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Task-specificity vs Ceiling Effect: Step-training in shallow water after spinal cord injury

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Abstract

While activity-based rehabilitation is one of the most promising therapeutic approaches for spinal cord injury, the necessary components for optimal locomotor retraining have not yet been determined. Currently, a number of different activity-based approaches are being investigated including body weight-supported treadmill training (with and without manual assistance), robotically-assisted treadmill training, bicycling and swimming, among others. We recently showed, in the adult rat, that intensive rehabilitation based on swimming brought about significant improvements in hindlimb performance during swimming but did not alter the normal course of recovery of over-ground walking (Smith et al., 2006; 2009). However, swimming lacks the phasic limb-loading and plantar cutaneous feedback thought to be important for weight-supported step training. So, we are investigating an innovative approach based on walking in shallow water where buoyancy provides some body weight support and balance while still allowing for limb-loading and appropriate cutaneous afferent feedback during retraining. Thus, the aim of this study is to determine if spinal cord injured animals show improved overground locomotion following intensive body-weight supported locomotor training in shallow water. The results show that training in shallow water successfully improved stepping in shallow water, but was not able to bring about significant improvements in overground locomotion despite the fact that the shallow water provides sufficient body weight support to allow acutely injured rats to generate frequent plantar stepping. These observations support previous suggestions that incompletely injured animals retrain themselves while moving about in their cages and that daily training regimes are not able to improve upon this already substantial functional improvement due to a ceiling effect, rather than task-specificity, *per se*. These results also support the concept that moderately-severe thoracic contusion injuries decrease the capacity for body weight support, but do not decrease the capacity for pattern generation. In contrast, animals with severe contusion injuries could not support their body weight nor could they generate a locomotor pattern when provided with body weight support via buoyancy.

Introduction

The isolated mammalian lumbar spinal cord is capable of generating patterns of rhythmic locomotor activity that underlies stereotypic walking. Spinal cords isolated from rat and mouse pups and maintained in vitro have been used to demonstrate that locomotor-like activity can be induced pharmacologically (Cowley and Schmidt, 1994) or by electrical stimulation of putative descending locomotor command pathways (Magnuson and Trinder, 1997; Liu and Jordan, 2005). The capabilities of the isolated lumbar enlargement also have been explored using the fully transected cat model (Lovely et al., 1986; Barbeau and Rossignol, 1987) illustrating that the lumbar enlargement can be retrained to generate high quality, weight-supported hindlimb stepping, and that the afferent input associated with limb loading (Edgerton et al., 1992) and cutaneous paw contact (Bouyer et al., 2003) is critical to successful retraining. Links between trophic ("growth factor") support and retraining also have been made in this (Boyce et al., 2007) and other models (Ying et al., 2008) suggesting that appropriate growth factor production/application may reduce the need for intensive retraining.

To date, retraining strategies aimed at improving locomotor function following more clinically relevant incomplete spinal cord injuries, whether contusion or laceration injuries, have met with very limited success. Fouad et al (2000) showed that treadmill training following dorsal hemisections could not improve upon what was already substantial functional recovery and concluded that spontaneous recovery and "self-training" occurred to such an extent that treadmill training was ineffective (i.e., a ceiling effect). Subsequently, Multon et al., (2003) used a microballoon thoracic compression injury model to show that acute weight-supported treadmill training with manual step-assistance, brought about a statistically significant (1.5 point) improvement in BBB scores at 8 – 12 weeks post-injury, with the trained group scoring a 10. In this study, treadmill trained animals achieved occasional weight supported plantar steps without coordination while untrained animals achieved plantar placement without weight support or weight support at stance only. More recently, Heng and de Leon (2009) showed that treadmill training with weight support and robotic assistance, initiated 6 weeks after a severe thoracic contusion, could bring about improvements in limb trajectory and a reduction in the number of steps with foot drag during stepping on the treadmill. Importantly, they noted that, as a group, animals with severe thoracic contusion injuries were able to generate some weight-bearing, plantar steps when first placed on the treadmill at 6 weeks post-injury. However, their training strategy did not bring about significant improvements in overground stepping (de Leon, R. personal communication). To date, treadmill or other activity-based rehabilitation strategies have not been able to bring about significant changes in overground locomotion in clinically relevant rodent models of spinal cord injury.

