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Original Article

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Reactions of the rat musculoskeletal system to compressive spinal cord injury (SCI) and whole body vibration (WBV) therapy

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Abstract

Traumatic spinal cord injury (SCI) causes a loss of locomotor function with associated compromise of the musculo-skeletal system. Whole body vibration (WBV) is a potential therapy following SCI, but little is known about its effects on the musculo-skeletal system. Here, we examined locomotor recovery and the musculo-skeletal system after thoracic (T7-9) compression SCI in adult rats. Daily WBV was started at 1, 7, 14 and 28 days after injury (WBV1-WBV28 respectively) and continued over a 12-week post-injury period. Intact rats, rats with SCI but no WBV (sham-treated) and a group that received passive flexion and extension (PFE) of their hind limbs served as controls. Compared to sham-treated rats, neither WBV nor PFE improved motor function. Only WBV14 and PFE improved body support. In line with earlier studies we failed to detect signs of soleus muscle atrophy (weight, cross sectional diameter, total amount of fibers, mean fiber diameter) or bone loss in the femur (length, weight, bone mineral density). One possible explanation is that, despite of injury extent, the preservation of some axons in the white matter, in combination with quadripedal locomotion, may provide sufficient trophic and neuronal support for the musculoskeletal system.

Keywords: Spinal Cord Injury, Rehabilitation, Whole Body Vibration, Muscle, Bone

Introduction

Spinal cord injury (SCI) disrupts ascending and descending connections below the lesion. Depending on the nature and level of the injury, patients can suffer severe disability and a high economic burden with rehabilitation being the only current treatment option[1]. Although severe injuries lead to permanent disabilities, less catastrophic accidents can be followed by some spontaneous functional recovery, with patients mostly benefiting from rehabilitation although high quality clinical evidence is often lacking. Whole-body vibration (WBV) has attracted increasing attention as a potential therapy. In humans, WBV delivers mechanical stimuli that are conveyed to the entire human body via the feet when standing on a vertically oscillating platform[2]. It is hypothesized that WBV results in an increased sensitivity of muscle spindles, which results in synchronization of motor units[3].

Whole body vibration is thought to improve some aspects of neuromuscular performance and increase bone mass and density[4,5]. It is being widely used to enhance sports training[6,7] by improving muscle power and force[8] and force-velocity relation[9] with positive training effects being achieved during a relatively short time period[10].

The authors have no conflict of interest.

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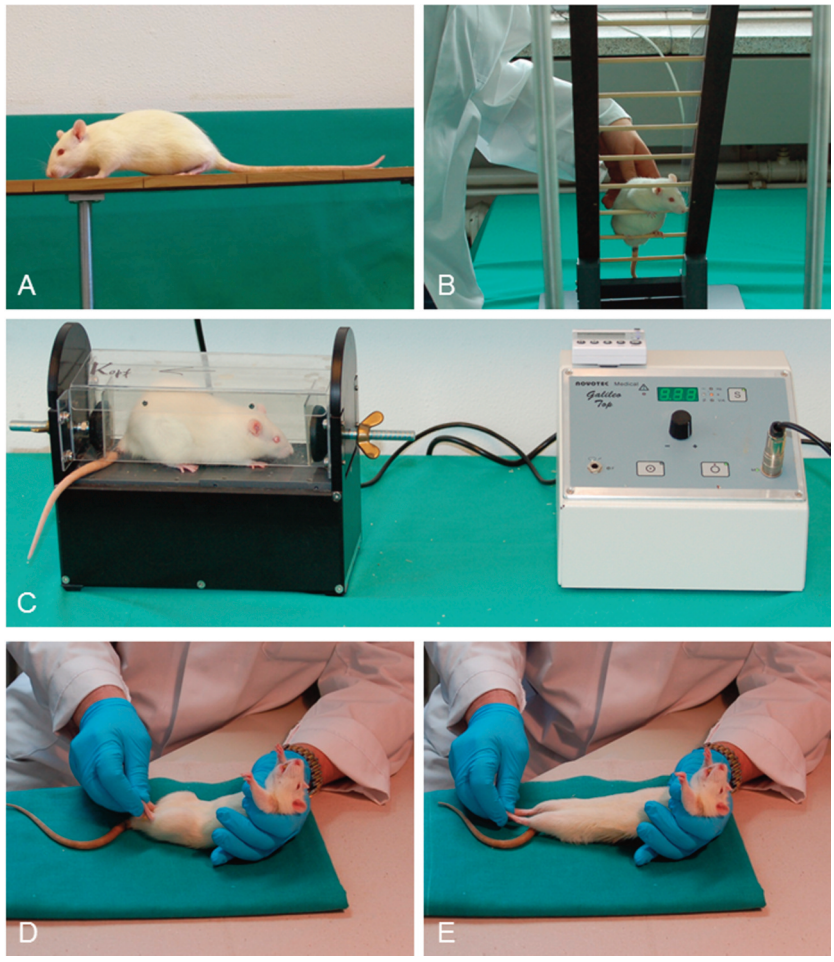

Figure 1. Conditioning of the rats was performed daily over two weeks before SCI. **A:** Walking on a wooden beam. **B:** Climbing on an inclined ladder. **C:** Setup of the rat Galileo-device for whole-body vibration (WBV) training: during conditioning intact rats received WBV at 15 Hz for 5 minutes daily. Figs. 1 A, B and C have been adopted from Manthou et al., 2015. **D, E:** Passive flexion and extension (PFE) of the hind limbs during manual therapy.

Following SCI in humans, WBV has been shown to inhibit soleus muscle H-reflex and thus reduce muscle spasticity[11]. Whole body vibration also increases bone mineral density[12]. Improvements in blood flow velocity and neuromuscular activity have also been reported[13]. A recent study also reported benefits for lower limb peripheral blood flow but not cardiovascular health[14]. Surprisingly, we identified only one earlier experimental study that examined vibration-induced stepping[15], the study being undertaken in spinal cats. Recently, we examined the effects of WBV in rat after thoracic compressive SCI[16]. Compared to sham-treated rats, WBV significantly improved body weight support as assessed by rump height index, bladder function and overall recovery. This was attributed to restoration of synaptic terminals in the lumbar (below lesion) spinal cord. However, it was unclear whether WBV also had an effect on the musculoskeletal system. While positive effects of WBV on muscle and bone health have been reported in human patients, no studies have been conducted thus far in experimental animals.

