A New Acute Impact-Compression Lumbar Spinal Cord Injury Model in the Rodent

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Abstract

Traumatic injury to the lumbar spinal cord results in complex central and peripheral nervous tissue damage causing significant neurobehavioral deficits and personal/social adversity. Although lumbar cord injuries are common in humans, there are few clinically relevant models of lumbar spinal cord injury (SCI). This article describes a novel lumbar SCI model in the rat. The effects of moderate (20 g), moderate-to-severe (26 g) and severe (35 g, and 56 g) clip impact-compression injuries at the lumbar spinal cord level L1-L2 (vertebral level T11-T12) were assessed using several neurobehavioral, neuroanatomical, and electrophysiological outcome measures. Lesions were generated after meticulous anatomical landmarking using microCT, followed by laminectomy and extradural inclusion of central and radicular elements to generate a traumatic SCI. Clinically relevant outcomes, such as MR and ultrasound imaging, were paired with robust morphometry. Analysis of the lesional tissue demonstrated that pronounced tissue loss and cavitation occur throughout the acute to chronic phases of injury. Behavioral testing revealed significant deficits in locomotion, with no evidence of hindlimb weight-bearing or hindlimb-forelimb coordination in any injured group. Evaluation of sensory outcomes revealed highly pathological alterations including mechanical allodynia and thermal hyperalgesia indicated by increasing avoidance responses and decreasing latency in the tail-flick test. Deficits in spinal tracts were confirmed by electrophysiology showing increased latency and decreased amplitude of both sensory and motor evoked potentials (SEP/MEP), and increased plantar H-reflex indicating an increase in motor neuron excitability. This is a comprehensive lumbar SCI model and should be useful for evaluation of translationally oriented pre-clinical therapies.

Key words: hindlimb; electrophysiology; injury model; lumbar; spinal cord injury

Introduction

SPINAL CORD INJURY (SCI) is a devastating affliction defined by motor and sensory deficits, with significant emotional and social disturbances, neuropathic pain, and cardiovascular complications. The highest incidence of SCI is in patients between the ages of 18 and 35 years, with total lifetime expenses in the millions of dollars.^{1,2} Outcomes have improved considerably in recent decades, but despite these advances, critical knowledge gaps remain in accurately modeling SCI at different levels of the spinal cord. Because 20–25% of SCIs occur in the thoracolumbar region of the spine, there is a significant need for clinically relevant SCI models.^{3,4} Only a small number of models have been described for this location, however.^{5–10}

Injuries to the thoracolumbar region of the spine differ from injuries to the midthoracic or cervical regions because each results

in distinct motor and sensory deficits.^{3,4} It has been shown that interneurons involved in the hindlimb central pattern generator (CPG) are predominantly localized in the L1 and L2 spinal cord segments of the rat,¹¹ with injuries to this region resulting in significant locomotor deficits. Patients also lose circuits of neurons that are essential for locomotion, with thoracolumbar injuries of the spine (T10-L1) typically inducing paraplegia, intractable pain, and a loss of bladder and bowel function.^{4,12–14}

In addition to a central nervous system (CNS) injury, the spinal nerves are often involved because of their obliquely descending course and position in the lumbar spinal cord, thereby producing a peripheral nervous system (PNS) injury as well (Fig. 1). Thus, this injury level represents a large clinical population, and an injury model that replicates the dorsal-ventral injury forces commonly observed in human SCI would be an important complement to the existing lumbar SCI literature.

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FIG. 1. Schematic diagram of the thoracolumbar rat spinal cord. The disparity between the vertebral levels and the spinal cord segments is shown. The lumbar spinal cord segments begin approximately at the junction between the T11 and T12 vertebrae and end midway through the L1 vertebra. The lumbar enlargement is present in all lumbar spinal cord segments (outlined in blue), and the location of the injury is marked with red "X's" at the junction of the L1 and L2 spinal cord segments. The diagram shows that three pairs of intradural roots (T12, T13, and L1) are potentially injured by clip-compression at L1-L2. Color image is available online at www.liebertpub.com/neu

In general, numerous models of traumatic SCI have been reported, using many different mechanisms of trauma, such as weight-drop, impactor-contusion, transection, distraction, excitotoxicity, clip-compression, and forceps-compression.¹⁵ With respect to lumbar SCI, several models have been described in the literature. Magnuson and associates⁶ in 2005 investigated behavioral and histological

outcomes after weight-drop contusion injury to various lumbar segments in the rat and provided the first description of traumatic SCI to the lumbar cord. The authors described moderately severe (12.5 g-cm) versus severe injuries (25 g-cm) with the NYU impactor at T13-L1, L2, and L3-L4 segments of the spinal cord.

An analysis of spared white and gray matter determined that gray matter loss is critical in the lumbar spinal cord, while considerable white matter sparing did not impact the persistent locomotor dysfunction observed. Two other groups investigated variations of the weight-drop mechanism and found similar results to those of Magnuson and associates.^{6,7,14}

Chemical injuries to the rat lumbar cord at the L2 spinal cord segment have been reported, although they are not considered clinically relevant.^{5,8} In contrast to the clip impact-compression model, which simultaneously applies dorsal-ventral forces that likely includes the spinal roots, contusion-only models cause trauma to the dorsal CNS alone. Impact-compression injury produced by the modified aneurysm clip is considered clinically relevant and reproducible with low interoperator variance.^{16,17} The clip closes rapidly producing bilateral, dorsal, and ventral impact followed by persisting compression injury with severity related to the closing force of the clip, and the duration of compression, both of which can be readily altered.

To relate our pre-clinical experimental injury to the clinical management of SCI, we have used a suite of imaging techniques to image the bony anatomy, visualize progressive abnormalities in white and gray matter over time, as well as observe cavitation *in vivo*. Computed tomography (CT) imaging was executed to image the bony anatomy to confirm that our meticulous pre-operative anatomical landmarking protocol was sufficient to locate the injury site. Further, we used intraoperative spinal sonography (ultrasound) to view the fluid-filled cavity after injury. Magnetic resonance imaging (MRI) was used to monitor the evolution of compression injury over time.