The present study used shallow-water walking, developed as a translational rehabilitation tool, to test the hypothesis that quadrupedal, voluntary step training with partial weight support and cutaneous feedback will bring about improvements in overground locomotion in a rodent model of spinal cord injury. The study used adult female SD rats with moderately-severe or severe thoracic contusion injuries in two different six week training regimes, one involving a constant level of weight support and a second involving decreased weight support over time. Stepping was assessed using kinematic and gait measures, both overground and in shallow water, in addition to the BBB Open Field Locomotor Scale. The results show that step-training in shallow water allows animals with moderately-severe thoracic contusion injuries to maintain their ability to generate quality plantar hindlimb stepping in shallow water, observed at 1 week post-injury, out to at least 9 weeks. However, step-training in shallow-water had no influence on the recovery of overground stepping suggesting that these animals experience a functional ceiling of locomotor recovery, brought about by in-cage activity, that cannot be improved upon using a model of partial body weight supported locomotor rehabilitation.

Methods

All animal care and surgical procedures were performed in accordance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and with the approval of the University of Louisville Institutional Animal Care and Use Committee (1996).

Spinal Cord Injury

Young adult female Sprague-Dawley rats (200-250 g, Harlan) were anesthetized with Pentobarbital (50mg/Kg, ip) and received either moderately-severe (25g-cm) or severe (50g-cm) contusion injuries at the T9 spinal cord segment using the NYU Impactor as previously described (Magnuson et al., 1998; Young W., Rutgers, New Jersey). After injury, muscle layers were closed with sutures and the incision was closed with staples and a topical antibiotic was applied. Body temperatures were maintained at 36-37°C and each animal received a prophylactic antibiotic, Gentomycin sulfate (15 mg/kg, im), immediately before the surgery and again 3 and 5 days later. Each animal received 10ml of normal saline, IP, for fluid replacement. Bladders were emptied after injury by gently massaging the lower abdomen twice daily until reflexive bladder emptying occurred (usually 10-14 days post-injury). The animals were kept on a 12:12 light/dark cycle and received water and food ad libitum. Starting at 1 week post-injury, animals were housed 2 per cage.

Experimental Design

Prior to the experiments we determined that a young adult female SD rat with a body weight of 225g placed in 5cm of water displaces 120-140mls and therefore experiences 50-60% body weight support from buoyancy when sitting quietly.

The experiments were completed in 3 phases (Table 1). In brief, Phase 1 involved comparing how injured animals walk overground and with the aid of buoyancy provided by 5cm of water during the acute (to 1 week) and early sub-acute time period. The second phase involved using shallow-water walking as a partial body weight-supported training strategy for animals with moderately-severe and severe contusion injuries. Finally, in Phase 3, we used only animals with moderately-severe contusion injuries to determine if progressive increases in the load on the hindlimbs (decreased water depth) during training could bring about improved hindlimb function during overground stepping.

Prior to each study, all the animals involved were acclimated to the training tank and were taught to receive treats of sweetened cereal each time they walked the length of the pool and part way up the exit ramp. They learned rapidly to move back and forth along the entire length of the tank for the treats and they were much more active as a small group than when placed in the tank alone. The animals were acclimated to the tank with 5cm of water also, but would not, even with repeated efforts, walk lengths of the pool in shallow water for treats. After injury, however, the presence or absence of shallow water in the pool made no discernable difference to the animals; they rapidly resumed the back and forth activity for cereal treats.

Phase 1: Stepping in Shallow Water

The animals were randomized into moderately-severe (n=9) and severe (n=7) groups and were acclimatized to the walking tank before injury. After injury, each animal was assessed individually in the walking tank with and without 5cm of water once each week for 3 consecutive weeks following the injury. Digital video recordings were made of a minimum of 6 complete lengths where the animals walked without stopping. Simultaneous video recordings were made of the sagittal and ventral views for kinematic and gait assessment. In addition, each animal was assessed weekly using the BBB Open Field Locomotor Scale. The training tank was made of plexiglass (150cm long × 18cm wide × 30cm deep), the bottom of which was covered by a 6mm layer of clear Sylgard 184 Silicone Elastomer (Dow Corning Corporation, Midland, MI) to provide grip.

