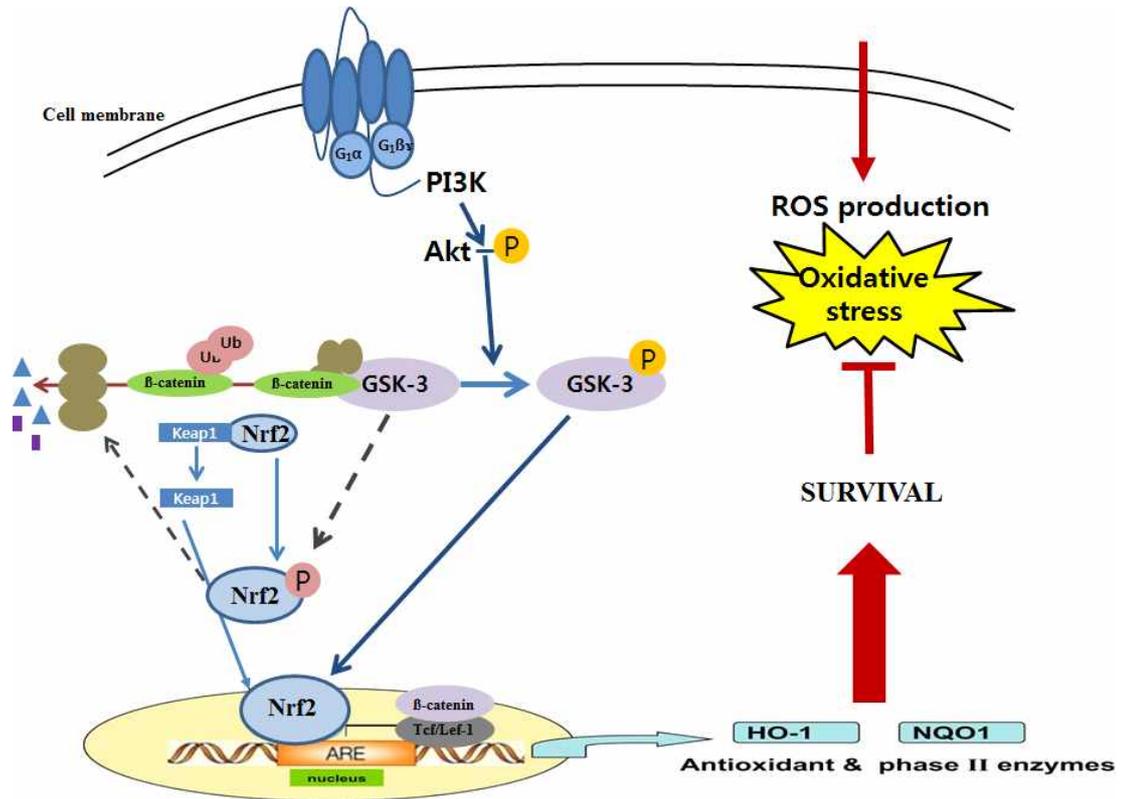


Figure 7. Schematic illustrations of relationship between GSK-3 β and Nrf-2 expression.

3. MATERIALS AND METHODS

Animals

We used female Sprague–Dawley rats (200–250 g, 7–8 weeks of age) (OrientBio Inc., Kyunggido, Korea). All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Jeju National University. The animal protocols also conformed to current international laws and policies national institutes of health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). Every effort was made to minimize the number of animals used and their suffering.

Surgical procedures

Clip compression injury was inflicted using a modification of previously published methods (Jung *et al.*, 2003; Kim *et al.*, 2003; Ahn *et al.*, 2012). Animals were anesthetized via intramuscular injection of Zoletil®50 (Virbac, France) and subjected to laminectomy at T9/T10. Immediately there after, the spinal cord was compressed with a vascular clip (Stoelting, WoodDale, IL, USA) applied vertically to the exposed spinal cord at a nocclusion pressure of 15–20g for 1min. After compression, the muscles and skin layers were closed. Sham-operated control rats under went laminectomy only. Spinal cord tissues from the surgical sites were harvested either fixed in 4%(v/v) paraformaldehyde in phosphate-buffered saline (PBS, pH7.2) for histological examination or stored at –80°C prior to Western blot analysis.

Antibodies

We used a rabbit anti-Iba-1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) antibody to immunohistochemically stain the ramified microglia and macrophages of rat spinal cords, and for Western blotting. We employed monoclonal rabbit anti-phospho-GSK-3 β (Ser9) (p-GSK-3 β) and monoclonal rabbit anti-GSK-3 β antibodies (Cell Signaling Technology, Beverly, MA, USA) to detect GSK-3 β . Rabbit polyclonal anti-Nrf-2 and -HO-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to stain the respective proteins. We also employed a mouse monoclonal anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO, USA).