In this study, we characterize an acute clip impact-compression injury to the lumbar spinal cord segments at L1-L2 in the rat. The main aim of this work is to generate a consistent, reproducible clip impact-compression lumbar SCI model for use in subsequent translationally oriented studies.

Methods

Animals

All experiments were performed using adult female Wistar rats (Charles River, Quebec, Canada, $n = 80$) weighing 250–280 g. All procedures involving animals were approved by the Animal Care Committee of the University Health Network, in accordance with the policies established in the Guide to the Care and Use of Experimental Animals made by the Canadian Council on Animal Care. For injured rats, bladders were expressed three times daily until spontaneous voiding returned. Food and water were provided *ad libitum*. Post-operative buprenorphine (0.03 mg/kg, Temgesic) was given subcutaneously before the animals awakened and then every 12 h for 7 days. Rats were given Clavamox (amoxicillin trihydrate/clavulanate potassium) in their drinking water for 7 days post-injury to prevent urinary tract infection.

The following humane end points were determined *a priori* for exclusion and/or euthanasia: weight loss exceeding 20% pre-operative weight, not eating or drinking for more than 24 h, anorexia, and chronic urinary tract infection or hematuria that did not respond to antibiotics. Autophagia of the paws was treated with meloxicam and buprenorphine, but if ineffective, was followed by euthanasia.

Tables 1 and 2 describe animal usage across all experiments. The number of animals that met the following exclusion criteria are

TABLE 1. INJURY SEVERITY GROUPS

Clip force	Group size	Exclusions*	Number included
20g	22	2	20
26g	18	4	14
35g	16	6	10
56g	14	6	8
Sham	10	0	10

*See Methods for exclusion criteria

indicated as well as the force of the clip used to produce the SCI: 20 g UTI (2), 26 g UTI (2), autophagia (2), 35 g UTI (3), autophagia (2), weight loss (1), 56 g UTI (1), autophagia (3), weight loss (2).

SCI model

Rats were anesthetized with 2% isoflurane carried by a 2:1 of nitrous oxide and oxygen. We determined that the iliac crests, which can be located by palpation through the intact skin, are a useful landmark to locate the adjacent L6 vertebra and spinous process, which lie immediately between the iliac crests. Then, the last rib is palpated and its medial most aspect is at the junction with the T13 vertebra. Counting the spinous processes sequentially rostrally from L6 to T13 was then used to confirm the location of the T13 vertebra (Fig. 1–2).

Under aseptic conditions, the spinal cord was exposed through a midline skin incision, and the muscles retracted to expose the spinous processes and laminae of the T9, T10, T11, and T12 vertebrae. The specific relative angulation of the spinous processes of the T9, T10, and T11 vertebrae was used as an important intraoperative landmark: T9 points caudally, T10 points directly dorsal, and T11 points rostrally. This produces a reliable “triangle” orientation that can be readily seen if the animal is positioned flat on the operating table.

After the T11 and T12 vertebrae had been identified, a laminectomy was performed at T11 and T12 with Friedman-Pearson rongeurs (Fig. 1–2). The clip was then held open with a clip applicator, with the lower blade of the clip passed extradurally completely around the spinal cord and nerve roots at the junction between the T11 and T12 vertebrae, corresponding to the L1-L2 spinal cord segmental level. The clip was then rapidly released from the applicator to produce a bilateral impact force and sustained dorsal-ventral compression (Fig. 1–2).

The compression of the spinal cord was maintained for 60 sec before removal of the clip. The muscles were then sutured using 3–0 polyglactin sutures, and the skin was closed with Michel clips. Before use, the force of the clip was measured with a force calibrator that measures the peak closing force of the clip to ensure reproducibility.

Open-field locomotor scale

Locomotor function was evaluated using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale weekly for 6 weeks.¹⁸ Rats were video recorded for 4 min to assess the animal's motor

TABLE 2. OUTCOME MEASURES FOR 20G CLIP FORCE

Outcome measure	Injured (n)	Sham (n)
Histopathology	12	8
Immunohistochemistry	8	4
Electrophysiology	8	4
Ultrasound	3	2
CT and MRI	6	4
Sensory testing*	12	8
Open field locomotion	20	10

*Von Frey and tail-flick.

function including joint movements, coordination, paw placement, and toe clearance. A score of 0 indicates no hindlimb movement, whereas a score of 21 indicates unimpaired locomotion as observed in normal uninjured rats.

Two evaluators blinded to groups performed the rating of the BBB scores. Five groups of rats were used in this study: one group underwent laminectomy-only sham operation ($n = 10$); four groups underwent injury with clip closing forces of 20 g, 26 g, 35 g and 56 g ($n = 22, 18, 16, 14$ respectively), with mortality and exclusion as reported (Table 1). Clips of varying closing force were used to determine the sensitivity of the lumbar spinal cord to impact-compression injury, and the duration of compression was 1 min in all groups.

Sensory testing

At-level mechanical allodynia: von Frey filaments (VFF). Cutaneous sensitivity to normally innocuous mechanical stimulation was evaluated weekly using VFF. We compared animals in the 20 g injury group versus the sham control group. A series of VFFs with stiffness of 1.4 g, 2.0 g, and 4.0 g were applied to T13, L1, and L2 dermatomes as described by Takahashi and colleagues¹⁹ in 2003 to determine at-level mechanical allodynia. Probing was performed when the animals were calm and not moving. A positive response was recorded when the filament bends, and the animal responded with an aversive action, including vocalizing, flinching, licking, turning away, or overt behavioral cue corresponding to discomfort. A total of 10 applications of each VFF were scored as positive or negative to obtain a total response percentage.

Thermal hyperalgesia: tail-flick. To measure thermal nociceptive response, we assessed 20 g and sham groups bi-weekly for 6 weeks. Animals were wrapped in a soft, dark material to calm and distract them. The dorsal surface of the tail between 4 and 6 cm from the tip was exposed to a beam of light calibrated to 50°C generated from an automated machine (IITC Life Science, Woodland Hills, CA). The timer was stopped when the animal flicked its tail away from the beam of light, indicating an aversive response. Latency was measured at 5-min intervals over three consecutive trials, with mean latency reported.