The purpose of the present study, therefore, was to determine whether WBV influenced various aspects of the musculo-skeletal system over a 12-week period following compression SCI. It is well known that sharp transection results in complete severing of axons and is ideal for studying regeneration. This approach has yielded important insights into the mechanisms responsible for forelimb recovery after cervical injury in the rat[17]. Similar models, however, do not allow to study additional mechanisms involved in incomplete SCI and reflect only a small proportion of human SCI[18]. Accordingly, a complete transection of the cord has been observed in less than 40% of the cases[19,20,21]. Thus, our model provides a clinically relevant paradigm that will allow for greater insight into the pathophysiology of incomplete thoracic injury and the identification of therapies targeted at improving functional recovery.

We also included a group that received hind-limb passive flexion and extension (PFE). We examined locomotor function using a variety of outcome measures (BBB score, foot stepping

angle, rump height index as a measure of body weight support and ladder stepping), soleus muscle (weight, muscle diameter and muscle fibre number and diameter), and femoral bone (morphological measures of bone appositions).

Materials and methods

Animals

A total of 70 adult (175-200 g) female Wistar rats (strain HsdCpb:WU, Harlan) were used. We used females because recent work showed that testosterone prevents muscle and bone loss after SCI[22]. Ten animals were used as intact controls and the remaining 70 were randomised to seven groups, each consisting of 10 rats. Animals were fed standard laboratory food (Ssniff, Soest, Germany), provided tap water *ad libitum* and kept in a conditioned animal room (23°C, 12-hour artificial light-dark cycle). All experiments were conducted in accordance with the German Law on the Protection of Animals.

Training prior to surgery

Prior for surgery, all 80 rats were trained to walk on a wooden beam (Figure 1A) and to climb up an inclined ladder (Figure 1B). Each training session was short (about 5 min per animal) and performed 5 days/week for 2 weeks. Animals that were randomised to receive WBV after SCI were also trained for 5 minutes daily for 2 weeks to remain still in the vibrating chamber at a frequency of 15 Hz (Figure 1C). Rats that were randomised to receive PFE after SCI were subjected to gentle passive flexions (Figure 1D) and extensions (Figure 1E) of the hind limbs once a day for 2 weeks.

Spinal cord injury

We used a technique previously established in our lab[16,23,24] to induce spinal cord compression injury[25]. A laminectomy was performed at T8 level and the exposed spinal cord compressed using electromagnetically controlled watchmaker forceps. The intact spinal cord was lesioned by compressing its diameter by 50%[24] with velocity of 100 mm/sec for 1s using a timed current. Rats were then housed individually in standard cages and the bladder voided manually three times a day.

Following surgery, all animals were randomised to 6 experimental groups: sham-treated (no WBV-therapy, although as a "handling control" rats were placed on the vibrating platform for the same period of time as the experimental groups but without activating it; N=10), WBV1 (WBV-therapy started on day 1 after SCI), WBV7 (WBV-therapy started on day 7 after SCI, N=10) WBV14 (WBV-therapy started on day 14 after SCI; N=10), WBV28 (WBV-therapy started on day 28 after SCI; N=10). A final group received passive flexion and extension of the hind limbs starting on day 14 after SCI (PFE).

Whole-body vibration

The use of this therapy was previously established in our lab[16]. We used a custom Galileo-device designed specifically for rats by Novotec Medical GmbH (Pforzheim, Germany). Rats could move inside the chamber and also turn around (Figure 1C).

In groups WBV1, WBV7, WBV14, WBV28, rats were subjected to WBV once daily always in the morning for 5 days/week with the amplitude, frequency and duration being controlled electronically.

Every WBV session comprised 5 sequential trials, each lasting for 3 minutes. During the first minute, the floor plates were vibrated at 15 Hz and in the following 2 minutes at 30 Hz. For the next trial, WBV was again at 15 Hz for 1 minute and 30 Hz for 2 minutes etc.

Passive flexion and extension (PFE) of the paralyzed hind limbs

In group 7, starting on the 14th postoperative day, animals received manual stimulation of the hind limbs twice a day. Each session consisted of 80 passive flexions and extensions (PFE) in the course of 1 minute (Figures 1 D, E). The rationale for including PFE was to examine whether low frequency stretching of the intrafusal muscle spindles in the paralyzed muscles would provide some benefit. The release of the so called "extension withdrawal reflex" was considered as a sign of successful extension of the intrafusal muscle spindles[26-28].

Locomotor tests

All assessments were undertaken by investigators blinded to treatment allocation. Assessments were performed prior to SCI (0 weeks) and 1, 3, 6, 9 and 12 weeks after injury.

BBB

Locomotor function was evaluated using the Basso, Beattie, Bresnahan (BBB) rating scale[29]. Scoring was undertaken from videos by 2 independent investigators, who were initially trained on the BBB scoring system to ensure high inter-reliability. The walking platform ("beam") and inclined ladder tests were performed as described previously[16,24]. Video recordings were made (see below) and observed at slow playback speed for single frame motion analysis.

Beam walking

Video recordings from the left and right side during beam walking were repeated 1, 3, 6, 9 and 12 weeks after SCI. Video sequences were examined using VirtualDub 1.6.19, a video capture/processing utility written by Avery Lee (free software available at http://www.virtualdub.org). Selected frames in which animals were in defined phases of locomotion were used. ImageTool 2.0 software was used to measure foot-stepping angle (FSA) and rump-height index (RHI) as described previously[16,24]. Analyses were performed by two identically trained but independent investigators.

Ladder climbing test

After the beam-walking test, rats were video recorded using the same equipment as above during climbing an inclined ladder as described previously[16,24].