Phase 2: Shallow-water Walking as a Retraining Strategy

For this experiment, animals were randomized into trained and untrained groups prior to receiving 25g-cm (moderately-severe) injuries, and at 1 week post-injury were assessed using the BBB Open Field Locomotor Scale. At 10 days post-injury the animals were re-introduced to the shallow water training tank, 2 or 3 at a time, and were trained for 4 × 10 minute sessions daily, for 4 days each week. A major advantage of training 2 or 3 animals at a time was their comfort levels, which allowed them to be active (stepping) almost continuously for the duration of each training session. Video of training sessions showed that individual animals could complete 70-80 lengths of the training pool, moving in both directions, or more than 350 feet linear distance. During the training sessions the training tank was filled with water to a depth of 5cm and the bottom was covered with Lego™ to provide texture. Training continued for 8 weeks. Untrained animals were transported to the training room with the trained animals and were placed, 2 or 3 at a time, into a large breeding cage, where they remained for the duration of the daily training. This provided a novel social environment, without training, as a control for the training sessions. Grouping of the animals for training was changed each day in an effort to maintain social novelty and activity at a high level.

Phase 3: Shallow-Water Walking with a Load Progression

This experiment was designed to provide a load progression to the hindlimbs of injured animals during shallow-water training. Training was initiated as in Phase 2, at 10 days post-injury and involved 4 × 10 minute training sessions, 4 days each week for 8 weeks. Training sessions on days 10 through 13 were done in 5cm of water. The water depth was reduced to 3.5cm for training sessions on days 14 through 30 and to 3cm for training sessions on days 36 through 49. We intended to reduce the water depth to 2.5cm for training days 50 through 64 but found that many of the animals were not able to generate quality plantar steps with reduced weight support. Thus, for the last two weeks of training the water depth was maintained between 2.5 and 3cm and was adjusted on a session by session basis in an attempt to optimize the plantar stepping generated during training. As in Phase 2, the bottom of the training tank was covered with Lego™ to provide texture.

Behavioral Testing

Hindlimb movement during overground stepping was assessed weekly using the BBB Open Field Locomotor Scale (Basso et al., 1995). As described previously (Magnuson et al., 1998), each animal was placed in an empty plastic wading pool, 100cm in diameter, and was evaluated for 4 minutes by two investigators who were blind to the injury severity and training. The animals received a BBB score ranging from 0 (no hindlimb movement) to 21 (uninjured). The BBB scale is divided into three levels, with scores from 0 to 8 dependent on individual hindlimb joint movements, scores 9 to 13 dependent on body weight support at stance and during stepping, and scores 14 to 21 dependent on forelimb-hindlimb coordination, paw placement, and tail position (Basso et al., 1995).

Kinematic and Gait Analysis

Sagittal and ventral view digital video recordings were made of each rat walking in the training tank with the Lego™ removed. The Sylgard bottom surface provided grip and allowed for a clear view of paw placement in the video images. Each animal received a green dot tattoo on the skin overlying the anterior rim of the pelvis (iliac crest; I) and the head of the greater trochanter (hip; H) using an Aramis micro-tattoo punch. These tattoos were reinforced using a black Sharpie™ marker prior to each recording session. Additional Sharpie™ marks were made on the lateral malleolus of the ankle (A) and on the metatarsophalangeal joint of the toe (T). As described for the traditional foot-print analysis technique (Kunkel-Bagden et al., 1993), the plantar surface of each hindpaw was marked also. As described previously

(Magnuson et al., 2009; Smith et al., 2009), these markings allowed us to define the movement of the limb by three distances (I-H, H-A & A-T) and two angles (I-H-A & H-A-T), and to positively identify plantar hindlimb steps, paw placement order and many other gait characteristics (See Figures 2D and 4B).

High-speed (60Hz) videos of six passes of each rat were analyzed using the kinematic software MaxTraq and MaxMate (Innovision Systems, MI) to create stick figures of the right hindlimb and to identify plantar hindlimb steps, forelimb steps and foot placement order. The HAT and IHA peak angles (extension), trough angles (flexion) and excursion (range of motion) were calculated for each step or hindlimb movement for each pass. The primary measures determined from the ventral-view recordings were the Regularity Index (RI) (Koopmans et al., 2005) and a novel, related measure, the Plantar Stepping Index (PSI). The RI was developed by Hamers and Koopmans (Hamers et al., 2001; Koopmans et al., 2005) as part of the gait assessment done using the Catwalk™ system. It scores plantar steps according to paw placement order and represents the gait as the ratio of plantar steps that are in order (one of four different orders that normal rats exhibit) over the total number of steps. The RI thus scores plantar stepping and forelimb-hindlimb coordination and is an excellent measure for animals with mild to moderate injuries where treatment or training may bring about improvements in coordination. For moderately-severe and severe injuries where there is no real prospect for forelimb-hindlimb coordination, we developed the PSI, which simply represents a ratio of the number of hindlimb to forelimb plantar steps expressed as a percentage (hindlimb steps / forelimb steps × 100). These measures were calculated for walking overground in a dry tank and with the body weight support provided by 5cm of water. For kinematic analysis of shallow-water walking we were unable to use 3D kinematics due to the distortion caused by viewing the hindlimbs through the surface of the water with an appropriate camera angle. Single-camera, 2D kinematics were not affected with the camera placed to view the walkway horizontally.