Histology

Animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital before sacrifice. For lesion analysis, animals were perfused with 500 mL of 10% neutral buffered formalin after intravascular injection of 1 mL heparin (1000 IU/mL), and the spinal column was dissected. A 1.5 cm segment of the spinal cord centered on the injury site was excised and post-fixed in 10% neutral buffered formalin at room temperature. Spinal cords from each of the four injury groups were randomly selected (sham, $n = 7$; 20 g, $n = 10$; 26 g, $n = 7$; 35 g, $n = 6$, and; 56 g, $n = 5$), and 8 μ m serial cross-sections were made in a cryostat. The sections were stained with Luxol Fast Blue (LFB) and counterstained with hematoxylin and eosin (H&E) to visualize white and gray matter.

Image analysis was performed with a Nikon TE300 inverted bright field microscope, and images of LFB/H&E stained sections were captured with an Optronix CCD camera connected to the microscope. To assess the impact of a clip compression injury at the L1-L2 spinal cord segments, transverse sections for each animal were systematically sampled every 240 μ m over a distance of 1 cm. Tissue section areas were quantified into volumes using the Cavalieri method. Any necrotic tissue within the cavitation was counted as part of the lesion. The percentage of scar/lesional tissue and cavity (total lesion) for each section were calculated as percent of total section area. Preserved gray and white matter were calculated similarly.

Immunohistochemistry

For immunohistochemical analysis, rats underwent fixation with 4% transcardial perfusion in 0.1M phosphate buffered saline (PBS),

pH 7.4, and the tissue was excised and maintained in 30% sucrose at 4°C. A 1-cm segment of the spinal cord centered on the injury site was dissected and used for cryosectioning. The tissue was embedded in optimal cutting temperature (OCT) compound and cryosectioned in 20 μ m serial cross-sections collected on Superfrost slides.

Sections were then rehydrated in 0.1 M PBS and permeabilized with 0.1% Triton-X 100, blocked with either 10% normal goat (NGS) or normal donkey serum (NDS) for 1 h at room temperature, and then incubated with the primary antibody overnight at 4°C. We used the primary antibody anti-choline acetyltransferase (ChAT) (1:500, Millipore, AB144P) for motoneurons. After three washes, cells were incubated for 1 h with the secondary antibody goat anti-mouse Alexa 564 IgG (1:500). Slides were washed with PBS and then coverslipped with Vectashield mounting medium containing DAPI for nuclear counterstain. Immunofluorescent tissue was examined using a Nikon TE300 inverted fluorescent microscope.

Motoneuron quantification

For motoneuron quantification, polyclonal anti-ChAT (1:100, Millipore, AB144P) was used, and large cholinergic cells in the ventral horn of the spinal cord were counted. These motoneuron counts were performed at 20x magnification bilaterally. ChAT+ cells were readily visible in the anterior horn and only counted in Rexed lamina IX. Values were pooled and the number of motoneurons per section was reported at 1-mm intervals.

Electrophysiology

Hoffmann reflex (H-reflex). The H-reflex records the mono-synaptic excitation of alpha-motoneurons, and has been used as a surrogate for spasticity after SCI. In this study, H-reflex recordings were performed before SCI and weekly thereafter for 6 weeks. Under isoflurane anesthesia, electrodes were inserted transcutaneously in the left hindlimb. A pair of stimulating electrodes was inserted into the region of the posterior tibial nerve slightly above the ankle. For recording, a pair of silver needle electrodes was placed into the plantar muscles of the left hindpaw. The tibial nerve was stimulated using a 0.1 msec duration square wave pulse at a frequency of 1 Hz.

The recordings were filtered between 10 and 10,000 Hz. Recordings of the H-reflex typically consist of two electromyography (EMG) responses—an initial M-wave and a later H-wave. The M-wave is the result of direct activation of the motor axons and does not involve the spinal circuits. The later H-wave, or H-reflex, is a compound EMG response in the plantar muscle elicited by the synaptic activation of motoneurons by muscle afferents. The peak threshold for both the M and H waves was determined, and the Hmax/Mmax ratio was calculated.

Motor and sensory evoked potentials. Motor (MEP) and sensory evoked potentials (SEP) were examined to evaluate the electrophysiological integrity of the spinal cord after clip impact-compression injury to the lumbar spinal cord at 6 weeks post-injury. The rats were immobilized in the prone position and fixed in a stereotactic holder under isoflurane anesthesia. The conductivity of both descending motor tracts and ascending sensory tracts were analyzed. SEPs and MEPs were analyzed by peak amplitude measurements from the first positive peak to the first negative peak.

SEPs. The intervertebral ligaments between C1 and C2 were removed surgically using fine forceps. Two pairs of 1.0-mm ball electrodes were positioned extradurally over the spinal cord at C1 and C2 for recording evoked potentials. The sciatic nerve was exposed and placed on bipolar silver electrodes. A constant current stimulus of 0.1 msec in duration and 2.0 mA in intensity was applied at a rate of 1.13 Hz to the sciatic nerve. At a bandwidth of 10 to 3000 Hz, a total of 100 SEPs were averaged and replicated. SEPs peak latency was measured from the start of the stimulus to the peak

of first negative peak (N1). The evoked potential amplitudes were measured as the voltage difference from the peak of the first positive peak to the peak of the N1.²⁰ Recordings were acquired using Keypoint Portable (Dantec Biomed, Denmark).

MEPs. Stainless steel subdermal needle recording electrodes were inserted into the biceps femoris muscle in the hindlimb. Stimulation was applied to the midline of the cervical spinal cord between C1 and C2 vertebrae using a ball electrode as described above with the following specifications: 0.13 Hz; 0.1 msec; 2 mA; 200 sweeps. The amplitude was determined by the difference between the first positive and negative peak. Recordings were acquired using Keypoint Portable.

Imaging

Ultrasound imaging. Very high-resolution ultrasound (VHRUS) imaging was used to measure *in vivo* cavity volume and tissue morphology. Spinal cord VHRUS imaging followed the protocols developed previously by Soubeyrand and colleagues²¹ in 2014. In short, at 6 weeks post-injury, animals were placed under 2% isoflurane anesthesia as described above, and a midline incision was made in the skin followed by retraction of muscle and scar tissue to reveal the surgical site and spinal cord. Ultrasound gel (Medi-Inn, Canada) was then applied on the dorsal aspect of the dura mater.