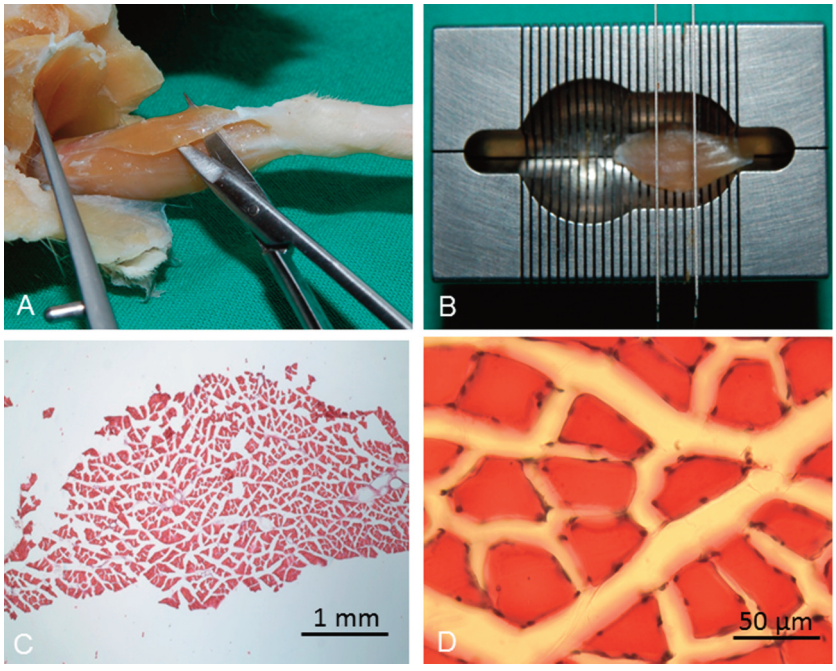

Figure 2. Following perfusion fixation the left soleus was gently removed (**A**), dried and weighed. Thereafter it was placed into a rat brain matrix (World Precision Instruments) and a 4 mm thick transverse slice through its widest part was dissected free (**B**). Each muscle slice was cut into 25 mm thick transverse sections and every 10th section was stained with hematoxylin-eosin (at least 10 sections per animal). Photographs at magnification x2,5 to determine the entire cross-sectional area and the number of its fibers (**C**) and at magnification x40 to determine the mean fiber diameter (**D**) followed.

Spinal cord lesion volume

This parameter was evaluated in longitudinal sections through the lesioned cord as already described[16,24].

Extent of white matter sparing evaluated in transverse sections

In two animals from each experimental group, we also evaluated white matter sparing at the lesion site from transverse sections. Following perfusion fixation (see above) 3-4 mm long thoracic spinal cord segments containing the injury site were dissected free and postfixed in 4% paraformaldehyde and 2,5% glutaraldehyde in phopsphate-buffered saline pH 7.4. Following postfixation in 1% OsO_4 with 1.5% $K3Fe^{III}(CN)_6$ in 0.1 M cacodylate buffer pH 7.2, samples were embedded in Epon 812 and after polymerization for 48 h at 70°C cut in 0,5 -1.0 µm thick transverse sections. A fractionator sampling strategy[30] was used and every 50th section (a total of at least 80 equidistant sections through the thoracic spinal cord) was stained with with 1% toluidine blue. Measurements of the spared white matter were performed as described above.

Morphological changes in the soleus muscle

Previous studies have shown that adaptive responses to SCI occur preferentially in slow twitch rather than fast twitch muscle fibers, and in extensor more than flexor muscle groups. Therefore, the soleus muscle, being a slow twitch postural extensor, is frequently evaluated for being particularly vulnerable to changes in weight bearing status[31].

Following perfusion fixation, the left soleus muscle was carefully exposed, removed (Figure 2A), dried and weighed. Thereafter, it was placed into a rat brain matrix (World Precision Instruments, Berlin, Germany) and a 4 mm thick transverse slice (specimen) through its widest part dissected free (Figure 2B). After cryoprotection (20% sucrose-infiltrated), the muscle specimen was cut into 25 mm thick transverse cryostat sections. After staining every 10th section with Hematoxylin-Eosin (at least 10 sections per animal), sections were photographed at magnification × 2,5 to determine the entire cross-sectional area and the total number of fibers (Figure 2C), and at magnification × 40 to determine the mean fiber diameter (Figure 2D).

Morphological changes in the femur

After fixation, the right femora of all intact animals and those of all rats from groups No WBV, WBV7, WBV14 were removed and cleaned carefully from all muscle insertions. Bones were then photographed, weighed and length measurements undertaken (Figure 3A).

X-ray examination

This technique was used to analyse bone density (Hewlett Packard Faxitron device, model No 43855A Mc Minville Di-

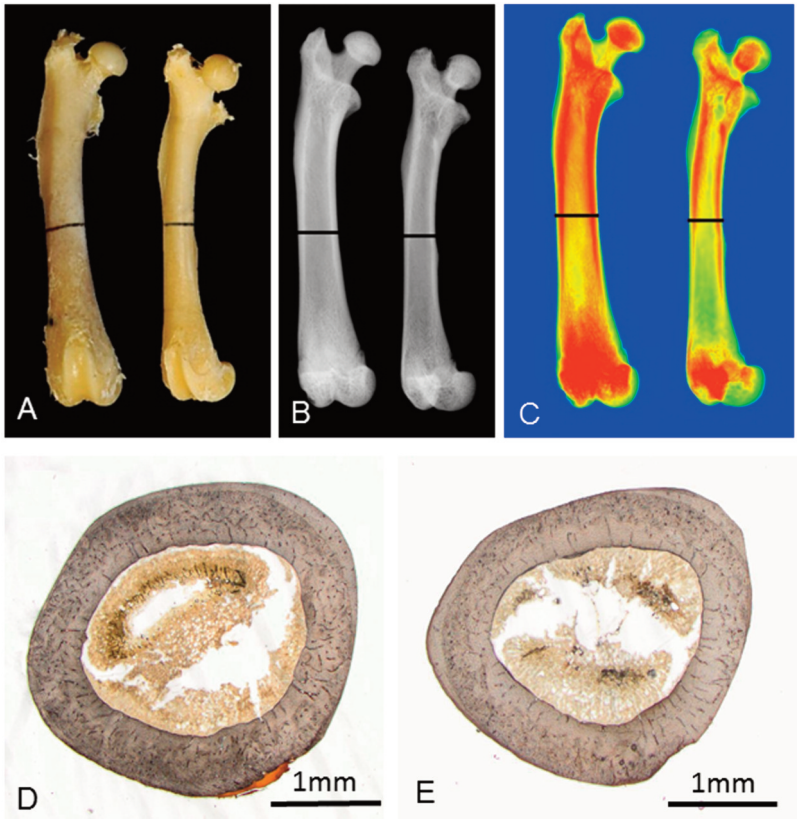

Figure 3. Photographing, weighing and length measurements of the right femora (**A**) were followed by X-ray analysis (**B**) and densitometry (**C**). After embedding in one-component metha-acrylate-based resin (Technovit 7200 VLC, Kulzer) femora were sectioned in the middle of the bone and thin ground sections (40 µm thick) were produced. The bones were not decalcified, which allows a better distinction between mineralized bone and unmineralized osteoid. Femoral length, weight, total-, corticalis portions-, and medullary cavity diameters were measured in intact (**D**) as well as in SCI-lesioned rats (**E**).

vision, USA). The frequency was 60 Hz, the power 600 VA, the exposure time 37s and the voltage 45 KV. (Figure 3B).