Lithium treatment

To assess the effects of lithium on SCI, rats were divided into the following three treatment groups (10 animals/group): sham control, vehicle, and lithium. To rapidly elevate the lithium level, the first dose of lithium chloride (Sigma-aldrich, USA) (80 mg/kg/day) was intraperitoneally injected into the lithium-treated group 30 min after surgery; identical doses were given on each of the next 3 d. Lithium is nontoxic to rats at this level; the serum levels are equivalent to those in human patients (De *et al.*, 2002). As in our previous study, serum lithium concentrations were measured using a lithium assay kit (catalog number LI01ME; MG Metallogenics, Chiba, Japan) (Ahn *et al.*, 2017). The body weights and behavioral features of all rats were checked daily.

Behavioral tests and histological examination

Locomotor function after SCI was examined using the Basso, Beattie, and Bresnahan (BBB) rating scale (Figure 5A and Table 3) and modified the sciatic functional index test (Figure 5B). All evaluations were performed in a double-blinded manner; average scores were calculated for each group and used to compare the severity of hind-limb paralysis.

Spinal cords collected at various time points (0, 4, 7, and 14 d post-injury [DPI]) were perfused with 4% (v/v) paraformaldehyde in PBS, pH 7.2. The T8–T10 regions of the spinal cords, including the sites of injury, were collected and post-fixed in 10% (v/v) neutral-buffered formalin for 48 h. Subsequently, sagittal sections (5 μ m thick) were stained with hematoxylin and eosin (H&E).

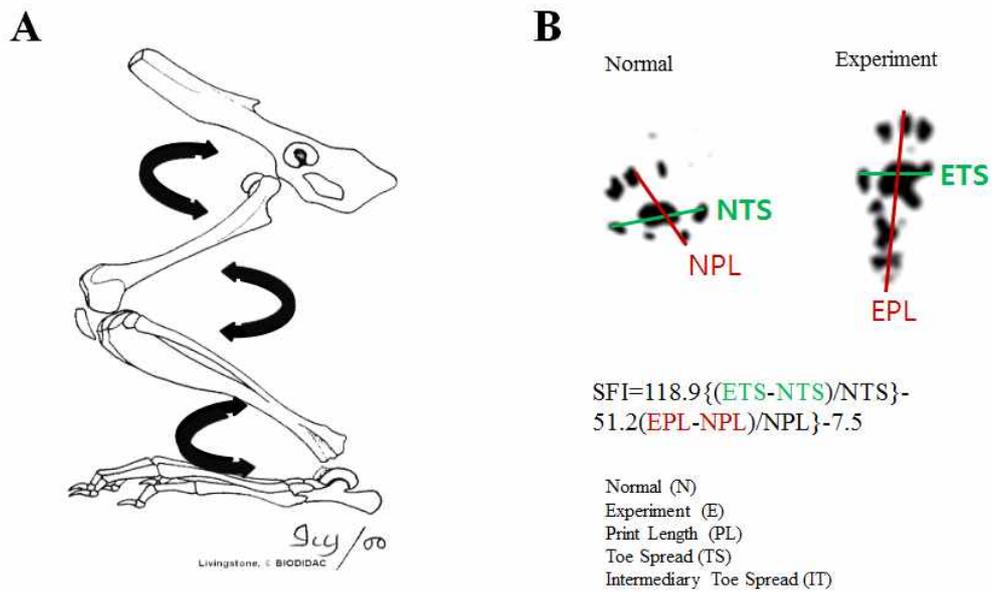


Figure 8. Schematic illustrations of behavioral evaluation of SCI. (A) BBB (Basso-Beattie-Bresnahan) Scoring (Basso *et al.*, 1996) and (B) sciatic functional index test (Luis *et al.*, 1982)

Immunohistochemistry

To assess early responses to treatment, we compared the microglial features of the vehicle- and lithium-treated groups at 4 DPI, as microglial reactions are prominent within the first week after SCI (Koshinaga and Whittemore, 1995; Dusart and Schwab, 1994; Loane and Byrnes, 2010). We immunostained the spinal cord for Iba-1 (a marker of activated cord microglia and macrophages) as described previously (Ahn *et al.*, 2016). Briefly, after incubation with matched blocking serum (10% [v/v] normal goat serum in PBS; Vectastain® Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), the samples were incubated with rabbit anti-Iba-1 (Iba-1; 1:800; Wako Pure Chemical Industries, Ltd. Osaka, Japan) for 1 h at room temperature (RT). After three washes in PBS, we proceeded as recommended by the manufacturer; the peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector, USA).

