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The Effect of Whole-Body Resonance Vibration in a Porcine Model of Spinal Cord Injury

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Abstract

Whole–body vibration has been identified as a potential stressor to spinal cord injury (SCI) patients during pre-hospital transportation. However, the effect that such vibration has on the acutely injured spinal cord is largely unknown, particularly in the frequency domain of 5 Hz in which resonance of the spine occurs. The objective of the study was to investigate the consequences of resonance vibration on the injured spinal cord. Using our previously characterized porcine model of SCI, we subjected animals to resonance vibration (5.7 ± 0.46 Hz) or no vibration for a period of 1.5 or 3.0 h. Locomotor function was assessed weekly and cerebrospinal fluid (CSF) samples were collected to assess different inflammatory and injury severity markers. Spinal cords were evaluated histologically to quantify preserved white and gray matter. No significant differences were found between groups for CSF levels of monocyte chemotactic protein-1, interleukin 6 (IL-6) and lL-8. Glial fibrillary acidic protein levels were lower in the resonance vibration group, compared with the non-vibrated control group. Spared white matter tissue was increased within the vibrated group at 7 d post-injury but this difference was not apparent at the 12-week time-point. No significant difference was observed in locomotor recovery following resonance vibration of the spine. Here, we demonstrate that exposure to resonance vibration for 1.5 or 3 h following SCI in our porcine model is not detrimental to the functional or histological outcomes. Our observation that a 3.0-h period of vibration at resonance frequency induces modest histological improvement at one week post-injury warrants further study.

Key words: cytokines; porcine model; resonance vibration; spinal cord injury

Introduction

The neurologic impairment resulting from spinal cord injury (SCI) is initiated by the primary mechanical damage and then followed by the delayed progression of pathophysiological processes that continue for weeks to months. This devastating condition has been the subject of considerable research efforts, and significant advancements in its medical, surgical, and rehabilitative management have been achieved. While this has resulted in greater life expectancy and improved long-term outcomes, much still needs to be done to improve the neurologic prognosis of this injury.[1,2]

The care of the spinal cord–injured individual begins with careful field extraction and expeditious transport to a hospital, typically by ground and/or airborne vehicles.[3–5] It is widely agreed that every effort should be made during such transport to minimize further mechanical damage to the spinal cord from the potentially unstable spinal column. Despite widespread adoption of standard spinal immobilization techniques during transport, one poorly understood factor that may impose additional mechanical stresses on the injured spinal cord is the vibration generated by the different modes of transportation (e.g. ambulance, helicopter, or fixed-wing aircraft). Such vibration may induce repetitive displacement of the spinal column, inducing further traumatic stress on the injured spinal cord, which may be tightly compressed by unreduced bone fragments.

Typically, transportation-related vibration occurs within a range of frequencies between 0.5 to 80 Hz and causes cyclical motion of the body at its interface(s) with the vibrating structure.[6] The frequency at which the amplitude of this displacement is at its maximum is defined as the ''resonance'' frequency. Because of this maximal displacement, the application of resonance frequency vibration has the greatest potential to cause further mechanical damage to the injured and compressed spinal cord.[7] The resonance frequency for vibration in the vertical direction is measured in the range of 4–8 Hz for both standing and seated individuals and 5.0 Hz lumbar spine of supine humans.[8–14]

The potential impact of vibration on acute SCI is unknown but is of great concern to first-responders and acute-care clinicians. For

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instance, previous studies on helicopter vibration have measured significant energy in some helicopter vibration signals at 5 Hz, which is close to the reported resonance frequency of the human lumbar spine.[8,9,15,16] Further, individuals who sustain an acute SCI are typically immobilized using a rigid collar and backboard during transport.[17] The litter or spine board supporting the patient is in direct contact with the transport vehicle, either by being placed on the floor or by being secured to a rack attached to the vehicle frame. Although, current immobilization designs may act as a low-pass filter to attenuate the overall transmission of high frequency vibrations occurring during transportation, they also may be ineffective in the resonance range of the spine and may even amplify the spine vibrations below resonance.[15] The absorption of such vibration by the spine, particularly at the resonance frequency, may result in increased motion in the local vertebrae and intervertebral tissues, and this may result in further motion of or mechanical damage to the spinal cord.[8]

Depending on its frequency, amplitude, and duration, exposure to vibration may have different but profound effects on specific parts and systems of the body. Most of the documented negative effects of vibration have been observed in a frequency range of approximately 5 to 25 Hz, or in frequencies that exceed 70 Hz.[6] Current data suggest that during neonatal transport, newborns are exposed to considerable mechanical vibration, which might be a potential risk factor for cerebral bleeding among very–low-birth-weight infants.[18–20] Grosek and colleagues found an association between ground transportation and increased heart rate and peripheral blood leukocyte counts in neonates.[21] Although the factors leading to these increased risks are complex, one possible cause is mechanical vibration encountered by the infant during over-ground ambulance transport. Such vibration is typically in the low frequency range of 3 to 18 Hz and overlaps the physiological resonance range.[22,23]

Within the setting of a compromised spinal cord, the effects of vibration on further injury remains poorly understood. Depending on the injury suffered, vibration during transport may have serious consequences for individuals who suffered SCI. However, to what extent and under what conditions this might occur has not been explored previously—particularly in an in vivo setting. Therefore, in this study, we investigated the effect of subjecting the traumatically injured spinal cord in a prone porcine model to resonance frequency vibration. We used our previously established Yucatan minipig model of SCI for this project. We have previously characterized the functional, histological, and biochemical effects of SCI in this model.[24]

While vibration around these frequencies is not always present during transportation, given that the amplitude of spine motion is greatest at frequencies of 5–7 Hz, even a short exposure to such vibration could plausibly result in some adverse effects. We hypothesized that resonance frequency vibration would be detrimental to the functional outcome after SCI and that this would be reflected in biochemical and histological analyses. In addition to analyzing the functional recovery and performing histological analyses of the spinal cords, we also measured a number of inflammatory cytokines and biomarkers of injury severity within the cerebrospinal fluid (CSF), as we have previously reported in human spinal cord injury.[25]

Methods

All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee and the US Army Medical Research and Materiel Command Animal Care and Use Review Office.

Animals and experimental design

Female miniature Yucatan pigs (Sinclair Bio-resources, Columbia, MO) weighing 20 to 30 kg were group housed at our large animal facility for five weeks before surgery. All animals were subjected to experimental SCI as described below. Animals were block-randomized into the following groups: i) non-vibrated control group ($n = 12$)—these animals were not subjected to vibration after SCI; ii) 1.5-h resonance group ($n = 8$)—these animals were subjected to 1.5 h of whole–body vibration at resonance frequency between 5 and 6.6 Hz after SCI; and iii) 3.0-h resonance group ($n = 12$)—these animals were subjected to 3.0 h of whole–body vibration at resonance frequency between 5 and 6.6 Hz after SCI. For both vibration groups, vibration at resonance frequency was commenced 30 min after contusion injury to the spinal cord to simulate a delay that would occur in deploying transport to the field to pick up the injured patient.

For the 3.0-h resonance group, four of the 12 animals were euthanized at 7 d post-injury (''acute 3.0-h'' group) and the remaining eight animals were euthanized at 12 weeks post-injury (''chronic 3.0-h'' group). Similarly, for the non-vibrated control group, four of the 12 animals were euthanized at 7 d post-injury and the remaining eight animals were euthanized at 12 weeks post-injury. See Supplementary Table 1, Supplementary Table 2 and Figure 1 for a more detailed overview of the experimental grouping and study design. (See online supplementary material at www.liebertpub.com.)

Porcine model of traumatic SCI

Exposure of dura and spinal cord. Animals were preanesthetized with an intramuscular (IM) injection of Telazol (4–6 mg/kg; Pfizer Animal Health, New York, NY) and xylazine (0.6 mg/kg; Rompun, Bayer Canada Inc., Toronto, Ontario, Canada), endotracheally intubated, and maintained on isoflurane (2 – 2.5% O_2) for the entire procedure. Mechanical ventilation was sustained at 10–12 breaths/min and the tidal volume at 10–12 mL/kg (Veterinary Anaesthesia Ventilator model 2002; Hallowell EMC, Pittsfield, MA) and hydration was maintained with intravenous (IV) Lactated Ringer's solution. Before and during surgery, pigs received buprenorphine (IV) injections (0.1 mg/kg) for analgesia. Body temperature was maintained at $\sim$37.0°C degrees throughout the surgical procedure.

Animals were placed in the prone position and a 13 cm dorsal midline incision was made between T8 and T14. The fascia was divided and the semispinalis, multifidus, and longissimus lumborum muscles were stripped from the dorsal spinous processes, laminae, and transverse processes of T9 to T13 using electro-cautery (Surgitron® Dual Frequency RF/120 Device; Ellman International, Oceanside, NY). Using anatomic landmarks, the T9, T10, and T11 pedicles were cannulated and instrumented with 4.5 × 35 mm screws (Select™ Multi Axial Screw, Medtronic, Minneapolis, MN). A T10 laminectomy was performed and widened to ensure that a circular window was made measuring at least 1.2 cm in diameter to expose the dura and spinal cord.