Densitometry

To measure bone mineral density and distribution, X-ray-pictures were scanned with Microtek Scan Wizard Pro (Version 7.20, driver: Scan Maker 9800XL) and analysed with the Morphomet (v.1.1.3, Kerpen 2004) image analyzing system. Different densities (different light absorbances are represented by specific gray scales) of X-ray images were measured and converted into distinct colours for better distinction by the human eye (Figure 3C) with red equating to high, and blue for low, bone mineral density.

Histological examination

The Cutting-Grinding Technique introduced by Donath and Breuner (1982)[32] allows production of thin ground sections (40 µm thick) with a smooth surface from very hard materials, such as bones, without decalcification[33]. For better penetration of dehydrating (alcohol in increasing concentrations) and infiltrating solutions, the right femora of all animals from groups intact, No WBV, WBV7 and WBV14 were cut transversely in the middle. After infiltration, embedding and polymerization in a photocurring one-component methaacrylate-based resin Technovit 7200 VLC (user instructions 7200, 7210, 7230 VLC, Heraeus Kulzer GmbH, Wehrheim), thin ground sections were produced with the Exakt-cutting-grinding-system 300 and Exakt-micro-grinding-system 400 CS (Exakt Apparatebau Norderstedt). Six different abrasive papers (P320, P500, P800, P1000, P1200, P4000) were sequentially used to smooth the surface. Sections were photographed on an Olympus Vanox AHBT-3 microscope equipped with a XC10 digital camera. In addition, an Olympus BX 50 microscope with a DP 21 camera was used at first for overview images (Cell Imaging Software 3.1) and thereafter for detailed images (Cell Imaging Software 2.3 by Olympus, Hamburg, Germany). Transverse and frontal diameters of the entire bone, the bone ring of substantia compacta (corticalis) and the medullar cavity were measured (Figures 3 D, E). Finally, the bone microscopic structure was analysed using a polarisation filter BX-POL (Olympus). The procedure does not involve decalcification and therefore allows a better distinction between mineralized bone and unmineralized osteoid.

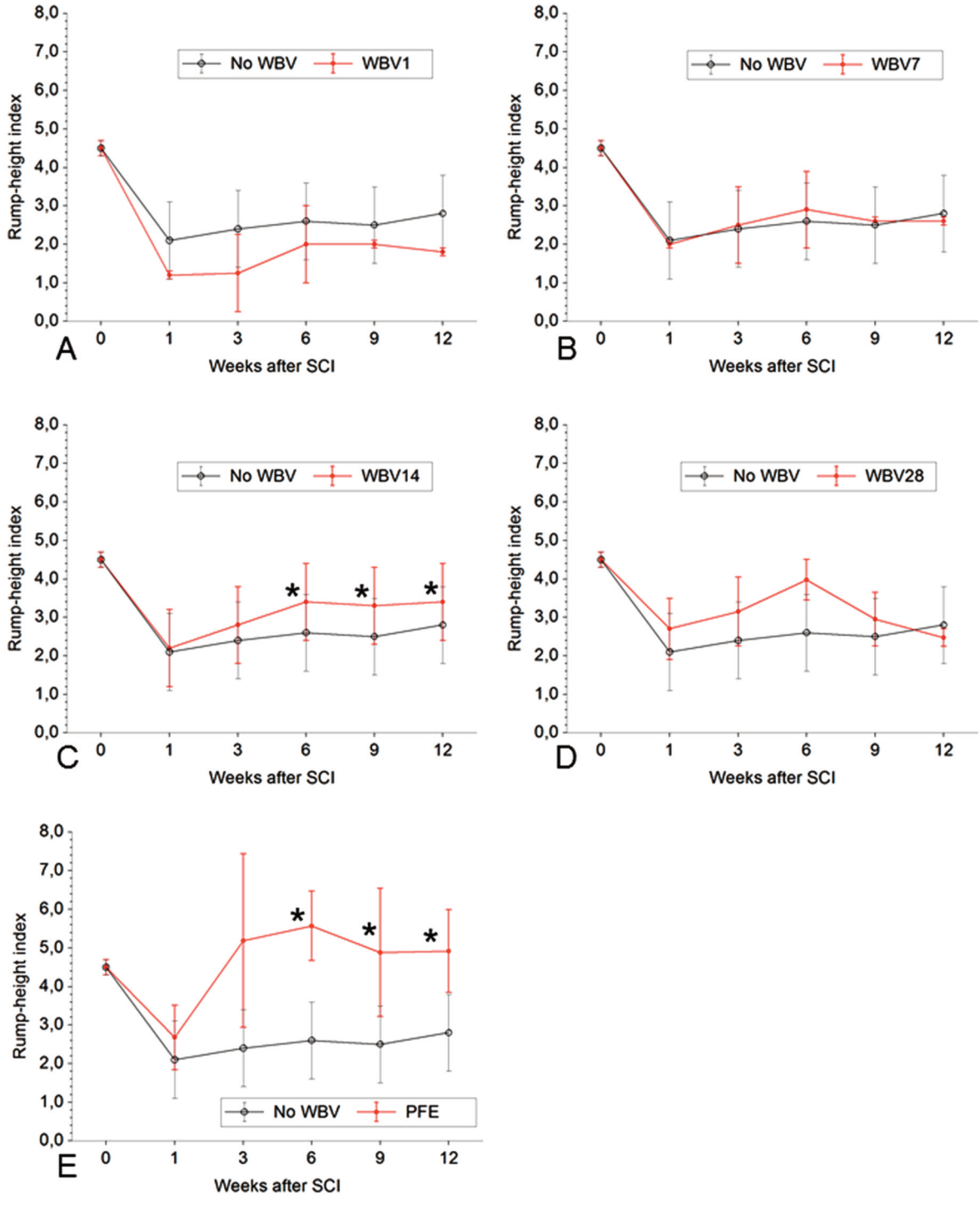

Figure 4. Graphical representation of the changes in RHI (in time course) in the 6 treated (WBV1, WBV7, WBV14, WBV28, PFE) depicted by the red lines as compared to the control group (SCI, but no WBV) shown by the black lines. Shown are group mean values ± S.E., N=10 rats per group.