Western blot analysis

We performed Western blotting as described previously (Kim *et al.*, 2009). Briefly, spinal cord tissue was homogenized in TNN lysis buffer containing protease and phosphatase inhibitors (1 mM Na₃VO₄, 1mM phenyl methane sulphonyl fluoride, 10µg/mL aprotinin, 10µg/mL leupeptin), centrifuged at 10,900g for 20 min at 4°C, and the supernatant was harvested. The cytosolic and nuclear fractions were separated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents as recommended by the manufacturer (Thermo Scientific, Rockford, IL, USA). Proteins (40 µg) were subjected to

10% (w/v) sodium dodecyl (or lauryl) sulfate polyacrylamide gel electrophoresis (SDS-PAGE or SLS-PAGE) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked by incubation with 5% (v/v) skim milk in Tris-buffered saline for 1 h and then incubated with primary antibodies (anti-p-GSK-3 β , 1:1,000 dilution; anti-GSK-3 β , 1:1,000 dilution; anti-Nrf-2, 1:1,000 dilution; and anti-HO-1, 1:1,000 dilution) for 2 h. After washing, the membranes were incubated with the appropriate secondary antibodies for 1 h. Bound antibodies were detected using a chemiluminescent substrate (in the WEST-one™ kit; iNtRON Biotech, Gyeonggi, Korea) according to the manufacturer's instructions. After imaging, the membranes were stripped and reprobed using an anti- β -actin antibody (1:10,000 dilution). The optical density (OD)/mm² of each band was measured using Image J software (NIH, Bethesda, MD, USA). To detect Iba-1, we used the Wes™ system (ProteinSimple, California, USA) as instructed by the simple western user manual (Chen *et al.*, 2013). All electrophoresis and immunoblotting steps were performed using a fully automated capillary system.

Statistical analysis

All measurements are averages of three independent experiments. All values are presented as means \pm standard error of the mean (SE). The results were analyzed using a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* testing for multiple comparisons. A p-value < 0.05 was considered to reflect statistical significance.

4. Result

Lithium-mediated behavioral changes

Locomotor function began to recover commencing on 3 DPI. By 8 DPI, the BBB scores was significantly higher in the lithium-treated group (10.4 ± 0.52 , $p < 0.05$) compared with those of the vehicle-treated group (6.78 ± 0.55); the improvements were maintained until 14 DPI (Figure 7).

The foot printed images that the modified the sciatic functional index test showed very sliding foot image in both groups to 1 DPI. on day 3 post-injury, the images in the lithium-treated group was enhancer than that in the vehicle-treated group. This difference persisted until 14 DPI (Figure 8).

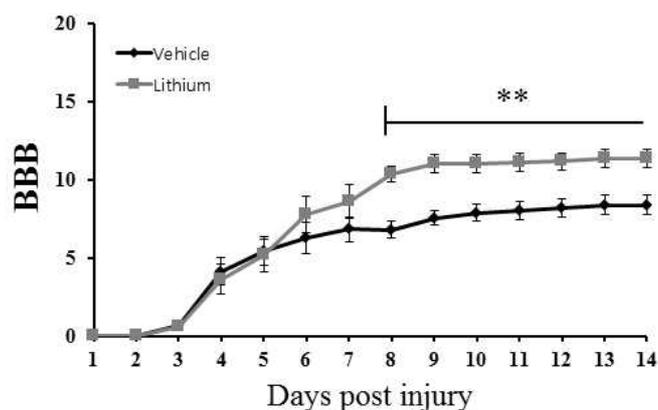


Figure 9. Locomotor outcomes as evaluated by Basso, Beattie, and Bresnahan (BBB) scoring ($n = 5$ /daily). The BBB scores were very low in both groups to 3 DPI; on Day 8 post-injury, the BBB score in the lithium-treated group was significantly higher than that in the vehicle-treated group. This difference persisted until 14 DPI. * $p < 0.05$ vs. vehicle-treated group.