CSF catheter placement

CSF collection was achieved using a 19 gauge epidural catheter (Perifix epidural catheter set; Braun Medical Inc., PA). A needle was used to puncture a hole in the dura approximately 5 cm caudal to the anticipated injury site. Immediately after, the catheter was inserted into the intrathecal space so that the catheter tip would rest 2 cm caudal from the injury epicenter. A small tapered collar was threaded onto the catheter prior to placement in order to act as a ''plug'' at the dural puncture site. A small amount of acrylic glue was applied to the collar and dura interface to prevent CSF leakage

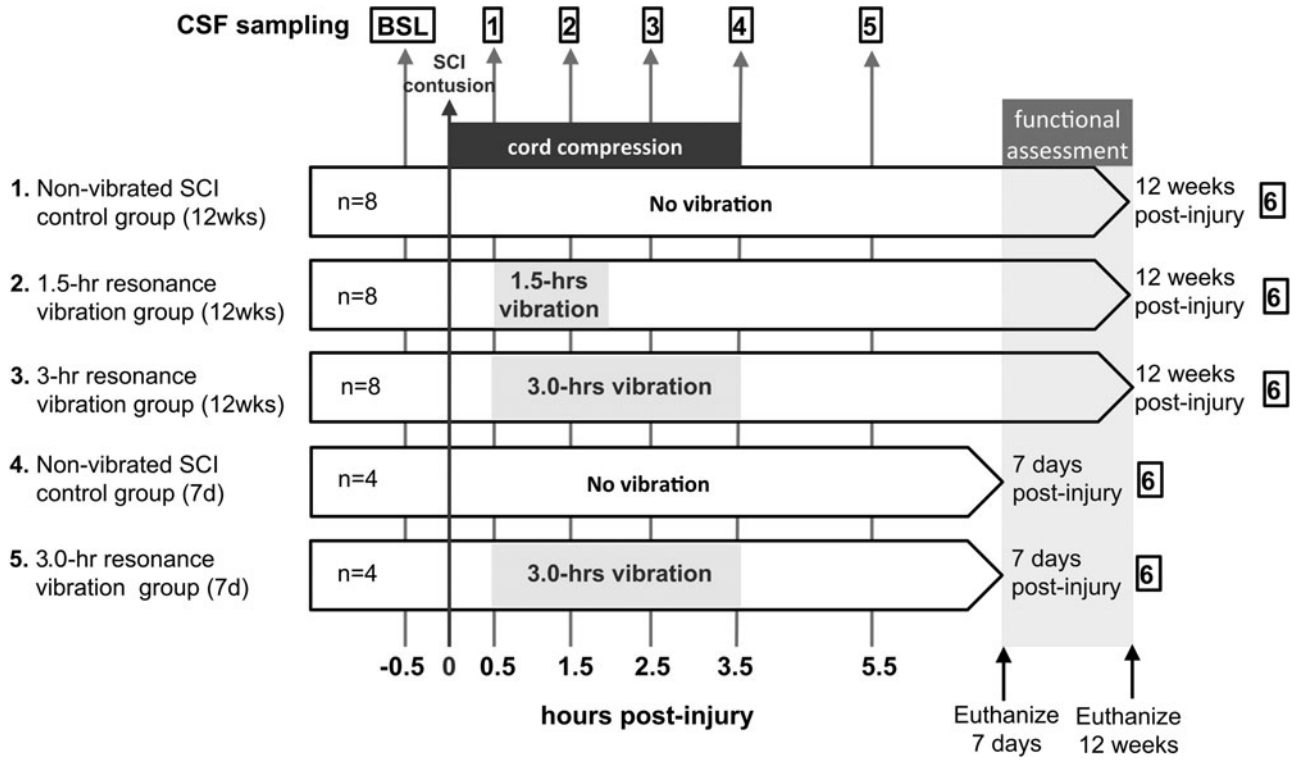

FIG. 1. Schematic of experimental design. Following contusion of the spinal cord, animals were either: 1) non-vibrated spinal cord injury (SCI) group ($n=8$); these animals remained undisturbed after SCI; 2) 1.5-h resonance vibration group; animals were subjected to 1.5 h of whole–body vibration at resonance frequency at 5.21 ± 0.02 Hz (total $n=8$); or 3) 3.0-h resonance vibration group ($n=8$); animals were subjected to 3.0 h of whole–body vibration at resonance frequency at 5.72 ± 0.17 Hz. For group 1–3, animals were euthanized at 12 weeks post-SCI. To determine the acute consequences of 3.0-h resonance vibration, two additional groups were added: 4) non-vibrated SCI control group ($n=4$) and 5) 3.0-h resonance vibration group ($n=4$)—both euthanized at 1 week post-SCI. For all groups, vibration was commenced 30 min after contusion injury to the cord. Before SCI (baseline [BSL]) and multiple time-points after SCI (samples 1–6), cerebrospinal fluid (CSF) was collected for the analyses of different cytokines, chemokines and spinal cord proteins. The final CSF sample was collected at the end of the study, either at 1 week or 12 weeks post-SCI.

and the catheter from being pulled out. The other end of the catheter was attached to a low volume titanium CSF port (LOVOL MID port; Instech Solomon, PA) and placed outside of the surgical site for CSF sample collection. A sterile 22-gauge Huber needle (Instech Solomon, PA) was used to access the port for withdrawal of CSF (collection times: 0 min, 30 min, 1.5 h, 2.5 h, 3.5 h, and 5.0 h post-injury and at the end of the study, either 7 d or 12 weeks post-injury; Fig. 1).

Spinal cord injury. The spinal cord was injured with a custom weight-drop device.[24] The rail that guides the weight drop impactor to the spinal cord was rigidly secured to the pedicle screws. The rail was adjusted and vertically positioned so that the impactor (50 g, flat cylindrical-shaped tip, with a diameter of 9.53 mm) would fall directly on the exposed dura and spinal cord. The tip of the impactor was instrumented with a load cell (LLB215; Futek Advanced Sensor Technology, Irvine, CA) to record the force at impact. Thirty minutes before impact, another half dose of buprenorphine was given (0.005 mg/kg). Just prior to injury, mechanical ventilation was ceased briefly to minimize movement of the animal and spinal cord within the intrathecal space during the actual drop. Then, the weight was dropped from a height of 20 cm (Supplementary Fig. 1A; see online supplementary material at www.liebertpub.com). Immediately following the contusion injury, compression was applied by placing a 100 g mass on top of the impactor for 5 min. After the compression, the weight-drop apparatus was removed and a curved plastic spacer was inserted between the dura and the laminae to simulate ongoing extradural compression (Supplementary Fig. 1B–C). The contact area of the spacer and spinal cord was approximately 50 mm^2. After exposure to vibration had ceased (described in detail below), the spacer was removed.

Accelerometers and vibration application

Each pig was placed in a prone position on a long spine board (50-0013, NSN# 6530-01-490-2487; North American Rescue LLC, Greer, SC; Supplementary Fig. 2A-B; see online supplementary material at www.liebertpub.com). Three tri-axial accelerometers (CXL04GP3; Crossbow Inc., San Jose, CA) were mounted to the T9, T10 and T11 pedicle screws (Supplementary Fig. 2C). Two straps (50-0027, North American Rescue LLC) were positioned over the shoulder and hip area of the animal to secure the animal and spinal board to a standard military litter (100047; Arizona Industries for the Blind, Phoenix, AZ). Tension in the seatbelts was maintained across animals. Vibration was controlled by an electromechanical shaker (LDS V455; Bruel & Kjaer, Nærum, Denmark).

Briefly, two rectangular frames made from steel unistrut were mounted on top of each other. The lower frame supported the weight of the upper frame, pig, spine board and litter. The upper frame was attached to the shaker through a threaded rod and rested on four springs located at each corner of the lower frame. The litter was attached to the upper frame. One single-axis accelerometer (786A Wilcoxon; Meggitt Sensing Systems, Germantown, MD) was attached to the upper frame as feedback to the shaker. Four accelerometers were attached to the upper frame to verify that the

direction of the vibration was mostly vertical. One accelerometer was attached to the bottom of the spine board to assess the vibration that was delivered to the litter.

The resonance frequency of the animal spine board–litter system was established for each animal based on the signal from the spine-mounted accelerometers. The animals were subjected to a range of vibration frequencies from 4 to 7 Hz. The acceleration amplitudes of the spine-mounted accelerometers were monitored in real-time. When the maximum amplitude was observed, that frequency was determined to be the resonance frequency for that animal. This resonance frequency was used to vibrate the animals for 1.5 or 3.0 h, beginning 30 min after SCI. The control group went through the exact same surgical and handling procedures but was not subjected to any vibration.