Histological Analysis

At the end of the study, the animals were euthanized with an overdose of Pentobarbital (100mg/Kg, IP) and perfused transcardially with calcium free tyrodes and 4% paraformaldehyde. The spinal cords were removed, post-fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in a 30% sucrose solution (0.1M PB, pH 7.2) for 4 days for cryoprotection. Then the spinal cords were blocked in OCT tissue freezing medium and stored at -20°C. The thoracic segments containing the injury epicenter were cut transversely at 30 μm on a Zeiss Microm cryostat, mounted onto charged glass microscope slides, and stored at -20° C. The sections were stained with eriochrome cyanin (Scheff et al., 2003) and were coverslipped with Permount (Fisher Scientific, PA). Five micrographs, each separated by 150μm, were taken of sections bracketing the contusion epicenter. The micrographs were taken using a Spot Cooled Digital camera (Diagnostic Instruments, MI) attached to a Nikon Labophot microscope and a G4 Macintosh computer. The images were imported into Appleworks 6.0, traced using a Wacom Intuos drawing tablet (Vancouver, WA), and saved as .tif files. The .tif files were opened in NIH Image J (rsbweb.nih.gov/ij/) where the spared white matter was identified and measured as cross-sectional area (mm^2). Only densely stained, compact white matter was included as spared. The single section with the smallest area of spared white matter was used to represent the injury epicenter. These area calculations were converted to percent spared white matter by comparison with an uninjured control as described previously (Magnuson et al., 2005).

Statistics

Terminal spared white matter, PSI and joint excursion data were analyzed statistically using Independent t-tests. BBB scores and joint excursions (baseline, week 1 and terminal) were assessed using repeated measures analysis of variance (ANOVA) followed by a Tukey's HSD post hoc t-test comparison when appropriate. All data are expressed as mean ± SD (n) unless otherwise noted.

Results

Phase 1

The goal of this study was to assess whether animals with spinal cord injuries walk better with partial weight support in shallow-water than overground at relatively early time points. As expected, 25g-cm injured animals had significantly more spared white matter at the epicenter than the 50g-cm injury group (9 and 4.5%, respectively) and both groups experienced improved hindlimb function during overground walking over the first 3 weeks post-injury (Figure 1A). The 3 week BBB scores of 10.06 ± 0.92 and 7.86 ± 0.75, respectively, indicated that the 25g-cm group occasionally achieved plantar, weight-supported stepping (BBB = 10) while the 50g-cm group could only achieve sweeping movements involving all 3 joints (BBB = 7) or plantar placement of the hindpaw without weight support (BBB = 8; Figure 1A). The Plantar Stepping Index (PSI) showed that the 50g-cm injury group was unable to achieve plantar stepping at any time point without partial body weight support and with weight support achieved hindlimb plantar placement during stepping at a rate of 10% of the number of forelimb steps (Figure 1D). In contrast, the 25g-cm injury group achieved plantar stepping at a rate of 15% without partial weight support, in keeping with a BBB score of 10, and a significantly improved level of close to 50% of the number of forelimb steps at each time point, with partial weight support (Figure 1C). They exhibited a full range-of-motion for the proximal and distal limb segments as illustrated by the excursion graph in Figure 1B. These data indicate that the partial body weight support provided by 5cm of water allowed the 25g-cm injury group to achieve plantar, partial weight-supported stepping at 1 week post-injury in the absence of any manual or robotic assistance. These data also show that the PSI did not improve, with weight support or overground, over the first three weeks despite the significant improvement in BBB score over the same post-injury time period.

Phase 2

The goal of this study was to utilize partial weight supported walking in shallow water as an activity-based rehabilitation strategy during the acute and sub-acute (1-9 weeks) post-spinal cord injury time period with the goal of bringing about improved overground stepping.