Statistical analysis

Statistical analyses were performed using Sigma Plot 11 software (SPSS, Chicago, IL, USA). Data were analyzed for distribution by the Kolmogorov-Simirnov test. Equal variance and appropriate parametric - nonparametric tests (one way ANOVA with Tukey test as post hoc test or Kruskal Wallis with Dunn test as post hoc test) were used. Data were expressed as mean and standard error. The threshold value for acceptance of differences was 5%.

Results

Functional parameters: Improved recovery only of the rump-height index (RHI) in the WBV14 and PFE-groups

We first analyzed locomotor function in SCI rats using the BBB rating scale. At 1 week after SCI, mean BBB scores in the six experimental groups (sham, WBV1, WBV7, WBV14, WBV28 and PFE) were similar (ranging from 1.1 to 2.4), indicating that the groups were equivalent in terms of lesion severity. A trend (but no statistically significant difference) for better recovery at 3-12 weeks was seen in rats with WBV initiated at 14 days post-injury (WBV14 group.

Analysis of stepping using FSA revealed, as for BBB scores, similar degrees of impairment in the five groups 1 week after SCI. In the sham group, the angle changed from an average of about 20° prior to injury to 130° at 1 week and only gradually and slightly decreased thereafter to an average of 93° at 12 weeks. The results for the WBV1, WBV7 and WBV28 groups were better than those obtained for sham animals with a mean recovery of plantar stepping between the 3rd and 6th weeks. As for BBB scores, we observed a trend (but no statistically significant difference) for best recovery of plantar stepping in WBV14 rats at 3-12 weeks (p=0.10-0.12 compared with sham rats, *t* test).

The third functional parameter analyzed was the number of correct ladder steps during inclined ladder climbing. Proper placement of the paws and maintenance of a stable paw position on the rungs to support body weight during climbing require higher levels of motor and sensory control than over-ground locomotion. Prior to SCI, the average number of correct ladder steps was 7 in all groups. One week after SCI, no animals were able to step on the rungs. Improvement with time after injury was minimal but could be detected as early as 6 weeks after SCI. On the average, at 12 weeks 1 correct step was achieved in the sham and WBV7 groups and 2 correct steps in the WBV14 and 3-4 correct steps in the WBV1, WBV28 and PFE groups 12 weeks after SCI. There were no significant differences among the groups.

The RHI estimates the ability of the hindlimbs to support body weight during over-ground locomotion. This ability was significantly impaired 1 week after SCI in all animal groups (Figures 4 A-E). Rats moved along the beam using the forelimbs while the hindlimbs were dragged behind and could not elevate the trunk above the beam surface. In the following weeks, RHI improved moderately, and to the same degree, in the sham, WBV1, WBV7 and WBV28 groups. At 12 weeks, the rump could be lifted to about one-fourth of the pre-operative rump height. Compared with these four groups, higher RHI was found in WBV14 and PFE rats at 6-12 weeks (Figures 4 C, E). At 12 weeks, the WBV14 and the PFE rats were able to lift their trunk on average to more than half the normal rump height ($p<0.05$).

WBV does not lead to alterations in lesion volume and white matter sparing

We next tested whether WBV affects the lesion volume in the thoracic spinal cord, a parameter known to be positively correlated with functional outcome. At 12 weeks after injury, there were no significant differences in lesion volume among the six experimental groups, sham, WBV1, WBV7, WBV14, WBV28 and PFE.

Cross sections through the epicenter of the injury (T_8-T_9) demonstrated a central lesion devoid of normal spinal cord tissue surrounded by a peripheral rim of preserved white matter containing the lateral and ventral funiculi. The amount of preserved white matter was 45-47% of the cross sectional area regardless of experimental group.

Thus, in our spinal cord compression model, some axonal sparing occurs at the lesion epicenter with loss of myelinated axons. This is consistent with our previous reports[16,24], as well as with several studies describing tissue sparing following spinal cord contusion injury[29,34-40].

No signs of atrophy in the soleus muscle after compressive SCI

Next we examined the degree of atrophy of the soleus muscle. After staining every 10th section with Hematoxylin-Eosin (at least 10 sections per animal) we determined:
(1) the entire cross-sectional area of the muscle,
(2) the total number of muscle fibers and
(3) the mean fiber diameter.

In line with earlier observations[31,41] our values of muscle weight (Figure 5A), muscle diameter (Figure 5B), number of fibers (Figure 5C) and fiber diameter (Figure 5D) were not significantly different at 12 weeks after SCI, i.e., there were no signs of muscle atrophy regardless of spinal cord compression and animal group.

No changes in length and weight of the femur after SCI and no effect of WBV

Basic measurements of weight and length of all femora revealed no significant differences ($p>0.05$) between the animal groups. The mean weight of femora in the intact group was 0,8348±0,05 g and that in the groups No WBV, WBV7, WBV14 0,68±0,05 g, 0,66±0,05 g, 0,64±0,03 g, respectively. Likewise, the mean length of femora in the intact group was 3,55±0,05 cm and that in the groups No WBV, WBV7, WBV14 3,51±0,09 cm, 3,44±0,11 cm, 3,43±0,05 cm respectively.

Histological structure - qualitative assessments

Studies on the human femur show that during erect stance or gait, long bones are "stressed" by bending due to gravitational forces. This bending is represented by tensile stress in the anterior part of the femur and compression in the posterior region with both acting as stimuli for bone metabolism.

In normally moving intact animals, physical bone bending is reduced and moderated by muscle forces. After SCI, however, both the total amount of stress as well as muscle activities are reduced. We therefore determined whether there were any changes in bone formation after SCI and subsequent WBV.