CSF collection

A total of 300 μL of CSF was collected 15 min before injury and 30 min following injury. Once the electromechanical shaker was switched on, additional CSF samples were collected at 1.0, 2.0, 3.0, and 4.5 h after vibration had commenced (i.e., 1.5, 2.5, 3.5, and 5.0 h post-injury; Fig. 1). Animals were euthanized either at 7 d or 12 weeks post-injury, at which point the last sample was collected. CSF samples were collected at the same time frame for the non-vibrated control groups. Immediately after collection, samples were spun at 1000 g for 10 min at room temperature. The supernatant was immediately frozen on dry ice and stored at -80°C until examined.

Post-surgical treatment and care of the animals

Post-operatively, the animals received diazepam (0.5–1.0 mg/kg) to calm them at recovery. Buprenorphine (0.01 mg/kg) was administered to the pigs every 8 h for 3 d. Morphine (0.25 mg/kg) was administered subcutaneously, and tramadol (1 mg/kg) orally as analgesics for the first two days. Tramadol was continued for up to 5 d or as needed. Enrofloxacin was administered once a day (IM) for 5 d as an antibiotic, and ketoprofen (3 mg/kg) was given once a day (IM) for 3 d as an anti-inflammatory agent. Animals were monitored every 2 h for the first 48 h. Typically, the animals were housed individually for the first 24 h, after which they were housed in pairs. The urinary catheter was removed 7 to 10 d after SCI, after which the animals were able to reflexively empty their bladders.

Porcine Thoracic Injury Behavior Scale

To assess hindlimb motor function, the Porcine Thoracic Injury Behavior Scale (PTIBS) was used, as previously described by Lee and colleagues[24] (Supplementary Fig. 3; see online supplementary material at www.liebertpub.com). Briefly, one week after the animals arrived at the facility, they were trained to walk cross a pathway on a rubber mat (4×1.22 m) at a constant speed without hesitation. A food reward was given after each run for motivation. To obtain baseline behavior for each animal, one week before surgery, five runs were recorded with three high-definition camcorders placed 30 cm above the ground and behind the animals. Functional assessment resumed one week post-injury and continued once weekly for 12 weeks. Later, the video footage was played in slow motion and the hindlimb functions were rated on a scale of 1 to 10 by two observers (a score of 1 is no movement of hindlimbs and 10 is normal ambulation).[24]

Enzyme-linked immunosorbent assay

CSF samples were analyzed for interleukin 6 (IL-6; Cat. No. P6000, R&D Systems, Inc., Minneapolis, MN), IL-8 (Cat. No. P8000; R&D Systems, Inc., Minneapolis, MN), monocyte chemotactic protein-1 (MCP-1; Cat. No. E101-800; Bethyl Laboratories Inc., Montgomery, TX) and glial gibrillary acidic protein (GFAP; Cat. No. RD192072200R; BioVendor, Candler, NC) levels using enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Immunohistochemistry

Tissue preparation. At the end of the experiment, animals were euthanized with an overdose of Sodium Pentobarbital (107 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). A 10 cm segment of thoracic spinal cord centered at the injury site was collected, post-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 48 h, and cryoprotected in steps of 12%, 24%, and 30% sucrose in PBS. Then, the spinal cord segment was further divided into 5×1 cm blocks in length (centered on the injury site) and frozen on dry ice and stored at -80°C. For each segment, 20-μm thick cryosections were taken every 400 μm and mounted onto a series of silane-coated glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA).

Quantification of white and gray matter sparing. For differentiating gray and white matter, Eriochrome Cyanine R histochemistry (EC; Sigma-Aldrich, St. Louis, MO) was performed on spinal cord sections, which specifically stains myelin sheaths blue.[26] Briefly, sections were dehydrated in an ethanol series, cleared in xylene, rehydrated in a reverse ethanol series followed by distilled water (dH_2O), then left in a solution containing 0.16% Eriochrome Cyanine R, 0.5% sulphuric acid and 0.4% iron chloride (in dH_2O) to stain myelinated fibers. Following staining, sections were differentiated in 0.5% ammonium hydroxide. After differentiation, the gray matter was counterstained in Neutral Red then rinsed in dH_2O. Finally, sections were dehydrated and cleared, as above, and then mounted using Permount mounting medium (Fisher Scientific, Fair Lawn, NJ).

EC-stained sections were examined using a Zeiss AxioImager M2 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) using a $5\times$ objective and pictures were taken of sections at 800-μm intervals throughout the lesion site. Images were analyzed using Zen Imaging Software (Carl Zeiss Canada Ltd., Toronto, ON, Canada) by manually tracing of the spinal cord perimeter and spared tissue for each image captured. This yielded the total area for each of these measurements. The lesion area was identified by the loss of EC staining, as well as severe tissue disruption. The lesion epicenter was defined as the section from each animal with the least amount of white matter sparing and quantification was carried out at 800 μm intervals from 14.4 mm caudal to 14.4 mm rostral to the epicenter. The spared white matter was defined as the areas that were stained for EC, whereas gray matter was considered spared when it was a stereotypic light gray color with a consistent neuropil texture containing neuronal and glial cell bodies. The percent white matter and gray matter were calculated by dividing the spared white or gray matter by the total area of the spinal cord on a given section.

Statistical analysis

All data are reported as mean $\pm$ standard error of the mean. Group differences in PTIBS, CSF cytokine expression, and amount of spared spinal cord white and gray matter within a single time-point were assessed with a Kruskal-Wallis test followed by Dunn's test for post-hoc analysis. Generalized Estimating Equation analysis was used to analyze the amount of spared spinal cord white and gray matter between groups.[27]

To determine the sample sizes necessary to uncover 1-point difference on PTIBS between non-vibrated control and vibrated animals, a post-experimental power analysis was run on the PTIBS scores (two-tailed t-test; α, 0.05; power, 80%). For all statistical analyses, we used the GraphPad Prism software (version 6.0; GraphPad Software Corporation, San Diego, CA). Values of $p \leq 0.05$ were considered significant.

Results

Injury force

All animals received a contusion injury by dropping a 50 g mass from a 20 cm height at the T10 level of the spinal cord. For the 12-week survival experiment, the impact force applied to the exposed spinal cord measured at the tip of the impactor was 3352.8 ± 114.5 kdynes, 3215.4 ± 173.7 kdynes, and 3314.6 ± 156.7 kdynes for the non-vibration control, 1.5-h resonance vibration, and 3.0-h resonance vibration groups, respectively (Supplementary Table 1 and Supplementary Table 2). Similar impact forces were recorded in the 7 d acute survival experiment (2706.2 ± 336.2 kdynes and 2930.6 ± 391.2 kdynes for the non-vibrated control and 3.0 h resonance vibrated groups, respectively). The impact force did not significantly differ between groups ($H = 4.439$; $df = 2$; $N = 32$; $p = 0.350$).

Resonance frequency

Whole–body vibration was provided by an electromechanical shaker. The spine resonance frequency for each animal was determined by testing a range of frequencies from 4 Hz to 7 Hz to identify the frequency at which the greatest vertical displacement (amplitude) of the spine occurred.

In the 12-week survival experiment, the average resonance frequency was 5.21 ± 0.02 and 5.72 ± 0.157 Hz for the 1.5-h and 3.0-h resonance vibration groups, respectively. In the 7-d survival group subjected to 3 h of resonance vibration, the average frequency was 5.23 ± 0.02 Hz (Supplementary Table 1 and Supplementary Table 2). No significant differences were observed for the resonance frequencies between the three vibration groups ($H = 2.05$; $df = 2$; $N = 20$; $p = 0.379$).

Resonance vibration resulted in 12.01 ± 1.21 mm, 8.49 ± 0.99 mm, and 17.84 ± 1.36 mm displacement of the spine in the anterior-posterior direction for the 1.5-h resonance vibration, 3.0-h resonance vibration 7-d survival and 3.0-h resonance vibration 12 week survival group, respectively. Between the two 3.0-h resonance vibration groups, a significant difference was noted in displacement ($H = 11.19$; $df = 2$; $N = 20$; $p = 0.001$) with a higher displacement in the 7-d survival group, compared with the 12-week survival group (Dunn's test; $p = 0.003$). There were no statistical differences found among the 1.5-h resonance vibration group and both 3.0-h vibration groups.