At the terminal time point (9 weeks post-injury), the spared white matter at the injury epicenter was approximately 6.5% and 3.5% for the 25 and 50g-cm injury groups, respectively, and was not different for the trained and untrained groups for each injury severity (Inserts in Figures 2A & B). Similarly, BBB scores were not different for the trained and untrained groups for each injury severity at any time point. Figures 2A and B show the BBB scores over time relative to a score of 10 where animals are able to achieve occasional plantar steps with weight support. Figure 2C shows that 25g-cm/untrained animals had a PSI of approximately 25 at week 9 in both assessment conditions (with and without partial body weight support) indicating that, without training, these animals were able to generate 1 plantar hindlimb step for every 4 forelimb steps. With partial body weight support, however, trained animals were able to generate almost 6 plantar hindlimb steps for every 10 forelimb steps (PSI = 59). This is a significant improvement over their PSI of 25 without the aid of weight support and over the untrained group, showing that trained animals could step better in shallow water than overground, and better in shallow water than the untrained group (independent t-test; $p<.05$). Figure 2C also shows that training appears to have had no influence on how animals with severe (50g-cm) injuries walk with weight support. While trained animals had a PSI of 15 walking with weight support and were not able to generate any plantar steps in the absence of weight support, the untrained group was only able to achieve PSIs of 22 and 9, with and without weight support, respectively. Figure 2D demonstrates the lateral and ventral views of one 25g-cm injured rat stepping in shallow water as an example of the images used to determine gait parameters.

Figure 3A shows that training in shallow water had no significant influence on the angular excursion of the Hip-Ankle-Toe angle for animals in the 25g-cm injury group. Both trained and untrained animals had HAT angular excursions close to normal when walking in shallow water despite the significant difference in PSI shown in Figure 2. When walking in dry tank conditions, untrained animals exhibited a mean peak HAT extension of approximately 175°, significantly greater than for normal animals, suggesting that they drag their hindlimbs with highly extended ankles and the dorsal surface of the paw in contact with the ground (Figure 3A). These observations are illustrated by the stick figures from one representative rat (25g-cm/trained), showing two plantar hindlimb steps in 5cm of water where the contact with the ground is stable throughout the stance phase (Figure 3B). In Figure 3C, the same animal is shown stepping in a dry tank. In this example the paw first drags with the ankle highly extended (dorsum of paw in contact with the ground), followed by a swing phase, which is in turn followed by a stance phase where the plantar surface of the paw is in contact with the ground, but is dragged with the ankle in a flexed position (Figure 3B & C). In B, the point of contact is stable for both weight-supported plantar steps in shallow water while in C, the paw moves relative to the ground throughout the step, with the ankle first extended and then flexed, in the dry tank.

Phase 3

The goal of this study was to utilize partial weight supported training in shallow water, and in addition to provide a progression of increased load to the hindlimbs as training proceeded. All the animals had 25g-cm contusion injuries. Starting at week 2, the water depth was reduced by 1.5cm, and starting at week 4 the depth was reduced an additional 1cm, and was adjusted within this range to provide a load progression while retaining sufficient weight support to allow for plantar hindpaw placement. As in phase 2, training was done with a textured bottom (Lego™).