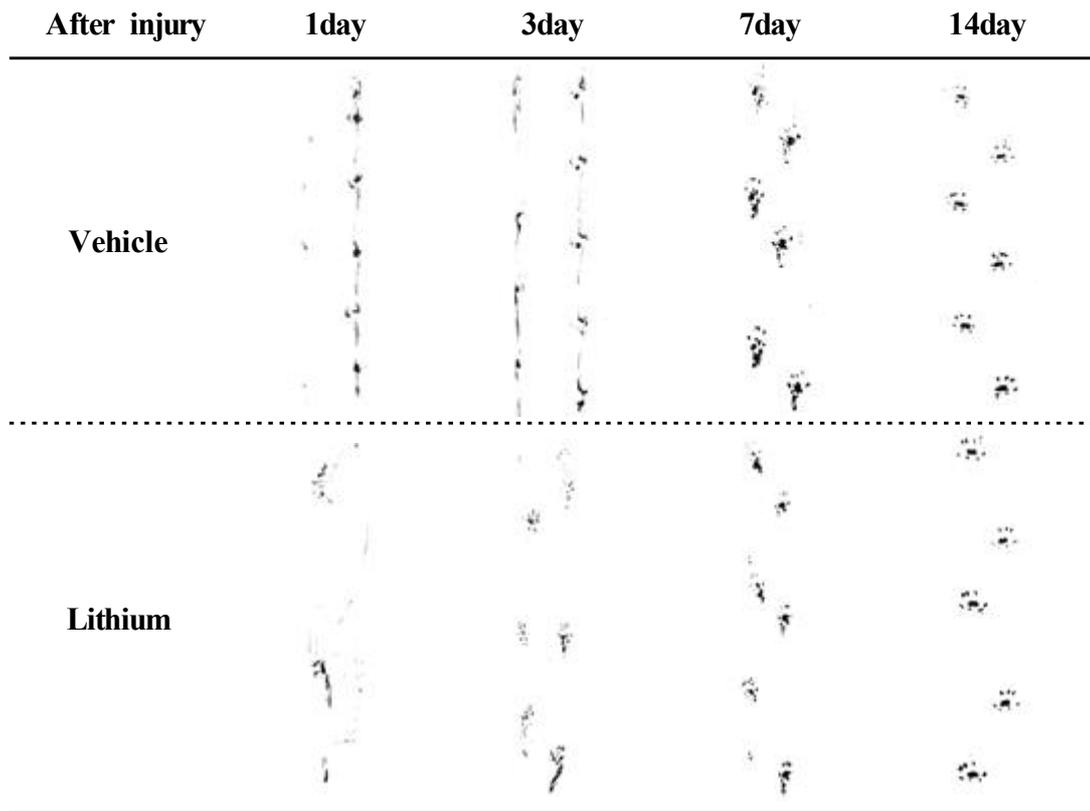


Figure 10. Locomotor outcomes as evaluated by modified the sciatic functional index test (n = 5/daily). The foot printed images were very sliding in both groups to 1 DPI. On day 3 post-injury, the images in the lithium-treated group was enhanced than that in the vehicle-treated group. This difference persisted until 14 DPI.

Histological findings

The sham-operated group exhibited no mechanical change in the core region of the spinal cord (data not shown), as found previously (Jung *et al.*, 2003; Ahn *et al.*, 2012). The cords of vehicle-treated rats exhibited reduced cellularity and edema in longitudinal sections of core lesions (Fig. 1A). Inflammatory cells had infiltrated by 4 DPI (Fig. 2C). In contrast, severe edema and hemorrhage were evident in the lithium-treated group (Fig. 2B). Additionally, accumulation of round-type inflammatory cells (Fig. 2G, arrowheads); activated microglia; and small, round vacuoles were evident in the core regions of spinal cords of the lithium-treated group by 4 DPI (Fig. 2D).

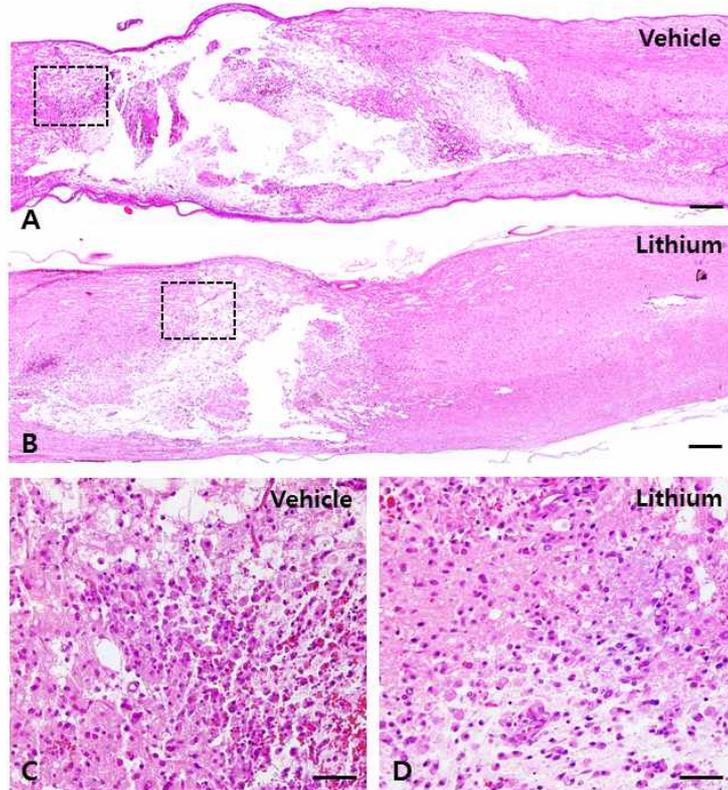


Figure 11. Histological profiles of the spinal cords of vehicle-treated (A and C) and lithium- treated groups (B and D) 4 DPI. (A and B) Low-magnification images of sagittal sections. (C and D) High-magnification images of the squares in A and B, respectively. A–D, hematoxylin and eosin staining. Scale bars: 100 μ m.