Effect of resonance vibration on locomotor function over 12 weeks

The 10-point PTIBS developed by Lee and colleagues[24] was used to assess locomotive recovery following SCI in our pigs (Supplementary Fig. 3). After SCI, there was a dramatic impairment of hindlimb function evident by the reduction of PTIBS scores in all groups. The mean PTIBS scores for the non-vibrated SCI control group, 1.5-h resonance vibration group and the 3.0-h resonance vibration group are shown in Figure 2. In the first five weeks

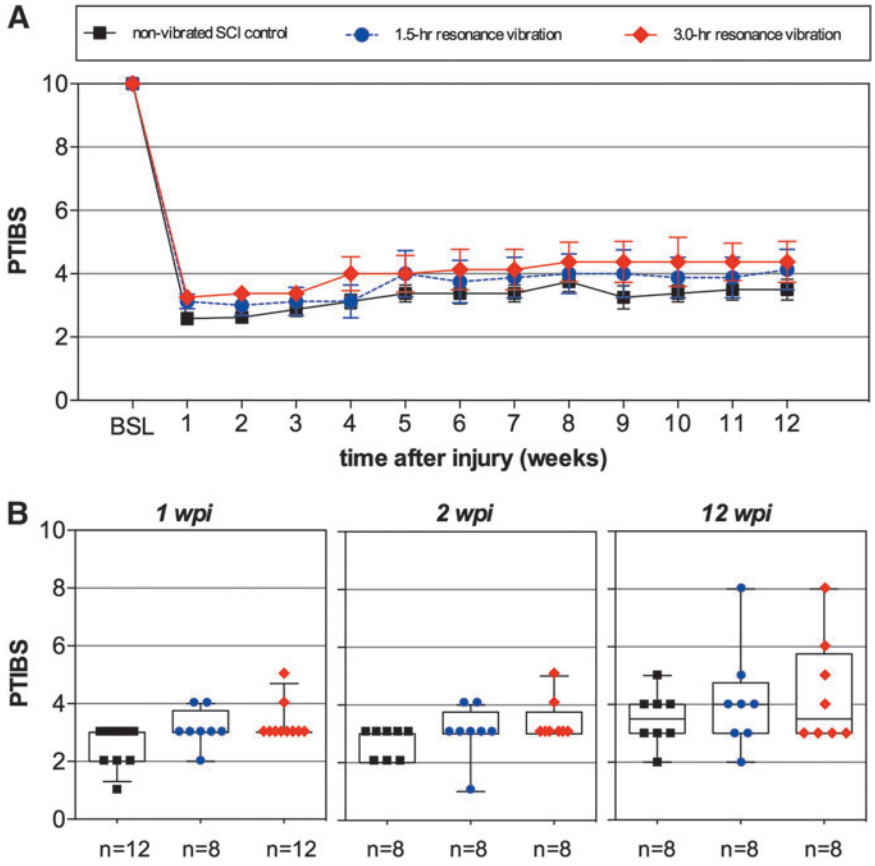

FIG. 2. Effect of resonance vibration on locomotor function during overground walking following spinal cord injury (SCI). **(A)** Locomotor function in the non-vibration SCI control group, 1.5-h resonance vibrated group and 3.0-h resonance vibration group as determined by PTIBS (Porcine Thoracic Injury Behavior Scale) testing. All values represent means ± standard error of the mean. **(B)** Scatter plots of individual values for PTIBS score for week 1, 2, and 12 post-injury. Box plots displaying the median value, 25th and 75th percentile. Whiskers represent 5th-95th percentile and dots the individual animals. Color image is available online at www.liebertpub.com/neu

after the injury, the non-vibrated SCI control group achieved a PTIBS score of 3.4 ± 0.26, indicating that most animals demonstrated active hindlimb movement with varying degrees of rump support but were unable to take steps (Fig. 2). At 12 weeks post-injury, the PTIBS score was 3.5 ± 0.33.

In the 1.5-h resonance vibration group and the 3.0-h resonance vibration group, the PTIBS score tended to be higher at one week post-injury (3.13 ± 0.38 and 3.25 ± 0.18, respectively), compared with the non-vibrated SCI control group (2.58 ± 0.19). Although the Kruskal-Wallis test revealed a significant group difference ($H = 6.220$; $df = 2$; $N = 32$; $p = 0.045$) at the one-week time-point, the difference between non-vibrated SCI control group and the 1.5-h or 3.0-h resonance vibration group did not reach significance by Dunn's test ($p = 0.19$ and $p = 0.06$, respectively). During the subsequent 11 weeks, no differences were observed between the three groups, with PTIBS scores at 12 weeks post-injury of 3.5 ± 0.33, 4.1 ± 0.64, and 4.4 ± 0.65 for the non-vibrated SCI control group, the 1.5-h resonance vibration group and the 3.0-h resonance vibration group, respectively ($H = 0.664$; $df = 2$; $N = 24$; $p = 0.718$).

Effect of resonance vibration on CSF markers of inflammation and injury severity

Interleukin 6. IL-6 is a 21–28 kDa cytokine with pleiotropic properties that has been shown to be a biomarker associated with various disease states and is secreted by various activated immune cells.[28,29] Before injury (baseline), in the majority of the samples, the IL-6 levels in CSF were below detectable levels. In the non-vibrated SCI control group, IL-6 levels were dramatically elevated in the CSF at 3.5 h post-injury (1.32 ± 0.80 ng/mL; Fig. 3). In the subsequent hours, IL-6 levels in CSF increased gradually, and at 5 h post-injury IL-6 levels were 1.90 ± 0.61 ng/mL. In the 1.5-h resonance vibration group, IL-6 levels followed a similar pattern at 3.5 h post-injury (0.40 ± 0.06 ng/mL) and 5.0 h post-injury (0.56 ± 0.11).

In the 3.0-h resonance vibration group, however, a high degree of inter-animal variability was observed (Fig. 3B) and as such, the mean IL-6 levels tended to be higher, compared with the non-vibrated SCI control group (3.5 h post-injury: 5.6 ± 2.6 ng/mL; 5.0 h post-injury: 6.40 ± 3.81 ng/mL). By 7 d and 12 weeks after injury, IL-6 levels were back to baseline levels for all three groups. No statistical differences were observed between groups at any of the time-points after SCI.

Interleukin 8. IL-8 is an 18-kDa pro-inflammatory peptide. This chemokine is a potent chemotactic factor for neutrophils and has been correlated with blood brain barrier dysfunction.[30] In non-vibrated SCI control animals, IL-8 levels were elevated within 3.5 h after injury, compared with their pre-injury levels. IL-6 levels increased to a mean value of 1.39 ± 0.59 ng/mL at 3.5 h post-injury, and 1.22 ± 0.35 ng/mL at 5.0 h post-injury (Fig. 4). In the 1.5-h

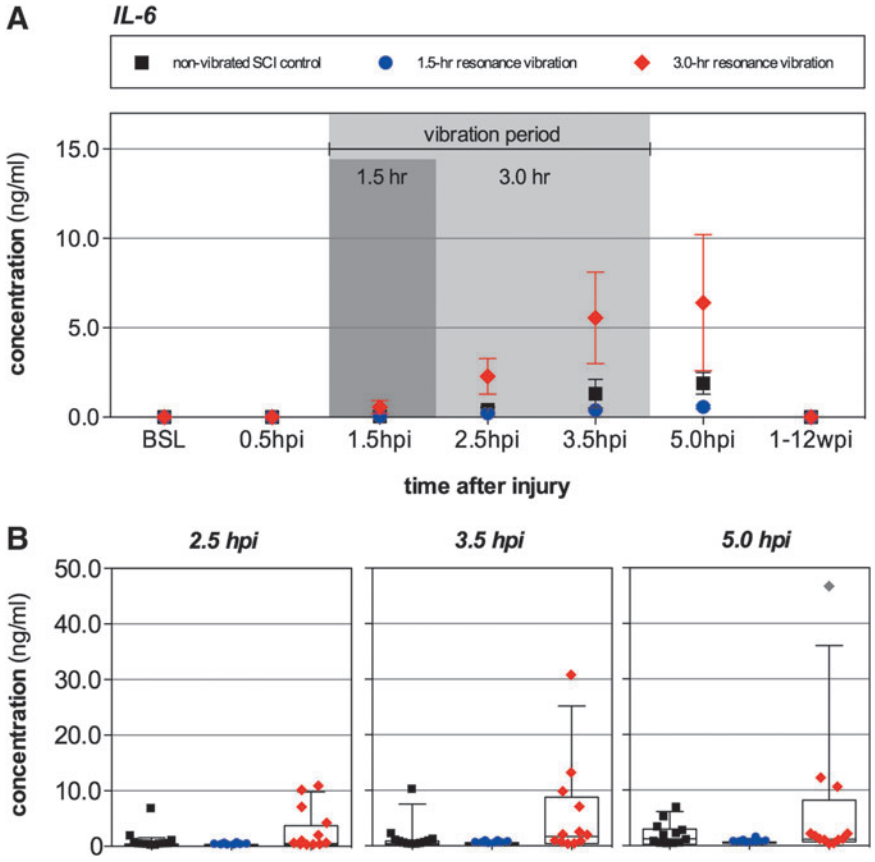

FIG. 3. Effect of resonance vibration on Interleukin-6 (IL-6) levels in cerebrospinal fluid (CSF) after spinal cord injury (SCI). **(A)** Average CSF levels of IL-6 measured in the non-vibrated SCI control group, 1.5-h resonance vibration group and 3.0-h resonance vibration group. The pre-injury levels (baseline [BSL]) of IL-6 in CSF were typically below detectable limits. **(B)** Scatter plots of individual values for IL-6 levels 2.5, 3.5, and 5.0 h post-injury. Box plots displaying the median value, 25th and 75th percentile. Whiskers represent 5th-95th percentile and dots the individual animals. All values represent means ± standard error of the mean. Color image is available online at www.liebertpub.com/neu