Our detailed histological examination of the femora revealed different kinds of appositions of new bone material: (1) small ap-

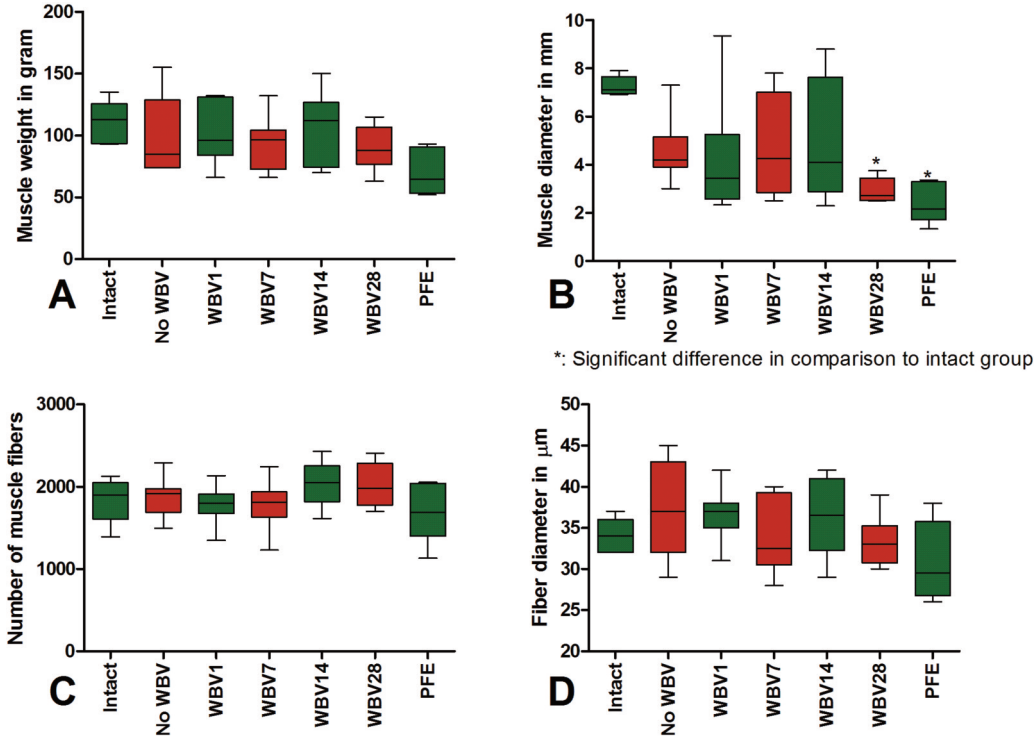

Figure 5. Graphical representation of muscle weight (**A**), muscle diameter (**B**), mean number of muscle fibers (**C**) and mean fiber diameter (**D**) in the soleus muscle of the intact and in the 6 experimental groups of rats (No WBV, WBV1, WBV7, WBV14, WBV28, PFE). Regardless of spinal cord compression and postlesional treatments, at 12 weeks after SCI, most values were not changed when compared to those obtained for intact rats. The only difference detected was the smaller mean diameter of the soleus muscle in groups WBV28 and PFE (indicated by asterisks). Shown are group mean values ± S.E., N=10 rats per group.

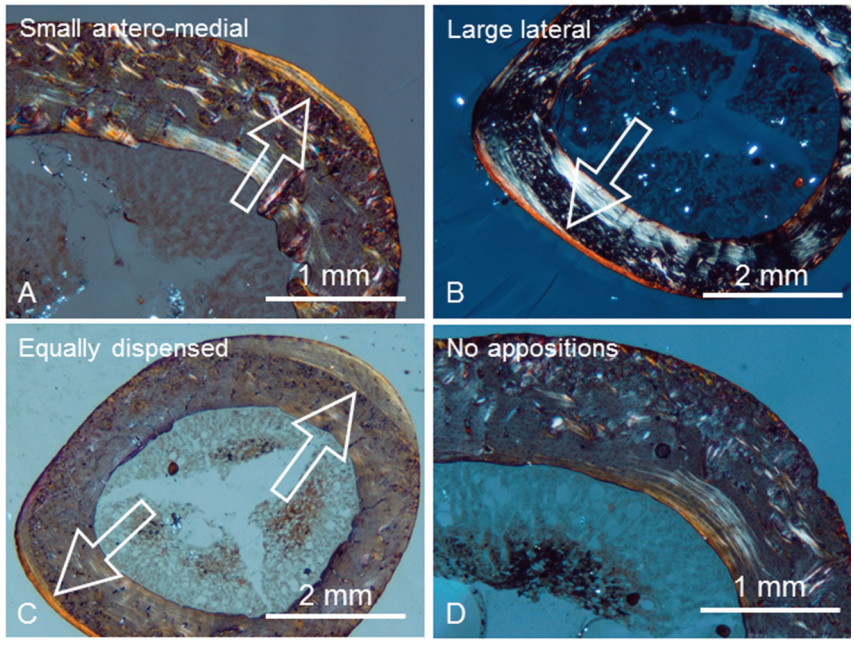

Figure 6. Observations through the polarisation filter BX-POL (Olympus) allow better distinction between mineralized bone and unmineralized osteoid. Representative pictures of cross-sections through the right femur depicting small bone appositions (**A**), large appositions especially on the anterior-medial and postero-lateral surface (**B**), equally dispensed appositions surrounding the whole bone or large parts of the bone (**C**) and no appositions of bone mass (**D**).

positions (Figure 6A); (2) large appositions especially on the anterior-medial and postero-lateral surface (Figure 6B); (3) equally dispensed appositions surrounding the whole bone or large parts of the bone (Figure 6C); (4) no appositions of bone mass (Figure 6D). There were no differences between the experimental groups: every kind of apposition was present in similar frequencies in each group.

Histology

Histological measurements on entire bones. The mean sagittal diameter of the entire femora in intact rats was 3,02±0,17 mm. In groups No WBV, WBV7, WBV14 diameters were 3,01±0,15 mm, 2,86±0,14 mm, 2,86±0,11 mm respectively. No significant differences between the groups were found.

The mean transverse diameter of the entire bones was 3,82±0,14 mm in intact rats. In the groups No WBV, WBV7, WBV14 we measured 3,53±0,13 mm, 3,53±0,13 mm, 3,51±0,23 mm respectively. No significant differences between the groups were detected.

Histological measurements on the surrounding corticalis. The mean diameter of the anterior corticalis in intact rats was 0,62±0,05 mm; in groups No WBV, WBV7, WBV 14 we measured 0,52±0,04 mm, 0,51±0,06 mm, 0,53±0,04 mm respectively. No differences between the groups were found.

The mean diameter of the posterior corticalis in intact rats was 0,63±0,03 mm; in groups No WBV, WBV7, WBV 14 we measured 0,59±0,06 mm, 0,58±0,04 mm, 0,60±0,03 mm respectively. No significant differences were found.