The VHRUS probe (44 MHz, Vevo 770, Visualsonics, Toronto, Canada) was attached to the rail mount of the Vevo Integrated Rail system III (Visualsonics, Toronto, Canada) by an integrated clamp. The rail mount was positioned 6 mm above the middle of the spinal cord. To obtain only sagittal acquisitions, the scanhead was tilted and locked. Both two-dimensional (2D) and three-dimensional (3D) acquisitions were obtained.

For 3D acquisitions, the motor stage travelled 6 mm from left to right, acquiring a 2D slice at each step, with a distance of 102 μ m. Slices were stacked and assembled as a 3D file. The 3D files were analyzed with ImageJ software (NIH). The analysis was then performed on a stack of 19 sagittal slices centered on the midline. On each slice, the dark cavitation within the spinal cord (known to be cavity by the absence of tissue to reflect high frequency sound waves) was delineated to obtain the area. Subsequently, the cavity area of each slice was combined to determine the cavity volume (using the known distance between sagittal slices).

CT. CT imaging was used to confirm the correct operative level (Fig. 2). As indicated above, the 13th rib articulates with the anterior aspect of the 13th thoracic vertebral body, and the L6 vertebra lies directly between the iliac crests, which can be seen clearly in the images. The bony anatomy was imaged with the GE Locus Ultra MicroCT system. The following technical specifications were used: 16-sec anatomical scan time with 150 μ m³ resolution, a 14-cm maximum transaxial field of view (FOV), 10.2 cm/16 sec maximum longitudinal FOV with 1000 volumes per rotation. The X-ray tube was used an 80 kV voltage, 50 mA current, and 0.15 mm Cu filter with a “standard” reconstruction filter. Animals were anesthetized with 2% isoflurane to avoid movement during scan acquisition and imaged at 7 days post-surgery.

MRI. Animals were anesthetized with 2% isoflurane for MRI imaging. The images were acquired on a 7T Biospec USR 70/30 (Bruker Corporation, Ettlingen, DE), with the B-GA12 gradient coil insert. A 7.2 cm inner diameter cylindrical linearly polarized RF coil was used for radio frequency (RF) transmission, and a 20-mm flat surface coil taped above the lesion was used for RF signal reception.

Coronal 2D-RARE T1-weighted images were acquired with the following parameters: echo time 12 msec, echo train length of 2, and repetition time was gated to the respiratory cycle (TR ~ 1300 msec). Images were acquired over a 40×40 mm FOV with a 320×320 matrix for 125-micron in-plane resolution. The effective readout

FIG. 2. Operative anatomy of the lumbar injury model. **A–C** are computed tomography (CT) images post-laminectomy. **D–F** are operative photographs and show vertebral levels in white, spinal cord segments in red, and spinal roots in black. CT imaging was used to verify consistent localization of the T11 and T12 vertebral levels. (A) is an AP view, while (B) and (C, higher magnification) show a lateral view. The last free floating ribs (13th rib) articulate with the last thoracic (T13) vertebra and are a useful anatomical landmark. Laminectomy is performed directly rostral to T13 to remove the T12 and T11 laminae, revealing the T13, L1, and L2 spinal cord segments (Fig. 1). (C) shows that the spinous processes and laminae of T11 and T12 have been removed. (D) After midline incision and clearance of connective tissue, the T9–T13 vertebrae are exposed. A useful landmark is the “triangle” of spinous process orientation of T9, T10, and T11: T9 is directed caudally; T10 is directed dorsally; and T11 is directed rostrally. (E) After removal of T11 and T12 laminae, three spinal cord segments are exposed (T13, L1, and L2). The clip is applied between T11 and T12 vertebrae and is indicated with a dotted red line in E and F. (F) A durotomy was performed to show that the injury at L1–L2 includes the T12 and T13 roots, which have been dissected to show their location (L1 cannot be seen in the photograph). Color image is available online at www.liebertpub.com/neu

bandwidth was 44.6 kHz, slice thickness was 1.1 mm for a total of 5 slices. The scan time was approximately 24 min.

Axial 2D-RARE T1-weighted images were acquired with the following parameters: echo time 14.4 msec, echo train length of 4, and repetition time was gated to the respiratory cycle (TR ~ 1300 msec). Images were acquired over a 16 × 16 mm FOV with a 128 × 128 matrix for 125-micron in-plane resolution. Out-of-FOV signal was suppressed using saturation bands. The effective readout bandwidth was 48 kHz, with a slice thickness of 1.1 mm for a total of 15 slices. The scan time was approximately 22 min.

Statistics

Tail-flick, VFF, and lesion analysis were analyzed using two-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* for multiple comparisons. Two-way repeated measures ANOVA was used to compare BBB scores between different injury groups with Bonferroni *post hoc*. T-tests were used to compare ultrasound quantification, H-reflex scores, and evoked potentials. Criteria for statistical significance was $\alpha=0.05$, $\beta=0.20$, and all statistical analyses were performed using SigmaStat software. All values are represented as mean ± standard error of the mean (SEM) except BBB scores, which are calculated as mean ± standard deviation (SD).

Results

Coordinated hindlimb locomotion did not recover after moderate impact-compression SCI

An initial experiment was performed to determine which clip injury force would produce an incomplete lesion with appreciable

sensorimotor deficit with sufficient tissue preservation to permit assessment of recovery for any future treatment studies.

In total, 80 animals were used for this experiment, and 18 animals were excluded: 9.1% 20 g, 22.2% 26 g, 37.5% 35 g, 42.9% 56 g, and 0% sham (Table 1). At 6 weeks, a significant hindlimb functional deficit was observed from moderate clip strength injury (20 g) with BBB score of 8.1 ± 1.5 , to moderate-to-severe clip strength (26 g) with BBB score of 4.6 ± 3.4 , and severe clip strengths (35 g and 56 g) BBB scores of 3.3 ± 1.7 and 2.3 ± 1.9 , respectively ($p < 0.05$, $n = 52$) (Fig. 3).