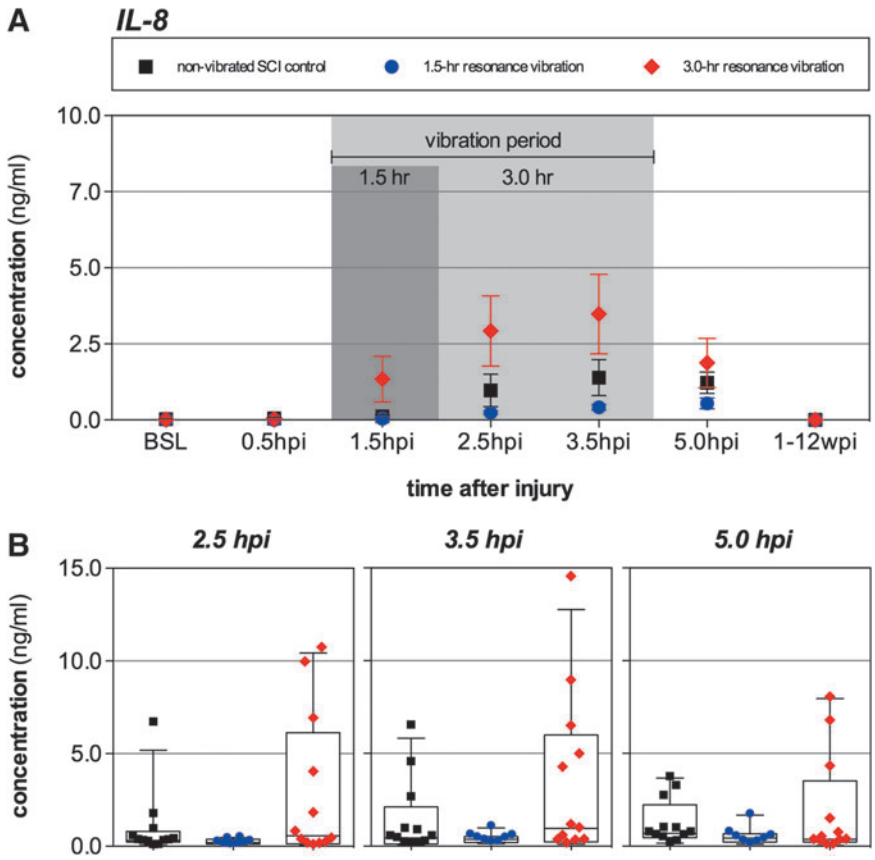

FIG. 4. Effect of resonance vibration on interleukin 8 (IL-8) levels in cerebrospinal fluid (CSF) after spinal cord injury (SCI). **(A)** Average CSF levels of IL-8 measured in the non-vibrated SCI control group, 1.5-h resonance vibration group and 3.0-h resonance vibration group. **(B)** Scatter plots of individual values for IL-8 levels 2.5, 3.5, and 5.0 h post-injury. Box plots displaying the median value, 25th and 75th percentile. Whiskers represent 5th-95th percentile and dots the individual animals. All values represent means ± standard error of the mean. Color image is available online at www.liebertpub.com/neu

resonance vibration group, IL-8 levels followed a similar pattern at 3.5 h post-injury (0.42 ± 0.09 n/mL) and 5.0 h post-injury (0.543 ± 0.174 ng/mL) as the non-vibrated SCI control group. IL-8 levels were approximately 2–3 times higher for the 3.0-h resonance vibration group at 3.5 h post-injury (3.48 ± 1.30 ng/mL) and 5.0 h post-injury (1.87 ± 0.81 ng/mL). However, due to the high inter-animal variability (Fig. 4B), these differences were not statistically significant. By 7 d and three months after injury, IL-8 levels were back to baseline levels for all three groups.

Monocyte chemotactic protein-1. MCP-1 (also referred to as chemokine [C-C motif] ligand 2) is a pro-inflammatory chemokine of ~12 kDa and a key mediator of early inflammation. It exerts its effect on perivascular transmigration and accumulation of monocytes, NK- cells and T-cells to sites of tissue damage.[31,32] In the non-vibrated SCI control group, a gradual increase in MCP-1 levels was detected in CSF following SCI (Fig. 5). At 3.5 and 5.0 h post-injury, MCP-1 levels in CSF increased to a mean value of 0.20 ± 0.07 μg/mL and 0.30 ± 0.06 μg/mL respectively. The 1.5-h resonance vibration group demonstrated similar MCP-1 levels at 3.5 h post-injury (0.16 ± 0.07 μg/mL) and 5.0 h post-injury (0.26 ± 0.04 μg/mL) to the non-vibrated SCI control group. In the 3.0-h resonance vibration group, the mean MCP-1 levels tended to be higher at 3.5 h post-injury (0.50 ± 0.13 μg/mL) and 5.0 h post-injury (0.52 ± 0.222 μg/mL), compared with the non-vibrated SCI control group. Again, there was a high degree of variability between animals (Fig. 5B) and as such, no statistically significant differences were observed between the three groups at any time-point after injury.

Glial gibrillary acidic protein. GFAP is a ~50 kDa structural protein expressed almost exclusively in astrocytes and released into the extracellular fluid and subsequently CSF upon cellular disintegration and degradation of the cytoskeleton.[25,33,34] In the non-vibrated SCI control group, mean GFAP level in CSF was elevated within 30 min after SCI and values continued to increase thereafter (Fig. 6). The non-vibrated SCI control group reached GFAP levels of 14.25 ± 4.95 μg/mL at 5.0 h post-injury.

In the 1.5-h resonance vibration group, GFAP values also were dramatically increased at 5 h after injury, with values similar to the non-vibrated control (14.86 ± 04.87 μg/mL). In the 3.0-h resonance vibration group, GFAP levels also were elevated; however, the increase occurred more slowly, compared with the non-vibrated SCI control group. By 3.5 h after injury, the 3.0-h resonance vibration group demonstrated 0.4 times lower GFAP values (5.31 ± 1.47 μg/mL), compared with the non-vibrated SCI control group. At 3.5 h post-injury, 10 of 12 animals in the 3.0-h resonance vibration group had values below 5 μg/mL, while such levels were rarely observed in the non-vibrated SCI control group (only one of 12 animals). The Kruskal-Wallis test ($H = 6.366$; $df = 2$; $N = 30$; $p = 0.042$) and Dunn's multiple comparison test confirmed a significant difference between the 3.0-h resonance vibration and non-vibrated control

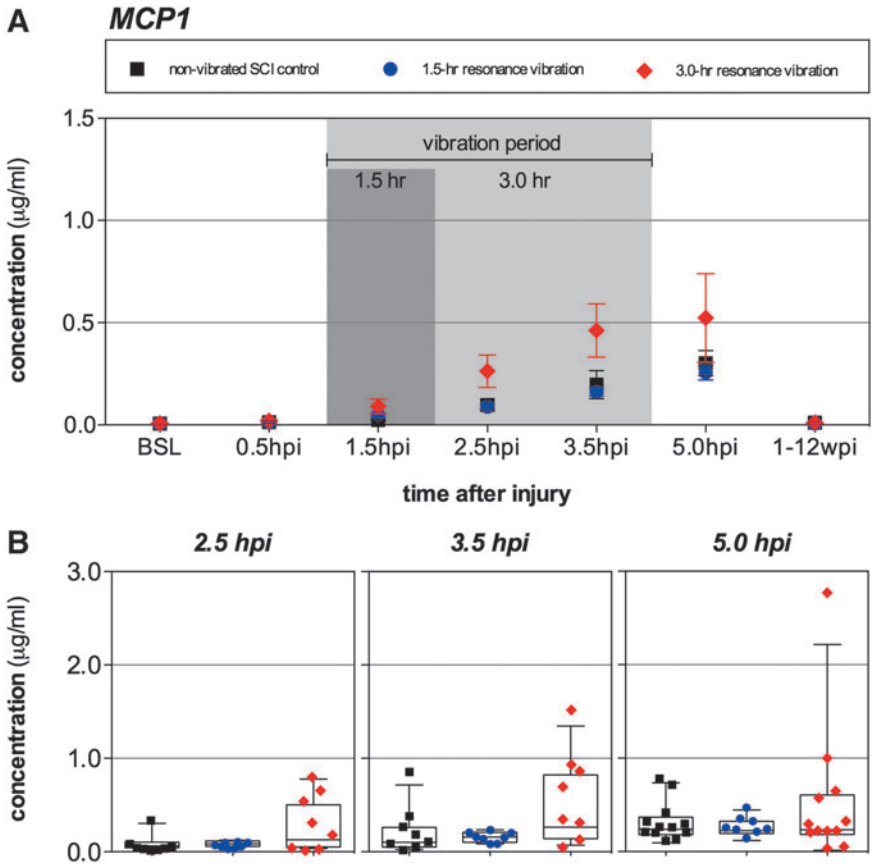

FIG. 5. Effect of resonance vibration on monocyte chemotactic protein-1 (MCP-1) levels in cerebrospinal fluid (CSF) after spinal cord injury (SCI). **(A)** Average CSF levels of interleukin 8 measured in the non-vibrated SCI control group, 1.5-h resonance vibration group and 3.0-h resonance vibration group. **(B)** Box plots displaying the median value, 25th and 75th percentile. Whiskers represent 5th-95th percentile and dots the individual animals. All values represent means ± standard error of the mean. Color image is available online at www.liebertpub.com/neu

group ($p = 0.046$) at 3.5 h post-injury but not with the 1.5-h resonance vibration group ($p = 0.285$). Notably, 2 h after the 3.0-h vibration period was stopped (5.0 h after injury), GFAP levels returned to values similar to those of the non-vibrated SCI control group (9.58 ± 3.12). By 7 d and three months after injury, GFAP levels were at baseline levels for all three groups.