Microglial reactions and infiltration of inflammatory cells

To assess microglial reactions and inflammatory cell infiltration, we immunohistochemically stained for Iba-1 and used a simple system to quantify the protein levels. Iba-1-positive microglial cells and macrophages were evident in all cord regions, including the white and gray matter (Fig. 3). Figure 3C illustrates a representative experiment; the data are displayed in pseudo-gel and electropherogram formats. By 4 DPI, the Iba-1 level in the lithium-treated group ($25.78 \pm 9.99\%$, relative OD) was significantly less than that in the vehicle-treated group ($50.93 \pm 6.39\%$, relative OD; $p < 0.05$) (Fig. 3C).

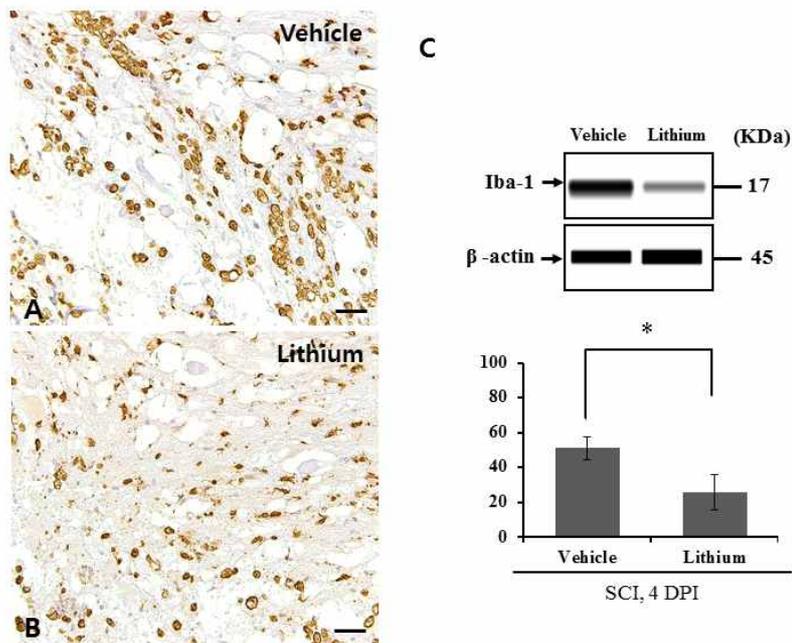


Figure 12. Ionized calcium-binding protein 1 (Iba-1) immunostaining of the core cord regions of vehicle-treated (A) and lithium-treated groups (B) on Day 4 post-injury. Iba-1-positive microglial cells were evident in all regions, including the white and gray matter. Ramified microglial cells and many inflammatory cells immunostained for Iba-1 in the core cord regions of both groups. Bar graphs: semi-quantitative analysis of Iba-1 levels (the ~17 kDa protein) using a simple Western blotting system. Normalization was achieved by reprobing the membranes with an anti- β -actin antibody. Means \pm SE (n = 5 per group) are shown. *p < 0.05 vs. the vehicle-treated group. Scale bars: 100 μ m.

Lithium-mediated modulation of the levels of GSK-3 β , Nrf-2, and HO-1 in the spinal cords of injured rats

We evaluated the GSK-3 β phosphorylation status via Western blotting to explore whether lithium inhibited GSK-3 activity in the spinal cords of injured rats (n = 5 per group). Lithium significantly increased the p-GSK-3 β expression level (relative OD values, 2.92 ± 0.44 -fold; $p < 0.05$) compared with vehicle (1.00 ± 0.13 -fold) at 4 DPI (Fig. 4A). We performed Western blotting to determine whether lithium influenced Nrf-2 levels in the cytosol and nucleus. Lithium significantly increased both the cytoplasmic Nrf-2 level (4.54 ± 1.76 -fold, relative OD/mm², $p < 0.05$) compared with the vehicle-treated group (0.21 ± 0.04 -fold) and also the extent of nuclear translocation (3.23 ± 1.08 -fold, $p < 0.05$) compared with the vehicle-treated group (0.14 ± 0.04 -fold) (Fig. 5A). In addition, the HO-1 protein level in the lithium-treated group was significantly greater (3.42 ± 0.46 -fold, $p < 0.01$) than that in the vehicle-treated group (1.00 ± 0.26 -fold) (Fig. 5B), reflecting enhanced Nrf-2 expression and translocation in SCI rats given lithium.