Rats injured with severe clip strengths of 35 g and 56 g exhibited a recovery plateau of two-joint movement (hip and knee), whereas animals injured with the moderate-to-severe 26 g clip recovered slight to extensive movement of three hindlimb joints (hip, knee, and ankle). In contrast, animals injured with the 20 g clip recovered extensive movement of all three hindlimb joints, with sweeping behavior consisting of patterned flexion/extension activity. No animals within injured groups regained coordinated hindlimb motion or weight supported plantar stepping.

Locomotion, determined by BBB scores, plateaued in all groups by 5 weeks post-injury, and by week 4 in the 20 g injury group. Further, rats in the 26 g, 35 g, and 56 g groups exhibited some observable atrophy of the hip muscles, with the hindlimbs continually held in extension and displayed a lack of flexor/extensor coordination. Moderate and severe clip-induced SCI produced severe deficits functionally similar to a full transection injury, which led us to evaluate the extent of spared functional ascending or descending tracts in the white matter and circuitry within the gray matter.

FIG. 3. Neurobehavioral outcomes. (A) The 35 g ($n=10$) and 56 g ($n=8$) clips produce severe locomotor deficits, and rats only recover to the level of slight movement of two to three joints in the hindlimbs. The 26 g ($n=14$) clip produces a moderately severe injury; however, these animals recover significantly more than animals injured with the 35 g or 56 g clips. In contrast, the 20 g ($n=20$) clip produces a moderate injury, and rats regain extensive movement of all three hindlimb joints. Note that none of the groups recover the ability to bear weight or to coordinate hindlimb motion. Data are represented as mean $\pm$ standard deviation. (B, C) The 20 g clip compression injury caused at-level mechanical allodynia and below level thermal hyperalgesia ($n=12$ injury, $n=6$ sham). Data are represented as mean $\pm$ standard error of the mean. *Statistical significance was accepted for values of $p < 0.05$. Color image is available online at www.liebertpub.com/neu

Injury to the rostral lumbar spinal cord caused pronounced sensory alterations

Marked sensory alterations were observed in both the tail-flick and VFF tests after 20 g injury to the L1-2 lumbar spinal cord. Sensory alterations were not observed in sham-operated animals at any time point. There was no difference in VFF response between the sham ($n=6$) and the 20 g impact-compression group ($n=12$) at 1 week post-injury (8% vs. 18% response, $p=0.62$) (Fig. 3C). Evidence of pathological changes in sensation occurred as early as 2 weeks post-injury (15% vs. 46% response, $p < 0.05$). The SCI group demonstrated significant progressive increases in both sensitivity to mechanical stimuli and temperature over the 6-week period, with the greatest difference at 6 weeks (5.7 sec vs. 16.4 sec latency, and 3% vs. 66% response, $p < 0.001$) (Fig. 3B, C).

Impact-compression injury produced significant cavitation and loss of gray and white matter

Histopathological quantification of all injury strengths was undertaken to determine the degree of tissue sparing between various injury severities in the rostral lumbar spinal cord. We observed a progressive lengthening of cystic cavitation with a concomitant increase in lesional tissue as clip force was increased, which confirms previous reports relating to injury strength and cavitation (Fig. 4A).¹⁸ All injury severities showed atrophy of both the gray and white matter and increasing cavitation at the lesion site with increased injury severity.

The moderate clip force (20 g) produced a lesion measuring 4.2 ± 1.7 mm in length, whereas more severe injuries (26 g, 35 g,

and 56 g) produced injuries of 6.2 ± 2.2 mm, 8.5 ± 2.6 mm, and 10.2 ± 3.1 mm, respectively (Fig. 4). The gray matter was largely obliterated at the lesion epicenter for all clip forces; however, the 20 g injury demonstrated an identifiable rim of spared white matter (Fig. 4B). In more severe injuries, the preserved subpial rim of white matter was absent or almost entirely demyelinated at the lesion epicenter.

The total volume of the lesion generated and the amount of lost gray and white matter were significantly less for the 20 g injured-animals versus the 26 g, 35 g, and 56 g injuries ($p < 0.05$). All injuries were significantly different versus the sham group ($p < 0.05$, $n=10$).

The moderate injury at 20 g was selected for further experiments.

Noninvasive imaging confirmed a defined cavity with tissue sparing in moderate injury at L1-L2

To compare with the histological outcomes, we examined ultrasound and MRI imaging after 20 g clip injury versus sham. At 6-weeks post-injury, ultrasonography revealed no cavitation in the sham injury group (laminectomy only, $n=2$) versus an average cavity volume of 2.64 mm^3 for animals injured with the 20 g clip ($n=3$) (Fig. 5A). Ultrasonography was capable of generating planar full-depth images and 3D reconstruction volumes of cavitation *in situ* (Fig. 5B).

Serial T1-weighted 2D-RARE MRI images were taken in both the sagittal and axial planes allowing complete observation of dynamic changes before and 48 h and 4 weeks after injury (Fig. 6). MR images revealed readily identifiable edema and/or cavitation 48 h post-injury. Axial MRI displayed clear dorsal column

FIG. 4. Histological analysis after clip impact-compression injury at L1-L2 spinal cord level. **(A)** Representative images from each clip strength (sham, $n=7$; 20 g, $n=10$; 26 g, $n=7$; 35 g, $n=6$; and 56 g, $n=5$) stained with Luxol Fast Blue and hematoxylin/eosin 6 weeks post-injury. R and C represent rostral and caudal to the injury site, respectively. As clip strength was increased, the total lesional tissue extended progressively with less preserved tissue. **(B)** Analysis of gray/white matter sparing and lesional analysis displayed as a correlation between clip strength and volume of both spared gray and white matter and lesional tissue, where lesional tissue is defined as fibrous tissue + cavitation. On the horizontal x-axis, (–) and (+) are rostral and caudal to the injury site, respectively. The vertical line at 0 distance represents the injury epicenter. Data are represented as mean $\pm$ standard error of the mean. Color image is available online at www.liebertpub.com/neu

FIG. 5. Ultrasound imaging after 20 g injury shows cystic cavitation *in vivo*. **(A)** shows cavity volume quantified by ultrasound. **(B, C)** shows representative images of rats injured with a 20 g clip-compression versus animals that underwent laminectomy only (“sham”). **(C)** The cavity is fusiform in shape and has a mean volume of 2.64 mm^3 , $n=6$. There is no cavity or damage to the spinal cord in the laminectomy only group (sham), $n=4$. SCI, spinal cord injury.