Effect of resonance vibration on white and gray matter tissue sparing

Eriochrome Cyanine was used to stain myelinated areas in the spinal cord and measure the amount of spared gray and white matter. The damaged spinal cord was characterized by large central damage, micro cysts formation, demyelination and loss of microstructural integrity of both white and gray matter (Fig. 7). At the lesion epicenters, intact gray or white matter was virtually nonexistent.

At 7 d post-injury (Fig. 8), the 3.0-h resonance vibration group demonstrated significantly greater white matter sparing caudal to the epicenter, compared with the non-vibrated controls, when expressed as a percentage of the total spinal cord area ($p < 0.001$). No differences were observed for the percentage spared gray matter. At 12 weeks post-injury (Fig. 9), there were no statistical differences in white matter and gray matter sparing between the non-vibrated control group and the resonance vibration groups (1.5-h: white matter $p = 0.063$, gray matter $p = 0.169$; and 3.0-h: white matter $p = 0.793$, gray matter $p = 0.140$). Tissue damage expanded approximately 14.4 mm and 22.0 mm in the white matter and gray matter, respectively.

Discussion

After traumatic SCI, the spinal cord may be vulnerable to further mechanical injury due to ongoing bony compression and instability of the spinal column. Motorized transport and the vibration associated with it may exacerbate such injury by inducing motion of the spinal column. It has been estimated that up to 25% of the spinal cord damage might be aggravated after the initial injury, either during pre-hospital transport or early in the course of treatment.[17,35] Despite this alarming suggestion, there remains a paucity of data available to guide paramedics, physicians and medical policy makers on the effects of various vibrations and how to safely transport individuals who have sustained an SCI.

In this study, we investigated how vibration at resonance frequency affected the traumatically injured spinal cord. In our studies, the frequencies that the resonance vibration groups were subjected to were in the range of 5.13–6.23 Hz and the resonance peak was confirmed for each animal in the vibration groups individually. The resonance frequencies that were used in this study are quite comparable to the values that were studied previously in seated and in supine humans.[8–11,15] The durations of vibration (1.5 and 3 h) were based on clinically realistic times that it might take to

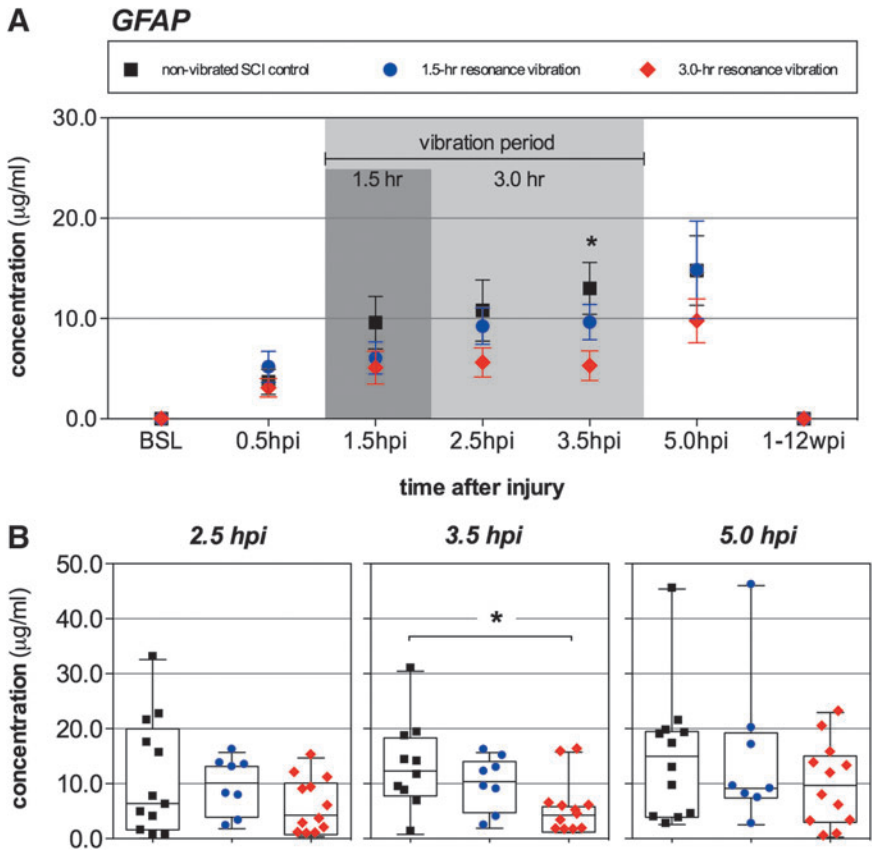

FIG. 6. Effect of resonance vibration on glial fibrillary acidic protein (GFAP) levels in cerebrospinal fluid (CSF) after spinal cord injury (SCI). **(A)** Average CSF levels of GFAP measured in the non-vibrated SCI control group, 1.5-h resonance vibration group and 3.0-h resonance vibration group. **(B)** Box plots displaying the median value, 25th and 75th percentile. Whiskers represent 5th-95th percentile and dots the individual animals. All values are means ± standard error of the mean. *Significantly different from non-vibrated SCI control group, $p < 0.05$. Color image is available online at www.liebertpub.com/neu

transport a patient to a medical facility. We used an extensively characterized porcine injury model in which SCI was inflicted by a weight drop impact followed by 3.5 h of mild compression simulating unreduced bone fragments. This resulted in consistent impact forces and deficits within each cohort of test animals.

Resonance vibration for 1.5 or 3 h after injury did not result in significantly worse functional outcomes—in fact, surprisingly, resonance vibration tended to have a beneficial effect on functional outcome. Spared white matter tissue was increased within the vibrated group at 7 d post-injury but this difference was not apparent at the 12-week time-point. A tendency towards higher CSF levels of inflammatory cytokines IL-6, IL-8, and MCP-1 was observed when SCI was followed by 3 h of resonance vibration, whereas GFAP expression, a marker of injury severity, was significantly decreased, compared with the non-vibrated control animals.

Hemorrhage and hemorrhagic necrosis have been shown to increase with the severity of an injury and are predictive of functional outcomes experimentally and clinically.[36–40] In addition, the infiltration of inflammatory cells through the release of pro-inflammatory cytokines precipitate free radical generation, causing oxidation of lipid membranes, proteins, and DNA, thereby exacerbating the damage.[41–44] During vibration, the thoracic spine exhibits vertical displacement and a flexion-extension motion, which may lead to additional soft tissue damage. Because CSF is in direct contact with the extracellular space of the spinal cord, it provides a reflection of the biochemical changes in the spinal cord in response to pathological processes. It has been suggested that the key factors that influence the concentrations of central nervous system (CNS) tissue proteins within the CSF are the extent of the primary lesion, the total pathological severity causing imbalance of CNS homeostasis, and the onset and duration of the injury.[45] Injured cells in the subarachnoid space can directly release protein into the CSF while protein from cells in the parenchyma must be transported to the CSF via interstitial fluid flow or edema.[46] All these factors may contribute alterations in CSF concentrations of cytokines and structural proteins at different times after injury.

After injury, pro-inflammatory cytokines are quickly and greatly upregulated.[25,47–50] Among the markers of inflammation, we focused on MCP-1, IL-6, and IL-8 to assess the potential injurious effects of resonance vibration. In the present study, peak concentrations of MCP-1, IL-6 and IL-8 were seen on average after 3 h of vibration. After injury, the most prominently-elevated cytokines and other inflammatory mediators are (IL-1β, tumor necrosis factor-α, and IL-6.[48,51,52] It is well known that these mediators can stimulate the production and release of specific chemokines, such as MCP-1, after cerebral ischemia (monocyte chemoattractant 1). These cytokines and chemokines are upregulated in the first 3 h after ischemic stroke and remain high for at least 6 h.[53] The earliest part of the production might be ascribed to activated microglia, followed by astrocytes and injured neurons.[54,55] We recently reported on the temporal pattern of expression of inflammatory cytokines and other structural proteins within the CSF of human