At the terminal time point (9 weeks post-injury) the mean spared white matter was between 5 and 6% and was not different for the two groups (Figure 4A). Similarly, the two groups had BBB scores of 10 with very low variability indicating that most animals achieved occasional weight supported, plantar steps without coordination. Both trained and untrained animals exhibited dragging of the hindlimb with the ankle extended and most animals also showed some dragging of the hindlimb with the ankle flexed resulting in an exaggerated angular excursion. Examples of both kinds of hindlimb dragging can be seen in Figure 4B. Using 3D Maxtraq with the VJR (Virtual Joint Recognition) plug-in to identify the position of the knee, significant differences in the angular excursion, peak (extension) and trough (flexion) were seen at week 1 post-injury, compared to baseline, for the complete set of animals prior to the initiation of training. Figure 5A shows that knee and hip excursions are significantly reduced at week 1 post-injury, being held in a more flexed position compared to baseline. In contrast, the ankle position is significantly extended at week 1 compared to baseline. These kinematic characteristics correspond to BBB scores of 2-5, as shown at week 1 in Figure 4A, indicating that these animals, at best, have extensive movement about 2 joints without sweeping movements. At 9 weeks post-injury the ankle shows significantly increased angular excursion compared to baseline while the knee and hip exhibited kinematic characteristics (excursion, peak flexion and peak extension) that were not different from baseline measures (Figure 5A). These kinematic characteristics correspond to the terminal BBB score of 10 indicating that most animals achieved occasional weight-supported plantar steps (5-50% of the time) regardless of training. Similar to phase 2, training did not bring about a significant improvement in stepping in a dry tank. The trained and untrained groups achieved PSIs of approximately 30 and 20% respectively (Figure 5B) illustrating that both groups could generate 2 or 3 weight supported plantar hindlimb steps for every 10 forelimb steps. Most of these steps appeared in an abnormal pattern, however, resulting in RIs of approximately 15 and 4, respectively, for the trained and untrained groups stepping in a dry tank (Figure 5B). Figure 5C shows stick figures, derived from MaxTraq 3D, of the right hindlimb of one trained animal stepping in a dry tank to illustrate a plantar drag (plantar contact with ankle in a flexed position), a plantar step with weight support (fixed contact point) and a hindlimb drag with the ankle in an extended position (dorsal contact, extended ankle).

DISCUSSION

Using swimming as a model of activity based retraining, we showed recently that swim trained animals had improved swimming characteristics; increased hindlimb activity, improved body position and near-normal hindlimb kinematics. However, hindlimb velocities remain significantly below normal (Smith et al., 2006a; Magnuson et al., 2009). Swim training appears to be task-specific because training does not bring about any improvements in overground stepping (Smith et al., 2006a; Magnuson et al., 2009). Alternatively, and as suggested previously by Fouad et al. (2000), animals with incomplete spinal cord injuries may retrain themselves to walk overground while moving about in their cages over the first few weeks post-injury (Smith et al., 2006a; Magnuson et al., 2009). Thus, activity-based training in this model may be unsuccessful because of a ceiling effect. In an effort to distinguish between task-specificity and a ceiling effect, we developed a model of voluntary, body-weight supported locomotor training based on walking in shallow water. It has the advantages of dynamic body weight support (buoyancy) with appropriate limb-loading and cutaneous feedback while allowing the animal to produce plantar steps voluntarily and quadrupedally. More than one animal can be trained at a time in a single training tank, loading of the limbs can be altered with water depth and a textured bottom can be used to improve grip and cutaneous feedback during the training.

Phase 1: Injury Severity vs Locomotor Capacity in the Acute Time Period

The relationship between injury location and severity and the functional capacity of the nervous system to generate stereotypic locomotor movements is complex, in particular during the post-spinal shock, acute and sub-acute time periods. We hypothesized that assessing hindlimb function using the BBB Open Field Locomotor Scale or kinematically, overground or on a treadmill, without providing some body weight support may underestimate the pattern-generating capacity of the nervous system simply because weight-support would appear to be a pre-requisite to allow pattern generation to be expressed and observed (Timoszyk et al., 2005). In this experiment, animals were assessed weekly for three weeks to compare stepping overground and with 50-60% body weight support in 5cm of water. As anticipated, both injury groups (moderately-severe [25g/cm] and severe [50g/cm]) showed improved BBB scores over the 3 week period (Basso et al., 1996; Smith et al., 2006a). However, gait analysis (the plantar stepping index or PSI) showed that the number of plantar hindlimb steps achieved by animals with 25g-cm injuries was significantly improved with partial weight support, compared to overground, but did not change over the three week period. In contrast, animals with 50g-cm injuries were unable to generate significant numbers of plantar hindlimb steps with or without partial weight support.

These data indicate a significant functional difference between the 25 and 50g-cm NYU Impactor injury groups that may not have been previously identified. The dramatic increase in PSI for 25g-cm injured animals placed in 5cm of water (from 15 to 50%) and near normal hindlimb kinematics, shows that their capacity to generate a functional locomotor pattern was present acutely post-injury, but was not observed when walking overground presumably because of a lack of capacity for weight support. In contrast, animals with 50g-cm injuries were unable to generate a locomotor pattern even with partial weight support, suggesting that they lack the capacity for both weight support and pattern generation at 1 week post-injury.