FIG. 6. Magnetic resonance imaging (MRI) of acute clip impact-compression injury at L1-L2 spinal cord segments. **(A–D)** Serial T1-weighted two-dimensional RARE MRI show changes *in vivo* after spinal cord injury. Red labeling represents vertebral bodies and white represents corresponding axial images. A and B are sagittal acquisitions pre-injury and 48 h post-20 g clip-compression injury of the same animal. The “triangulation” of the spinous processes T9–T11 can be seen in A. Vertebral bodies are labeled, and the injury is seen at the T12 vertebral level (B and C). C is a sagittal acquisition of the same animal 4 weeks post-injury with corresponding axial labels shown in D. **(D)** The distinction between gray and white matter can be seen both rostral and caudal to the injury site, but not at the injury epicenter.

FIG. 7. Acute clip impact-compression injury to L1-L2 caused a significant reduction in motor neurons. (A, B) SCI represents spinal cord injury (clip-compression) whereas sham represents laminectomy-only of T11 and T12 vertebral levels. (C) Choline acetyltransferase (ChAT) positive cells were significantly reduced in the injury group compared with sham over a distance of 7 mm centered on the injury epicenter. On the horizontal x-axis, (–) and (+) are rostral and caudal to the injury site, respectively. (D) Representative axial section stained with cresyl violet with Rexed laminae outlined with black. The area of staining is indicated with a red box. Data are represented as $\pm$ standard error of the mean. Color image is available online at www.liebertpub.com/neu

degeneration rostral to the lesion epicenter, including loss of distinction between gray and white matter at the lesion epicenter.

Clip impact-compression injury to the lumbar spinal cord caused a significant reduction in motor neurons

Animals from the 20 g compression group ($n=7$) and laminectomy-only group ($n=4$) were used to analyze motoneuron loss after clip impact-compression injury. The total number of motoneurons (ChAT-positive cells in laminae 9) within a 7-mm length of spinal cord tissue (3 mm rostral and caudal to the epicenter), corresponding to L1-3 spinal segments, were counted by blinded observers (Fig. 7). The total estimated number of motoneurons was significantly decreased after clip-compression injury compared with sham controls in the lumbar spinal cord ($p<0.05$).

Motor and sensory conduction was reduced, but not eliminated, across the injury site after 20 g injury

Hindlimb MEPs and SEPs were subsequently examined after L1-L2 injury (Fig. 8A, B). At 6-weeks post-injury, the peak amplitude of both MEPs and SEPs was reduced by over half ($n=8$, $p<0.05$) versus sham rats ($n=4$, $p<0.05$) (Fig. 8C). In addition, peak latency was significantly increased in both MEPs and SEPs after injury ($n=8$, $p<0.05$), in comparison with sham animals ($n=4$, $p<0.05$) (Fig. 8D).

L1-L2 injury resulted in an enhanced H-reflex

Animals injured at L1-L2 displayed enhanced excitability of the H-reflex measured by the H/M ratio (H_{max}/M_{max}) (Fig. 8E).

Significant differences between sham and injury groups were observed as early as 1 week post-injury ($p<0.05$) (Fig. 8F). The heightened H-reflex was maintained for 6 weeks. Sham animals showed no heightened H-reflex at any time point after injury.

Discussion

With the primary aim of establishing a clinically relevant and translatable model of lumbar SCI, we used a variety of techniques to test the relation between experimental lumbar injury to human lumbar cord injury. Neurobehavioral testing revealed lasting paralysis and no evidence of weight bearing or hindlimb coordination in any injury severity. Major tissue damage could be reliably observed, both histologically and with *in vivo* ultrasonography and MRI.

Evaluation of sensory outcomes revealed marked mechanical allodynia and thermal hyperalgesia. Deficits in spinal tracts were confirmed by electrophysiological outcomes that showed increased latency and decreased amplitude of both sensory and motor evoked potentials, confirming that the 20 g injury was neurologically incomplete as opposed to complete. An increase in the plantar H-reflex was indicative of an increase in motoneuron excitability, further highlighting the pathological changes after SCI that are indicative of human pathology.²²

Modeling human lumbar SCI

Making the injury at the targeted anatomical level is critical and may be difficult particularly at the thoracolumbar junction. For example, if the rat injury is performed one level rostral at the T10-T11 vertebral level, the injury would result in a lower thoracic SCI, which would not replicate a lumbar SCI.¹⁷ If the injury was made one vertebral level caudal at the T12-T13 level, the injury

FIG. 8. Acute clip impact-compression injury at L1-L2 results in a reduction of motor and sensory evoked potentials and a significant increase in hindlimb H-reflex ratio. Evoked potential studies were completed at end point, 6 weeks after SCI. Representative waveforms of sensory evoked potentials (SEP) are shown in (A) and motor evoked potentials (MEP) in (B). (C) revealed that SEP latency is increased, and the peak amplitude was significantly decreased after the 20 g injury ($n=8$) compared with the sham group ($n=4$). The same result is seen in MEP in (D). Peak amplitude was significantly lower in injured rats versus sham rats: $n=4$ in sham group and $n=8$ in injury group, t test; $p < 0.05$. Representative waveforms of Hoffmann-reflex are shown in (E). The maximal plantar H-reflex/maximal plantar M-response (HMax/MMax) ratios determined the excitability of the H-reflex. (F) revealed that the mean value obtained for maximal H-reflexes in sham-operated animals was significantly lower than those that underwent clip impact-compression injury at L1-L2. The H-reflex is heightened as early as 1 week post-injury and is maintained after injury for 6 weeks. Data for all electrophysiological outcomes are represented as mean $\pm$ standard error of the mean. Statistical significance was accepted for values of $p < 0.05$. Color image is available online at www.liebertpub.com/neu

would represent a caudal lumbar SCI, with animals recovering almost full function of the hindlimbs.⁶ Therefore, significant efforts were taken to ensure a consistent and accurate landmarking procedure.