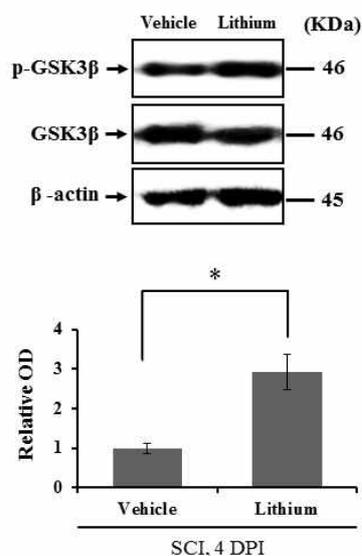


Figure 13. Western blotting to detect glycogen synthase kinase (GSK)-3 β in rats with spinal cord injury (SCI). (A) Representative immunoblots of phosphorylated GSK (p-GSK)-3 β (Ser9), total GSK-3 β (~46 kDa), and β -actin (~45 kDa). Bar graphs: The p-GSK-3 β level increased significantly in the spinal cords of lithium-treated rats. To quantify GSK-3 β phosphorylation, the levels of the phosphorylated form were normalized to those of total GSK-3 β . Normalization was achieved by reprobing the membranes with an anti- β -actin antibody. Means \pm SEM (n = 5 per group) are shown. *p < 0.05 vs. the lithium-treated group.

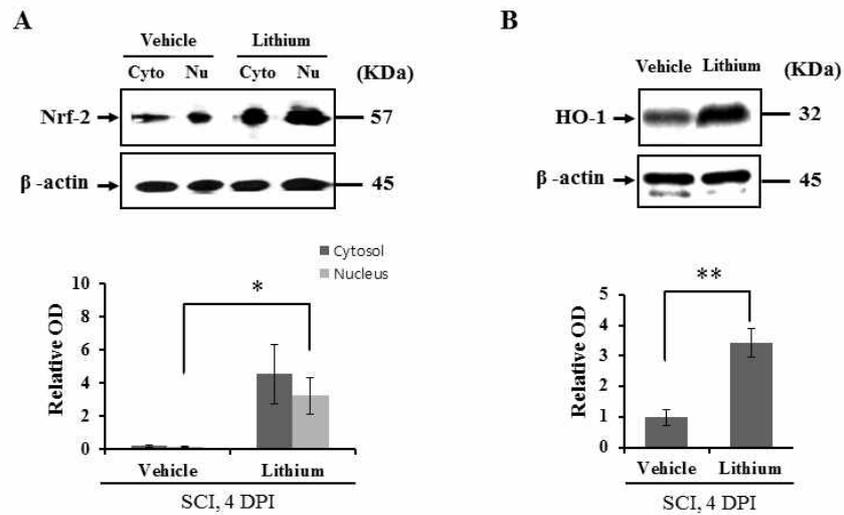


Figure 14. Western blotting to detect nuclear factor erythroid 2-related factor (Nrf)-2 and heme oxygenase (HO)-1 in the spinal cords of rats with SCI. (A and B) Representative immunoblots of Nrf-2 (~57 kDa), HO-1 (~32 kDa), and β -actin (~45 kDa). Bar graphs: Both the Nrf-2 and HO-1 levels increased significantly and Nrf-2 was translocated from the cytoplasm to the nucleus in the spinal cords of lithium-treated rats. Normalization was achieved by reprobing the membranes with an anti- β -actin antibody. Means \pm SEM (n = 5 per group) are shown. *p < 0.05 vs. the lithium-treated group.

5. Discussion

Many types of spinal cord injury models have been developed using rodents (Shin *et al.*, 2013). To obtain reliable data associated with impact power, computerized data analyses have been applied using the New York University impact device (Vijayaprakash and Sridharan, 2013), Ohio State University impact device (Koshinaga and Whittemore, 1995), and MASCIS impactor (Dusart and Schwab, 1994). Alternatively, for the present study, a clip compression technique was employed to induce spinal cord injury because there is a progressive improvement in BBB scores in rats following the use of this technique (Ahn *et al.*, 2015; Kim *et al.*, 2009; Basso *et al.*, 2006). Additionally, the clip compression technique is an alternative choice for the induction of spinal cord injury in the absence of digital analysis systems, such as MASCIS (Vijayaprakash and Sridharan, 2013).

We first showed that lithium exerted significant anti-inflammatory and anti-oxidative effects by inhibiting inflammatory cell infiltration, microglial activation, and Nrf-2 translocation (thus enhancing HO-1 synthesis), significantly improving functional recovery in rats. Earlier, we showed that lithium treatment reduced the extent of the Iba-1-positive area of the spinal cord, and reduced the serum TNF- α level, in an experimental rat model of EAE (Ahn *et al.*, 2017). Lithium suppresses both the level of circulating pro-inflammatory mediators and the number of CNS microglial cells, and enhances locomotor function in rats with SCI. A previous report indicated that GSK-3 β , a negative regulator of Nrf-2, influenced the relative Nrf-2 proportions in the cytosol and nucleus (Dusart and Schwab, 1994). Here, we show that lithium-mediated inhibition of GSK-3 β induced nuclear Nrf-2

accumulation, thus activating HO-1, in rats with SCI.