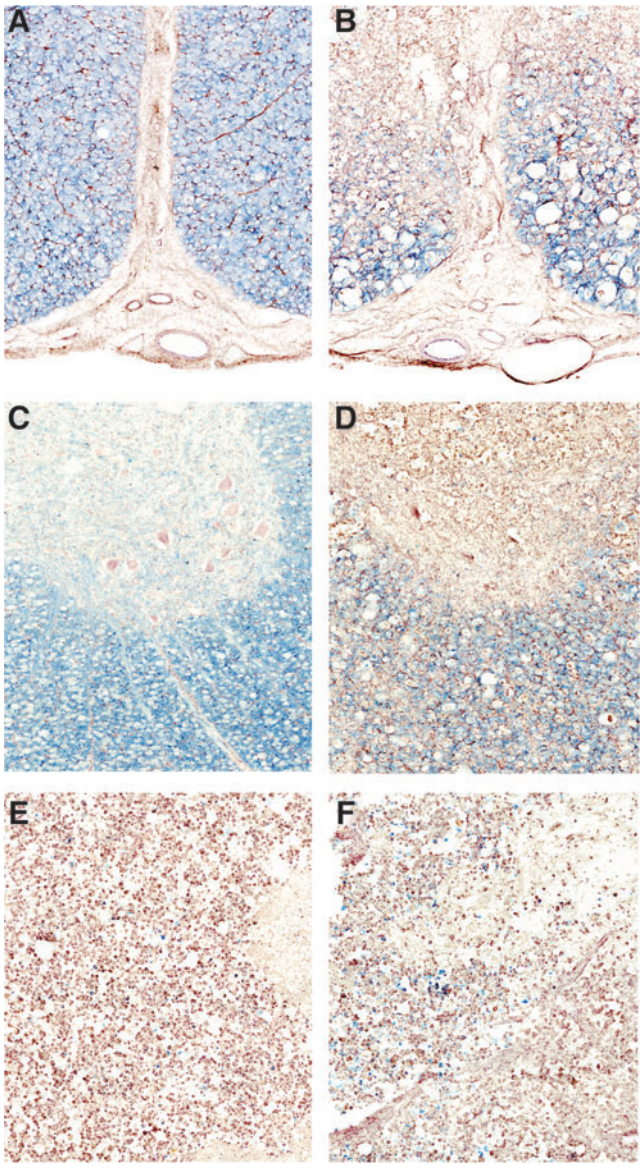

FIG. 7. Eriochrome Cyanine (EC)-stained cross-sections of the uninjured and injured spinal cord at 20× objective. Photographs showing representative EC staining of the ventral white matter of **(A)** uninjured and **(B)** injured spinal cords. Photographs showing representative EC staining of the ventral horn of **(C)** uninjured and **(D)** injured spinal cords. **(E-F)** Photographs showing representative EC staining at the lesion epicenter. Color image is available online at www.liebertpub.com/neu

patients who had suffered acute SCI.[25] IL-6, IL-8, and MCP-1 were elevated at 24 h post-injury in a severity-dependent fashion, such that they could be used to delineate the baseline American Spinal Injury Association (ASIA) Impairment Scale and predict outcome. We were encouraged by the demonstration that the same inflammatory cytokines (IL-6, IL-8, and MCP-1) that were elevated in an injury-severity–dependent fashion in human SCI patients also were elevated after SCI in our thoracic Yucatan SCI model.

In humans, it has been shown that inner organs and muscles are also in resonance at vibration frequencies around 5–8 Hz.[56] In multiple trauma patients without any injury to the CNS, both IL-8 and IL-6 are released into the circulation within 90 min post-trauma.[57] Further, Naghii and colleagues reported elevated blood levels of IL-6 in rats subjected to whole–body vibration.[58] As we did not measure serum IL-6 or IL-8 levels in matched blood samples, the present study does not indicate whether or not the increased IL-6, IL-8, and MCP-1 was due to neuroinflammation, or might be the result of a passive leakage of inflammatory protein across an impaired BBB by damaged peripheral tissues contributing to the high variations in cytokine levels.

In this study, we also measured expression levels of a non-inflammatory neuronal marker, GFAP, because of the potential use as a biomarker of injury severity.[25,59] GFAP samples around 24 h post-injury revealed substantial differences between human patients with ASIA A, B, or C injury severity, which illustrates the injury severity dependent pattern of expression for this protein. In this porcine SCI experiment, we observed lower CSF GFAP concentrations when the primary injury was followed by 1.5 or 3.0 h of resonance frequency vibration, potentially suggesting a lower degree of injury. However, the concentration of a specific CNS-derived protein in the CSF may not only depend on the concentration of the protein in the cell and interstitial fluid but also the rate of diffusion of the protein into the CSF. Passive diffusion via interstitial fluid flow or edema is dependent on properties such as molecular weight and time. Van den Berg and colleagues observed striking differences in the distribution of low–molecular weight (10 kDa) and high–molecular weight (70 kDa) dyes after vibration at 7 Hz.[60] They injected one-cell embryos with a mixture of two dyes of different molecular weights, which were then vibrated for 10 min or left untreated. In both vibrated and non-vibrated embryos, the low–molecular weight dye was similarly diffused throughout the cytoplasm. However, the high–molecular weight dye remained more localized around a single point in the embryo.

Taken together, these results suggest that vibration might affect the diffusion rate of different size molecules of different sizes differently. MCP-1 is a pro-inflammatory chemokine of ~12 kDa, IL-6 is a 21–28 kDa cytokine, IL-8 is an 18-kDa pro-inflammatory cytokine, while GFAP is a structural protein of 50 kDa. Since the tip of the catheter is 2 cm caudal to the injury epicenter, the discrepancy between the decreased GFAP levels and increased MCP-1, IL-6, and IL-8 levels might thus be the result of a complex interplay between vibration and diffusive movement of molecules in their environment. One limitation of this study is the lack of additional time-points for CSF collection after the vibration period has ceased. It is difficult to know whether a change in the pattern of cytokines occurs at later time-points. The trend of increased levels of MCP-1, IL-6, and IL-8 and the decrease in GFAP levels in the CSF might be a transient effect, with levels progressively recovering over time when vibration has stopped. However, the duration of the study was too short to generate a complete temporal expression profile.

Here, we demonstrated that resonance vibration at approximately ~5 Hz over a 1.5- or 3.0-h period did not significantly worsen nor improve behavioral outcome in our porcine model of SCI. PTIBS scores following a 20-cm weight drop were comparable to our previously published report results, which suggests that the degree of compression maintained by the spacer (not used in our previous study) did not exacerbate the injury at this level of exposure.[24] Although the contribution of prolonged compression mediated by inserting a spacer may have been mild, compression is one of the possible factors of the experiment that may have attributed to the variability. The rationale for adding a spacer to prolong compression came from recognizing the fact that clinically, compression to the injured spinal cord may be maintained if an unreduced bone fragment is compressing the cord or is against the cord and mobile during transportation. Even though every effort

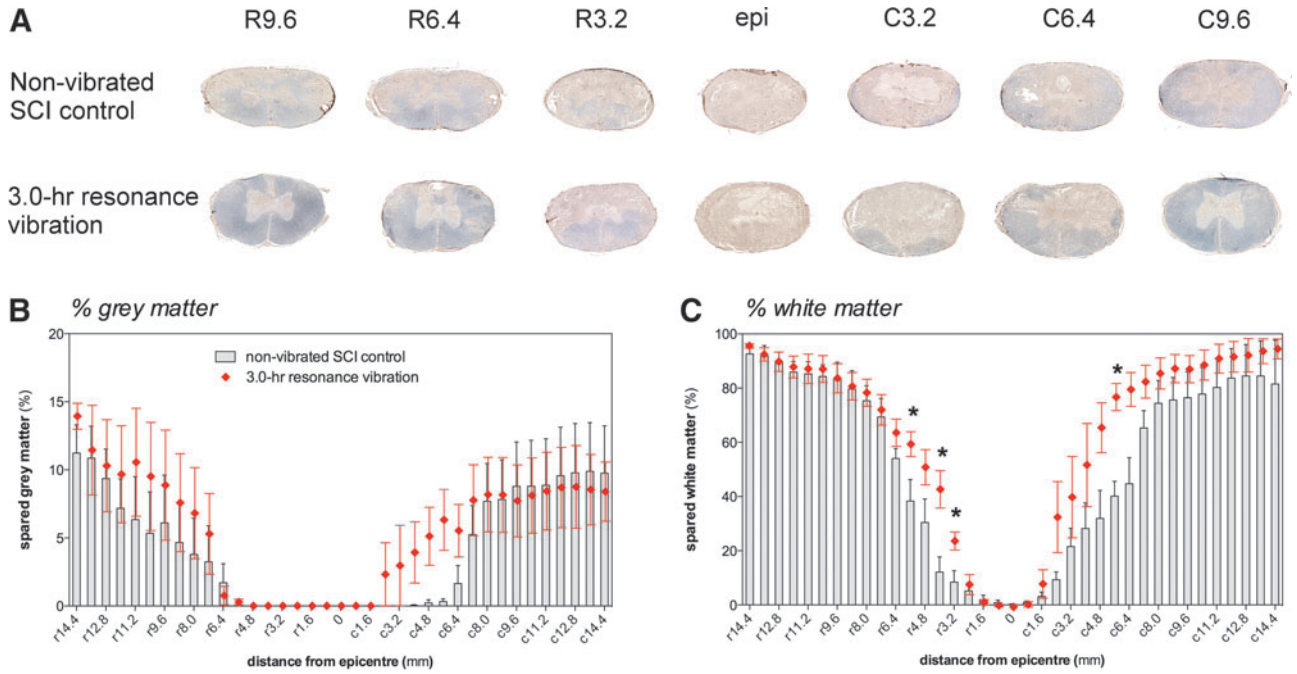

FIG. 8. The spinal cord lesion at 7 d post-injury, quantified as the percentage of gray and white matter sparing around the injury site. Here, there were only two experimental groups (3.0 h resonance vibration vs non-vibrated controls). **(A)** Series of EC-stained images of axially sectioned spinal cords illustrating the rostral-caudal extent of the injury at 7 d post-contusion of the non-vibrated spinal cord injury (SCI) control group and 3.0-h resonance vibration group (original magnification 5× objective). Injury was performed at the T10 spinal level. **(B)** Quantitative analysis of spared gray and **(C)** white matter expressed as a percentage of total cord area. *Significantly different from non-vibrated SCI control group ($p < 0.05$). All values represent means ± standard error of the mean. R3.2, 3.2 mm rostral to the injury; C3.2, 3.2 mm caudal to the injury. Color image is available online at www.liebertpub.com/neu