Using the last rib, iliac crests and the “triangle” of spinous process orientation of the spinous processes at T9-T11, we have generated a reliable landmarking protocol that allows the surgeon to reliably locate the targeted spinal cord location. In clinical management of traumatic SCI, CT is used for visualizing the vertebral column and verification of the injury site. In our pre-clinical model, CT imaging proved to be valuable in confirming the operative level, and we can conclude with confidence that the rats in these experiments were operated at the correct level.

Further to visualizing the bony vertebral column, MRI provides additional value for differential characterization of tissue abnormalities across acute and chronic phases of injury. At post-acute time points, regions of tissue scarring and cystic cavitation may appear with differential image contrast depending on the severity of hemorrhage.²³ In pre-clinical models of clip compression, MRI can

show biomarkers for longitudinal monitoring of injury evolution, including the increasing volume of lesional tissue, which is visualized as hypointense volume in our T1-weighted image sets at both 48 h and 4 weeks.²³ Quantitative biomarkers (e.g., magnetization transfer, diffusion tensor imaging) may also report on white matter quality, which has been reported.²⁴

Further, as intraoperative spinal sonography (ultrasound) is used to evaluate the gross pathology during surgical procedures, we used this technique for *in vivo* quantification of spinal cord cavitation.²¹ Quantitative ultrasonography revealed the heterogeneity of the *in vivo* spinal cord cavity (that consists of numerous interconnected cavity pockets; see Fig. 5 cross-sectional images) without the morphological alterations that may occur during tissue processing for histology. The injuries had a mean cavity volume of 2.64 mm³ for 20 g injury in comparison with no cavitation in the sham group.

Based on both the functional and neuroanatomical outcomes (Fig. 3–8), the 20 g clip injury produced a clinical injury related to an American Spinal Injury Association Impairment Scale (AIS)-C/D

human injury severity where some functional connections persist and there is not a complete loss of motor and sensory activity in S4–S5 spinal segments. Conversely, the 35 g and 56 g clips produced a “severe” injury, which can be clinically related to an AIS-B injury. Even with complete transection injuries, rats show considerably more hindlimb movement and functional recovery than patients do, suggesting that rats cannot be AIS-A.²⁵

Tissue alterations and lesion dynamics

Cystic cavitation was reliably produced by the 20 g clip, which is a common histopathological feature in patients with traumatic SCI. Similarly, some white matter was spared in the 20 g injury comprising approximately 35% of the remaining tissue area. Interestingly, the 26 g clip produced injured cords that, when observed post-fixation, maintained significant preserved subpial rim with either obvious cystic cavitation, or a collapsed cavity.

Severe injuries caused by the 35 g and 56 g clips produced injuries with significantly reduced subpial rim and consistently collapsed cystic cavities. The collapsed cavities appear to be caused by extensive atrophy involving most of the cross-section of the spinal cord and a loss of turgidity within the fluid-filled cavity. As other studies have shown, it appears that the loss of gray matter plays a more important role in determining the clinical effects of injuries to the lumbar spinal cord.⁶

Another histological finding is the substantial white matter loss in the dorsal funiculus (including the corticospinal tracts) and relative sparing of the lateral funiculus, even after application of the least severe 20 g clip force (Fig. 4). In rodents, the corticospinal tracts are located in the dorsal funiculus as opposed to the lateral funiculus in humans. Further, in rats, the rubrospinal tract does not descend as low as the lumbar spinal cord.²⁶ Both tracts have been shown to be involved in fine motor movement of the forelimbs, but have not been shown to be significantly involved in gross hindlimb movement.²⁶

A possible explanation for this histological finding: the orientation that the clip is applied to the exposed spinal cord. The forces are angled in the vertical orientation; therefore, the lateral funiculi are not exposed to as much force as the dorsal and ventral funiculi, resulting in an injury that spares the lateral funiculus more so than the dorsal funiculus.

Neurobehavioral recovery

Hindlimb activity was highly dysfunctional after clip impact-compression injury to the rostral lumbar spinal cord, resulting in enduring paralysis in animals injured by all clip forces. There was no evidence of recovery of coordinated hindlimb motion or weight-supported stepping in any of the injury groups (Fig. 3). The relationship between clip force and SCI level is summarized in Table 3.

Our laboratory has shown previously that injury caused by the same clip force at vertebral level T2 or T8 caused significantly less severe locomotor deficits as indicated by the BBB, with the majority of animals regaining coordinated hindlimb motion and weight-supported stepping.^{27–29} Impact-compression SCI induced by a 35 g clip applied to T2, T7, and L1–2 produced BBB scores of 10, 9, and 3.3, respectively.^{17,29} Lumbar SCI also produces clear dysfunction in the flexor/extensor pattern, as demonstrated in all injury groups.

Motoneurons within L1 and L2 supply flexor muscles of the thigh, with hamstring and gluteal muscle innervation starting at L3 and L4, respectively.^{30,31} By eliminating some flexor motoneuron pools in L1 and L2 without ablating extensor motoneuron pools

TABLE 3. LOCOMOTOR SCORE AFTER ACUTE CLIP IMPACT-COMPRESSION INJURY OF VARYING MAGNITUDE AT DIFFERENT SPINAL CORD LEVELS IN RATS

Reference	SCI level	End point	Clip force	BBB score
Nori ⁴⁹	C6	8 weeks	18 g	11.3
Rivlin ¹⁶	T2	4 weeks	20 g	12
			26 g	12
			35 g	10
			56 g	9
Kypers ²⁶	T7	8 weeks	35 g	9
Current study	L1–L2	6 weeks	20 g	8.1
			26 g	4.6
			35 g	3.3
			56 g	2.3

SCI, spinal cord injury; BBB, Basso, Beattie and Bresnahan.

more caudally, hindlimbs of injured animals were held in constant extension.^{30,32} These results serve to confirm the proper and specific localization of the injury, as well as the pre-supposition that an anatomically intact lumbar spinal cord is a pre-requisite to achieving weight-supported hindlimb stepping and coordinated hindlimb motion.