The mean diameter of the medial corticalis in intact rats was 0,65±0,04 mm; in groups No WBV, WBV7, WBV 14 we measured 0,51±0,02 mm, 0,50±0,08 mm, 0,53±0,10 mm respectively. No significant differences were found.

Finally, the mean diameter of the lateral corticalis in intact rats was 0,74±0,05 mm; in groups No WBV, WBV7, WBV 14 we measured 0,59±0,04 mm, 0,63±0,05 mm, 0,70±0,10 mm respectively. No significant differences were found.

Histological measurements on the medullar cavity. The mean transverse diameter of the medullar cavity in intact rats was 1,79±0,15 mm. In groups No WBV, WBV7, WBV14 we measured 1,91±0,1mm, 1,79±0,12 mm, 1,74±0,1 mm respectively. No significant differences between the groups were found.

The mean sagittal diameter of the medullar cavity in intact rats was 2,44±0,16 mm. In groups No WBV, WBV7, WBV14 we measured 2,43±0,14 mm, 2,41±0,22 mm, 2,29±0,18 mm respectively. No significant differences between the groups were found.

Densitometry. Bone mineral density (BMD) in X-ray pictures was decreased in the femora of the SCI-rats when compared to that in intact rats ($p<0.005$). However, we found no significant differences between the 3 experimental groups (No WBV, WBV7, WBV14). In each experimental group examined, there were some bones which appeared denser in the diaphysis, epiphysis and metaphysis. However, in total the red colour was equally distributed over the four experimental groups.

Discussion

The locomotor results of the present study confirm our earlier findings which showed that whole body vibration started on the 14th day after the compressive SCI (WBV14) as well as passive flexion and extension of the hind limbs (PFE) improved the rump-height index (body weight support). We also saw trends for improvements in the WBV14 group for the BBB score (over-ground locomotion) and in the foot-stepping angle in comparison to sham treated group.

We failed to detect accompanying morphological changes in the soleus muscle and femur. There were no signs of muscle atrophy or bone loss/remodelling. either in the sham-treated or WBV groups. The present study thus shows that, 3 months after compressive SCI, the rat musculoskeletal system does not atrophy. One possible explanation is that axons preserved in the white matter after SCI provide enough trophic support for muscles and bones.

Rump-height index (RHI): best recovery in both groups in which treatment started at 14 days after SCI

The RHI provides a numerical estimate of the ability to support body weight. We found a significant improvement at 6-12 weeks in the WBV14- and PFE-groups compared with sham-treated rats. This is an important finding from two points of view.

First, the simultaneous use of different outcome measures which assess specific aspects of locomotion (e.g., plantar stepping and body weight support ability) allows more insightful conclusions about treatment effects and reduces the possibility of missing such effects compared with a single measure (see also[42]).

Second, the ability to support body weight depends on coordinated force output of different lower limb muscles. Based on human data WBV is expected to improve muscle force and synchronous stimulation of muscle spindles[11,43]. Our RHI data suggest that WBV does indeed improve body weight support. The notion of task-specificity concurs with previous experimental work showing that a specific type of training, for example stepping versus walking, influences different spinal circuitries and their respective functional outcome[44]. We do not know the morphological substrates that influence body weight support in our experimental model. Nevertheless, the finding that WBV initiated at 2 weeks after injury partially restores synaptic input to the ventral horn[16] suggests that synaptic rearrangements in the distal spinal cord might be important for improving body weight support. Local blood flow changes in vibrated muscles might also lead to better performance[14].

Therapeutic timeframe for WBV and PFE

The positive effects of WBV and PFE were mostly expressed when treatments were initiated at 2 weeks after SCI. Treatment with an onset at 1 week after SCI had no measurable effects; furthermore, initiation of WBV at 1 day after injury appeared even harmful. These results suggest that WBV and PFE might be a feasible and effective therapy only when initiated sub-acutely after SCI[16]. Several reasons may be consid-

ered as essential for the failure of early-onset WBV treatment. One possibility is that wound healing and the recovery of vertebral column stability and function of the spinal musculature may require approximately two weeks. Second, within the first two weeks after contusion/compression SCI, the blood-spinal cord barrier is severely disrupted[45,46] and extravasation of inflammatory blood cells is prominent[47]. Third, it is possible that some remodelling of intraspinal and supraspinal circuitries, i.e. axonal sprouting and new synapse formation[48-50], a process that takes several weeks, must first occur to enable positive effects of WBV on the outcome of SCI.

We decided to use the passive flexion and extension (PFE) of the hind limbs as control because, following SCI, stretching of muscles and passive joint range-of-motion (ROM) are widely accepted in clinical practice worldwide[51,52]. Accordingly, The Consortium for Spinal Cord Medicine prescribes that physical therapy begin within the first week postinjury and continue throughout the acute phase. A recent review of clinical trials of SCI rehabilitation found these therapies, however, to be ineffective when compared with no intervention or conventional care[53,54].

The effects of stretching/ROM maneuvers in animal models of SCI remain largely unknown because only 5 studies mention the use of stretch or ROM therapies[55-59]. One reason to use them is the loss of soft-tissue extensibility which in turn could maintain function and allow for optimal neurological plasticity. As outlined by Ben & Harvey[60], numerous animal studies have shown a positive effect of stretch on muscle remodeling and extensibility, sarcomere length, collagen arrangement, and increased force production[61,62].

In contrast to our results, showing good recovery of body weight support (RHI) after SCI and PFE-treatment, two elegant recent studies[58,59] reported on declines in hindlimb function during overground stepping during the first 4 weeks in the stretched animals: BBB scores improved slightly but remained below the control group.

Our explanation of this discrepancy concerns the onset of PFE-treatment. Whereas in the studies of David Magnuson´s group stretching was initiated immediately after SCI, we started PFE 14 days thereafter. This is why, we propose that a timeframe, probably identical with that of the WBV-treatment, i.e. beginning at 2 weeks after SCI - is also present. As already mentioned, a lot of important events occur during the first two weeks post SCI: wound healing, restoration of vertebral column stability, partial recovery of function in the spinal musculature, closure of the disrupted blood-spinal cord barrier and remodelling of intraspinal and supraspinal circuitries (see above).