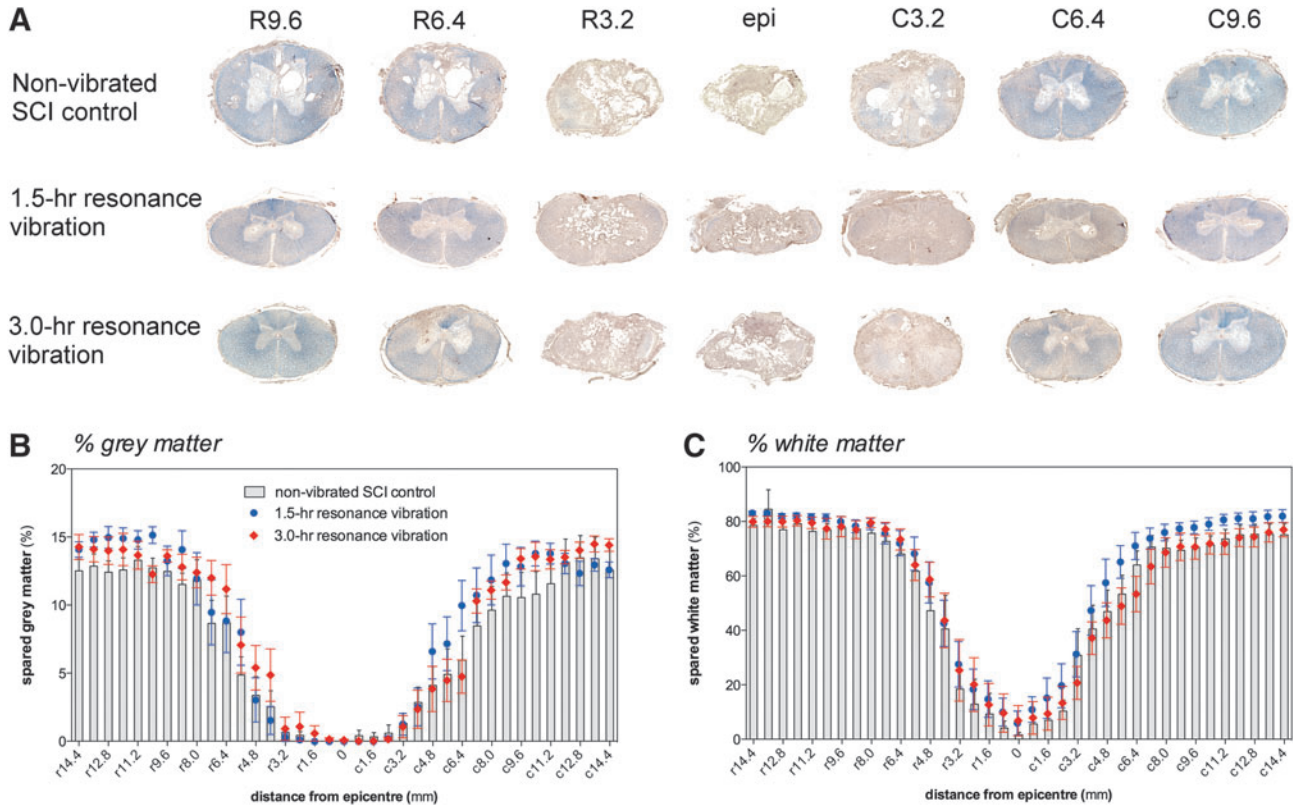

FIG. 9. The spinal cord lesion at 12 weeks post-injury quantified as the percentage of gray and white matter sparing. **(A)** Series of EC-stained images of axially sectioned spinal cords illustrating the rostral-caudal extent of the injury at 12 weeks post-impact in the non-vibrated spinal cord injury (SCI) control group, 1.5-h resonance vibration group, and 3.0-h resonance vibration group (original magnification 5× objective). Injury was performed at the T10 spinal level. **(B)** Quantitative analysis of spared gray and **(C)** white matter expressed as a percentage of total cord area. All values represent means ± standard error of the mean. R3.2, 3.2 mm rostral to the injury; C3.2, 3.2 mm caudal to the injury. Color image is available online at www.liebertpub.com/neu

was made to apply consistent compression to the injured spinal cord, the native anatomy, laminectomy, and size of the spinal canal may have differed between animals, thereby creating variability in the extent of compressive force applied by the spacer. It is possible that inconsistency in the applied compression to the spinal cord may have masked minor functional effects of resonance frequency.

It should be noted that the number of animals per group is small, and thus the reported findings may have been a reflection of inadequate power. We performed a post-experiment power analysis to determine the sample size needed to detect a significant difference between the groups, given the variability that we actually observed. The vibration groups exhibited an average standard deviation in PTIBS score of 1.52 across the 12 weeks of the study. The power analysis indicated that, assuming equal group sizes and variability, a sample size of 30 for each group would be needed to distinguish a 1-point difference on the PTIBS (power of 80% α set at 0.05). This dropped to sample sizes of eight animals per group for detecting a more severe 2-point PTIBS difference. We acknowledge that our study may not have been able to demonstrate subtle changes in behavioral changes due to vibration, although one might then question the clinical relevance of such changes.

Intriguingly, we observed that animals subjected to vibration for 3 h had more spared white matter at 7 d post-SCI (an effect that was not seen at the 12 week time-point). This somewhat surprising finding implies a possible beneficial effect of vibration. Various studies have examined the effect of passive low frequency vibration on blood circulation. The majority of research has focused on the negative effects of high frequency occupational vibration such as increased vasoconstriction as in ''vibration white finger'' and ''vibration- induced Raynaud's phenomenon'' associated with industrial tools that resonate at 80 to 100 Hz.[61–63] Similar effects also have been observed in the tail model of hand-arm vibration.[64–66] Only a few studies have been published on low frequency vibration (<80 Hz) and its effect on blood circulation. These studies demonstrated that vibration (between 30–60 Hz) could actually increase blood vessel vasodilation and blood flow within skin and soft tissues in healthy subjects.[67–71] Immediately after the vibration session stopped there was a rapid decrease in blood flow.

The question arises whether or not blood flow increases can be achieved with resonance frequency in the injured spinal cord. It has been well recognized that following SCI, profound vascular changes lead to ischemia and hypoxia of spinal cord tissue. It has been suggested that re-establishing blood flow with neuroprotective therapies such as N-methyl-D-aspartate receptor antagonists, granulocyte colony-stimulating-factor and granulocyte-macrophage colony-stimulating-factor, fibroblast growth factor 2, and erythropoietin may contribute to protective effects seen in animal models of cerebral and spinal cord ischemia and impact injuries.[72–76] To our knowledge, no studies have examined the effect of vibration at resonance frequency on blood flow and further research in this area is necessary.

In summary, this study suggests that exposure to resonance vibration after traumatic SCI does not result in significant behavioral or histological worsening of the injury. This is true even though we induced a rather dramatic degree of motion of the spinal column (between 8 to 18 mm of vertical motion) that occurred five times per second. While we employed a relatively uniform degree of sustained extradural compression and subjected the animal to vibration early after SCI, we acknowledge that the effects of vibration on the acutely injured spinal cord might be influenced by the extent of extradural cord compression and the degree of spinal cord swelling/edema. Characterizing this would require further experiments in which resonance frequency vibration would be applied with varying degrees of compression and at different time-points post-injury when cord swelling could be more extensive.

The experimental setting that we developed allowed us to apply vibratory stimuli with controlled frequency, amplitude, and duration. During ground or air transportation, vibrations present complex oscillatory motions, characterized by a wide frequency spectrum with variable acceleration amplitude and direction. Therefore, further studies are necessary to investigate the effects of duration and frequency that are generated by real-world modes of transportation, such as ambulance and helicopter. Results from these studies may provide valuable information as to the biological and physiological effect of vibration caused by transportation and in addition offer guidance on ways to improve transporting individuals that sustained an SCI in the future.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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