It is important to note, however, that the motoneuron pools for the thigh, hamstring, and gluteal muscle groups extend rostrocaudally over greater distances than are injured in the focal L1–2 clip impact-compression injury.³¹ Therefore, no muscle group loses significant enough motoneurons for complete denervation and loss of functionality.^{6,31} We hypothesize that partial motoneuron loss at L1–L2 (Fig. 7), in addition to the loss of interneurons of the central pattern generator, accounts for the persistent loss of coordinated hindlimb motion and plantar stepping in the hindlimb after clip impact-compression injury at this level.

The primary goal of this study was to produce a clinically relevant model of incomplete lumbar SCI. Using electrophysiology, we further demonstrated that MEPs and SEPs, although abnormal, were still transmitted through the spinal cord. In humans, only 5% of injuries to the thoracolumbar spinal cord are functionally complete, and preservation of MEPs and SEPs after 6 weeks post-injury is indicative of an incomplete lesion.³

Evoked potentials are an important complement to histological techniques in SCI research, because it is not possible to directly determine which region of the spared white matter, if any, is functionally propagating electrical signals. Further, in the lumbar model article by Magnuson and coworkers,⁶ the transcranial magnetic MEPs were absent in all injury levels and all injury severities. We have shown that in our model, SEP and MEPs can be used to quantify sensory and motor tract integrity.

The heightened H-reflex, and therefore H/M ratio, is indicative of disinhibition of the 1a alpha motor neurons and can indicate the presence of clinical spasticity in the hindlimbs.²² Spasticity, or the velocity-sensitive increase in resistance to manipulation, is a common side effect of SCI experienced in an estimated 65–78% of all patients with SCI.³³ Interestingly, the H-reflex is higher in the L1–L2 model than other thoracic or cervical injuries of similar severities and injury mechanisms.²² This may be because of a decrease in inhibition from upper motor streams in the brain as well as segmental inhibition from the L1 and L2 spinal cord segments that inhibit lower lumbar segments.⁸

In human patients with SCI, injury to the lumbar spinal cord typically involves many of the lumbar segments (L1–L5) because, in most persons, the entire lumbar cord is opposite the T12 vertebral

body, which is a common site of injury. Conversely, the experimental injury we have generated is at the T11–12 vertebral level, in which only two lumbar cord segments are injured.

Lumbar injury in humans results in flaccidity, because most of the motoneurons innervating the muscles of the legs originate in the lumbar segments that are damaged. In the rat, however, the hindlimb muscles are innervated by a longer motor pool spread throughout more spinal cord segments than in humans.^{31,34} The H-reflex confirms the presence of an upper motor neuron injury in this model, which is well described throughout the human and animal literature.²²

We observed significant at-level sensory alterations after clip impact-compression injury, which mimics similar outcomes seen in many lumbar root avulsion models.^{35–37} Root avulsion models are designed to replicate the rare clinical scenario where the root is avulsed without an accompanying SCI. Many peripheral nerve injuries are mixed injuries including both a CNS spinal cord component and a PNS peripheral component, such as those seen in injuries to the thoracolumbar spinal cord at T10–T12 vertebral level. Because the spinal cord in the rat extends over a larger number of vertebral segments in comparison with the human lumbar cord, it is reasonable to suggest that we have damaged some spinal roots in this model, although no other data support this conclusion.³¹

The Magnuson group⁶ was the first to describe a traumatic lumbar SCI in the rat, investigating behavioral and histological outcomes after traumatic injury to various lumbar spinal cord segments. Chemical lesions in the lumbar spine have been shown to produce similar exaggerated locomotor deficits; however, these models do not produce the forces or mechanisms of traumatic SCI and therefore do not produce many of the key components of human SCI and potential therapeutic targets.^{5,8}

The impact-compression lumbar SCI model we have described should be useful for therapeutic studies. Lumbar spinal cord segments are an active area of SCI research, including recent studies investigating therapeutic and curative epidural stimulation in both the rat and the human.^{38–41} In addition to epidural stimulation, treadmill and other rehabilitative therapies have harnessed the intrinsic lumbar spinal cord circuitry after injury to a higher spinal level (cervical or thoracic).^{42,43}

It would be of interest to examine these techniques in using spared gray/white matter after lumbar SCI to determine efficacy when the lumbar circuitry is the site of injury. This could provide a distinction between the capability of therapies to return lost function, or simply lower the activity threshold for circuitry that was not included in the injury. Further, because of the interneuron populations within the lumbar neuronal circuits and CPG, our model may be ideal for neuronal transplant studies as we and others have attempted previously.^{14,44–49}

The model we have characterized could also be a unique opportunity for pharmacological therapies attempting to reduce pain after SCI.^{50–52} It is possible that the described model replicates a more complete picture of the pathology occurring after traumatic SCI, potentially involving nerve roots, and would therefore represent an opportunity to test drugs in a more clinically relevant manner.

Conclusion

The above experiments demonstrate a bilateral clip impact-compression injury that reasonably mimics injuries to the lumbar spinal cord at the thoracolumbar vertebral level in humans. The current model addresses a gap in the literature by describing a clinically relevant lumbar SCI model that includes a comprehen-

sive neuroanatomical and neurobehavioral characterization of the injury. The lumbar spinal cord represents an ideal region to investigate clinically oriented therapies such as replacement and repair of functional neuronal circuitry with the use of stem cell therapies and/or rehabilitative strategies.

Acknowledgments

The authors would like to acknowledge Joe Moonen for his artistic contributions, Rita Van Bendegem and Linda Lee for their technical assistance, and Dr. Nicole Forgiione for her advice. We also thank the Crothers Family Fellowship in Neuroscience, the Unilever/Lipton Fellowship, the Krembil Foundation, Toronto General and Western Hospital Foundation, Spinal Cord Injury Ontario, and the Ontario Neurotrauma Foundation for support.

Author Disclosure Statement

No competing financial interests exist.

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