No muscle atrophy after compression injury of the spinal cord

After *complete* SCI in humans, skeletal muscles undergo extensive detrimental changes including profound atrophy and loss of muscle strength[41,63-67]. Such impairments, together with slower but progressing bone loss[68] [a steady-state in bone mass and density is reached after 5-8 years (Eser et al., 2004[69]; Frotzler et al., 2008[70]] increase greatly the risk of secondary health complications, such as type 2 diabetes, cardiovascular disease, osteoporosis and fragile fractures[71]. Development of effective therapeutic strategies to prevent the loss of muscle, as well as bone, and function after SCI is critically important[67,72].

Experimental contusion models closely reproduce important features of the pathophysiology observed clinically[29,35,67,73]. These models have been widely used to characterize locomotor and neuromuscular changes after SCI[74,75]. Such studies have shown that some muscles start to atrophy as early as a few days after SCI with a maximum loss of about 25% after 1 week. However, three weeks later the weight of the slow twitching soleus muscle is normal[31]. Thus, spinal cord contusion resulting in hind-limb paralysis with recovery of some locomotor function does not result in any significant changes in the soleus muscle after 10 weeks. Our present results are in line with these findings.

At first sight, our data seem to be contradictory to those from a very recent study[67]. The authors found that 63 days after moderate severity contusion SCI, there was modest, though significant, atrophy of gastrocnemius muscle and a significant decrease in expression level of two activity-dependent genes (PGC-1α and RCAN1.4). Thus, their "data are consistent with the expected diminishment of post-injury gastrocnemius muscle neuromuscular activity and indicate that even the mild decrease in motor function caused by a moderate severity contusion SCI is sufficient to induce significant, albeit small, deterioration of sublesional skeletal muscle[67].

One important difference between this report and our study, which might be responsible for this discrepancy, may lie in the different hindlimb muscles studied. Whereas Bramlett et al.[67] investigated the entire triceps surae and the gastrocnemius, we concentrated on the soleus muscle. We met this choice because the soleus is easy to identify, dissect and trim (the bordrline between muscle body and tendon is readily visible). In addition it is small and flat, qualities which provide important advantages when measuring the surface of the sections and counting the individual fibers. Finally, should we have found any significant differences between the groups, we could readily extend the study towards changes in the morphology of the motor endplates. In the soleus muscles they are concentrated in the middle of its anterior surface, where a branch of the tibial nerve enters the muscle body. However, the slow soleus muscle tends to normalize weight after several weeks in spinal cord contusion, while the fast gastrocnemius muscles atrophy persists[31]. In addition, spinal cord contusion does not result in any significant changes in MHC composition in the soleus muscle, and contractile properties returned to baseline levels within 10 weeks after lesion.

Another possible explanation of this discrepancy may be due to a difference in the intact circuitry remaining after incomplete SCI which is known to allow sufficient muscle activation[31]. This possibility is supported by our earlier results showing unlesioned white matter tracts in both longitudinal as well as transverse sections through the lesion site of the experimentally compressed rat spinal cord (data not shown; see however Figures 3 B, D and Figures 4 A-G in Ozsoy et al., 2012, Ref.[23]). In another study from our laboratory we meas-

ured the extent of white matter sparing in 1 μm thick transverse plastic ("semithin") sections through the epicenter of the injury (T_8-T_9). We observed a central lesion devoid of normal spinal cord tissue surrounded by a peripheral rim of preserved white matter containing the lateral and ventral funiculi. The amount of preserved white matter was 44-48% of the cross sectional area. Thus, whereas muscles appear to be sensitive to lack of effective innervation and motor performance immediately after SCI, due perhaps to a spinal shock, at more chronic stages, spared innervation surviving incomplete SCI may be sufficient to allow minimal muscle activity and preserve tissue[76,77].

Effect of whole-body vibration therapy on muscles

WBV is a relatively novel therapy used variously in sports and more recently in rehabilitation[43]. At a gross level, mild physical side-alternating movements cause pelvic movements similar to that which occurs during walking. High-frequency mechanical stimuli activate muscle spindles and elicit cyclic elongation and contraction of the muscles, which results in measurable increases in muscle strength[78,79], flexibility[80], but - astonishingly - no improvements in bone mineral density[81,82]. Increased muscle-strength, function and flexibility and bone mineral density are desirable therapeutic effects after SCI. The positive WBV effects on the musculoskeletal system also appear to benefit patients with muscle atrophy after SCI.

So far, however, it was not known whether vibration is well tolerated by atrophying and recovering muscles[31]. Our present results reveal that WBV had no harmful effects on non-active hindlimb muscles after severe thoracic SCI in rat. In this way our results are in line with those from a very recent report which revealed that vibration treatment did not appreciably alter muscle weights or expression of the activity-dependent genes PGC-1α or RCAN1.4 and thus id not appear to have benefits to skeletal muscle, at least in this model of SCI[67]. Accordingly, we completely accept their remark that improvements in neuromuscular function might increase muscle strength without any change in muscle volume or cross sectional area[67]. Consistent with this interpretation in both normal and activity-restricted mice, vibration increased maximal torque of anterior crural muscles without significant changes in muscle size[78].

No bone loss after SCI

No bone loss was detected in our study. Our findings are supported by a recent study investigating bone loss in rats following spinal cord contusion of varying severity (6.25 g-cm, 12.5 g-cm, 25 g-cm, 50 g-cm). Voor et al. (2012)[83] report that there appeared to be a threshold with only in the 50 g-cm group (most severe) incurring significant bone loss, while groups with less severe injuries were functionally incomplete and showed no severe bone loss. Alternatively, the 12 weeks post-operative survival time may coincide with or be an extension of a known period (4-6 weeks after SCI) of increased bone remodeling[84] or it may be a part of the ongoing bone growth[85].

The situation contrasts with human studies showing bone mineral density loss of 2 to 4 percent a month, particularly at sites rich in trabecular bone[86]. This leads to 50% loss in paralyzed limbs by 3 years after SCI reaching steady state with values 50 to 60 percent lower than in age-matched controls until 8 years after SCI[87]. The contrast between rat and human studies may reflect quadripedal versus bipedal locomotion and questions whether a rat model of incomplete injury such as we describe in the present study is suitable for examining bone loss after SCI.

In conclusion, the present results show that 3 months after compressive injury of the spinal cord in rats the disuse-induced atrophical changes in the structure of sublesional muscles (soleus) and bones (femur) are not as severe as expected. Application of physical therapy (WBV and PFE) lead to improved recovery of neuronal circuitry and synchronized motor functions but not of the musculoskeletal structure.

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