Lithium chloride reduces the disruption in the blood-spinal cord barrier and promotes the recovery of neurological function after SCI. This occurs partly due to decreases in the activation of endoplasmic reticulum stress, which plays an important role in SCI by inhibiting GSK-3 β activation (Loane and Byrnes, 2010). In the present study, lithium inhibited GSK-3 β phosphorylation and thus may have reduced inflammation in the spinal cord samples subjected to clip compression. Additionally, the regulation of GSK-3 β activity is generally mediated by phosphorylation of the amino-terminal domain (at Ser9) by any of several kinases, including Akt, protein kinase A, and/or protein kinase C, which inactivate the enzyme (Kim *et al.*, 2009). Furthermore, toll-like receptors mediate GSK-3 β phosphorylation at Ser9 via the regulation of pro- and anti-inflammatory cytokines (Chen *et al.*, 2013). Thus, it is possible that the inhibition of GSK-3 β relieves the clip compression SCI in rats.

Various drugs exert antioxidative neuroprotective activities in the spinal cord by activating Nrf-2 such as asiatic acid, rosmarinic acid and resveratrol (Jiang *et al.*, 2016; Shang *et al.*, 2017; Wang *et al.*, 2016; Keshewani *et al.*, 2013). In Wistar rats, carnosol protects against SCI-induced oxidative stress and inflammation by modulating nuclear factor- κ B, cyclooxygenase-2, and Nrf-2 levels (Wang *et al.*, 2016). Several drugs associated with Nrf-2 activation have been used to treat rat SCI; however, this is the first report to show that lithium-mediated inhibition of GSK-3 β protects against SCI by regulating Nrf-2 translocation and subsequent activation of the HO-1 target gene. Recent evidence indicates that GSK-3 β plays a critical role in regulating and degrading Nrf-2 in a Keap1-independent manner (Rada *et al.*, 2011).

Furthermore, it was postulated that lithium exerts neuroprotective effects via the activation of Nrf-2 in spinal cord-injured rats.

In conclusion, our findings suggest that lithium ameliorates rat paralysis caused by SCI, and that the molecular mechanism involves inhibition of GSK-3 β , increased nuclear translocation of Nrf-2, and subsequent upregulation of HO-1.

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척수손상모델에서 리튬의 치료 효과 및 그 기전에 대한 연구

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척수손상(SCI)은 기계적 및 염증반응에 의해 유발되는 손상을 특징으로 하며, 병리학적으로 부종, 축삭변성, 염증세포침윤, Fibronectin의 삼출 등을 특징으로 한다.

임상에서 척수손상은 대부분 외상성 손상에 의해 발생하며, 내원한 증례의 경우에도 밟힘에 의하여 후지마비 증상을 보였다. 혈액검사에서는 백혈구증가증의 소견을 보였고, 신경검사에서는 Proprioception 등에서 후지의 마비를 확인하였으며, 영상학적 검사에서는 Sagittal view에서 흉추10번의 골절을 확인하였고 Coronal view에서 흉추10번의 척수강 내 붕괴가 확인되었다.

Glycogen synthase kinase (GSK) -3 β 및 이와 관련된 효소는 척수손상 (spinal cord injury: SCI)을 비롯한 다양한 형태의 신경 염증과 관련되어있다. 전통적으로 양극성 신경질병의 치료제로 쓰이는 리튬은 최근 여러 연구에서 척수손상 후 신경원세포를 보호하고 신경 발생을 자극하며, 세포사멸을 억제하는 것으로 알려졌다.

본 연구에서는 GSK-3 β 의 비선택적 억제제인 리튬을 이용하여 랫트의 압박성 척수손상모델에서 효능을 평가하였다. 또한 그 기전연구로 GSK-3 β 관련 분자이며, 산화적 손상을 억제시켜 세포를 보호하는 것으로 알려져 있는 nuclear factor erythroid 2-related factor-2 (Nrf-2) 및 그 표적 단백질인 heme oxygenase-1 (HO-1)의 변화를 확인하였다. 랫트의 8-10번 흉추골을 절단하여 척수를 노출시킨 후, 혈관 클립을 이용하여 1분 동안 척수를 압박하는 방법을 이용하여 척수손상 모델을 유도하였고, 수술 후 뒷다리의 완전 마비가 유도된 랫트를 선별하여 대조군과 리튬 투여군으로 나누어 행동학적 평가, 조직학적 평가, 면역조직화학적 평가, Western blot analysis을 진행하였다.

행동학적 평가에서는 Basso, Beattie, Bresnahan (BBB) score와 sciatic functional index test 방법을 변형하여 보행정도를 평가하였고, 대조군과 리튬 투여군 모두 손상 후 3일부터 회복하기 시작하여 손상 후 8일부터는 대조군에 비해 리튬 투여군이 유의성 있게 행동학적 차이를 보였으며 손상 후 14일까지 유지되었다 ($p < 0.05$). 보행형태는 손상 직후에는 발등을 끄는 형태의 보행을 나타내다가 리튬 투여 후 3일째부터 보행정도가 점차 발바닥에서 발가락으로 옮겨 보행하는 형태를 확인할 수 있었다.

조직학적 평가에서는 척수손상 후, 척수 조직 내 출혈, 염증세포의 침윤 및 조직의 손실되었으나, 리튬을 투여한 경우에는 이러한 조직학적 소견이 향상되었다. 이러한 조직학적 소견을 바탕으로 척수 조직 내 염증정도를 확인할 수 있는 microglia의 활성정도를 ionized calcium-binding protein-1 (Iba-1) 항체를 이용하여 면역염색을 실시한 결과, 리튬을 투여한 경우에 microglia 양성세포의 수가 감소되었을 뿐만 아니라, 웨스턴 블롯을 이용하여 그 양을 측정된 경우에도 유의성 있게 감소되었음을 확인하였다. 또한 리튬은 억제성 GSK-3 β 의 인산화와 Nrf-2의 핵내 발현을 증가시켰고, 표적단백질은 HO-1의 발현도 증가시켰다. 이는 척수손상으로부터

산화적 손상을 리튬이 완화시켰음을 의미한다.

본 연구는 리튬이 척수손상이 유도된 랫트의 마비를 개선시켰고, 척수손상에 의한 염증세포의 침윤 및 조직 내 출혈, 조직의 손실을 완화시켰으며, GSK-3 β 의 억제, Nrf-2의 핵 전이 증가로 인한 표적 단백질인 HO-1의 활성화를 통해 신경세포를 산화적 손상으로부터 보호하는 효과가 있었다. 이러한 리튬은 척수손상의 치료에 보조적인 역할을 할 것으로 기대된다.

주요어 : 척수손상, Lithium, Glycogen synthase kinase (GSK)-3 β , nuclear factor erythroid 2-related factor 2(NrF-2), heme oxygenase-1 (HO-1)

감사의 마음

존경하는 신태균교수님.

기껏해야 학문과 지식을 깊이와 넓이로만 생각하던 저에게 학문과 지식에도 품격이라는 것이 존재한다는 것을 몸소 보여주시고 “나무와 소년”의 이야기처럼 모자란 제자인 저에게 학문적인 그루터기를 기꺼이 내주신 신태균교수님께 다시 한 번 깊은 감사와 존경을 바칩니다.

존경하는 정종태교수님과 이주명교수님.

석사를 잘 마무리했기에 박사라는 자리를 맞이할 수 있었습니다. 석사 시절 많은 지도편 달과 가르침에 다시 한 번 감사와 존경을 바칩니다.

존경하는 지영훈교수님과 문창종교수.

모자란 저의 논문 심사에 많은 조언을 주시고, 먼 거리에서 발걸음을 옮겨주신 존경하는 두 교수님께 진심으로 감사의 마음을 전합니다.

고마운 안미정박사와 김정태박사를 비롯한 실험실 식구들.

생소한 실험실 생활에 물심양면으로 도와주고 아낌없는 조언과 협조를 해준 실험실 식구들에게 모자라지만 이 지면을 통해 진심어린 감사의 마음을 전합니다.

존경하는 부모님(故김진일, 원화자)

세상에 빛을 보여주시고 정성과 사랑으로 키워주신, 지금은 하늘에서 저를 보고 계신 아버지님과 건강히 곁에 계셔 주시는 어머니께 존경 그 이상의 마음을 바칩니다.

존경하는 장인어른(이창훈)과 장모님(박향숙).

사랑으로 키운 외동딸을 부족한 저에게 감히 주신 장인어른과 장모님께 일생동안 갚지 못한 빛이라 생각하고 열심히 늘 노력하는 모습 보여드리겠습니다.

세상에 하나뿐인 인생의 반려자.

인공위성이 지구궤도를 벗어나지 않게 항상 수정을 하듯이 내 인생에 있어 곁에서 언제나 궤도를 수정해 주고 인생의 조력자로서, 반려자로서 서 있는 아내 이소연에게 진심을 담아 고마움을 전합니다.

내 삶의 별인 호진이와 가현이에게.

언제나 든든하고 책임감 있는 아들 호진이와 늘 명량하고 긍정적인 딸 가현아.

앞으로 어렵고 힘든 일들이 많더라도 잘 극복하리라 믿는다.

언제나 아빠는 너희들을 곁에서 지켜주고, 항상 응원 할게.