Phase 2. Locomotor Training with Partial Weight Support

In clinical rehabilitation, weight-supported treadmill training is gaining wider acceptance despite the fact that our understanding of what components comprise an optimal strategy is far from complete. There is general agreement that loading, cutaneous feedback and step-assistance are critical (Edgerton et al., 2001), and that training of individuals with incomplete ASIA C and D injuries is generally more successful than training of persons with ASIA A (complete) injuries (Dietz and Colombo, 2004). However, the success of weight-supported treadmill training is variable and hard to predict. Furthermore, the majority of clinical studies focus on chronic patients that are at least 6 months post-injury. While this strategy is sound in terms of avoiding potential harm and detecting improvement in functionally stable subjects, Norrie and colleagues showed in the rat dorsolateral laceration model that early initiation of activity-based rehabilitation results in higher overall functional gains (Norrie et al., 2005). The fact that much of the animal literature is based on task-specific improvements in hindlimb stepping following a complete transection, which comprise only a small minority of clinical injuries, must also be considered when interpreting the outcomes of both clinical and basic animal studies.

In this experiment, we utilized the dramatically improved stepping exhibited by 25g-cm injured rats when placed in 5cm of water as a training strategy, initiating the training at 10 days post-injury. The buoyancy of the water provided partial body weight support that allowed the animals to achieve high numbers of plantar hindimb steps with loading, and supplementary cutanous feedback was provided by Lego™ sheets placed on the bottom of the pool. The 2 and 3 week BBB scores suggest that the training influenced the initial rate of improvement in overground stepping, but the early differences were not maintained later into the study. Rapid improvements in overground stepping of trained rats with moderately-severe contusion injuries have also been reported by Stevens et al. (2006). This group utilized one week of treadmill training with manual weight support to show that improvements in overground locomotion were accompanied by increases in the peak forces generated by the soleus muscle, retained muscle mass and improved muscle fiber size (Stevens et al., 2006). In the current study, at 9 weeks post-injury 25g-cm/trained animals had a PSI of almost 60 in shallow water, significantly higher than the 25 achieved during overground stepping, while the 25g-cm/untrained animals scored around 25 both in the water and overground. No differences were observed in BBB scores for the trained and untrained groups. One interpretation of these data is that training with partial weight support in shallow water brought about a task-specific improvement in shallow water stepping in trained animals compared to untrained controls. Task-specificity is a hallmark of many training studies using both human and animal models. For example, Heng and DeLeon showed recently that weight-supported treadmill training brought about specific improvements in ankle trajectory during stepping on the treadmill, and in keeping with the current study, that both trained and untrained rats could successfully generate partial weight-supporting plantar hindlimb steps at 6 weeks post-injury (Heng and DeLeon, 2009).

Keeping in mind that the animals with 25g-cm injuries in the phase 1 experiment had PSIs of 50 at week 3 post-injury, with no training, an alternative interpretation of these data is that the untrained animals experienced a decrease over time in their capacity to generate plantar paw placement (pattern generation). The untrained animals with 25g-cm injuries in phase 2 achieved a PSI in shallow water at 9 weeks post-injury of approximately 25, while the animals with similar injuries in phase 1 achieved a PSI of approximately 50 at much earlier time points (weeks 1, 2 and 3). Spared white matter was slightly less for the animals in phase 2 (6.5 vs 9%) which may account for this difference, but terminal BBB scores were almost identical. This alternative explanation suggests that the effect of shallow-water training of animals with 25g-cm injuries was to maintain the capacity for plantar stepping with weight support, rather than to retrain a capability that was lost following SCI.

Phase 3. Locomotor Training with Increased Load

A number of both animal and human studies have shown that the proportion of body weight support required during treadmill training decreases with training time (Cha et al., 2007; Harkema et al., 1997) arguing that the capacity for weight support is improved through training. It also has been hypothesized that decreasing the amount of weight-support provided during training (i.e. a load progression) is an important component of a successful training regime (Timoszyk et al., 2005). To test the "load progression" hypothesis, we used the shallow water model starting with 5cm of water and decreased the water depth progressively over time, attempting to maintain plantar stepping and to maximize loading of the hindlimbs. As previously reported (Thota et al., 2001), significant changes in the angles of all three hindlimb joints were observed following injury. Lack of capacity for weight support was observed at week 1 as extended ankles (dragging), while the hips and knees were flexed. At 9 weeks post-injury both trained and untrained groups exhibited hip and knee angles that were not different from controls, although the ankle excursions were significantly greater than either baseline or week 1 post-injury due to the presence of both plantar paw placement (relatively flexed ankle) and dragging with the ankle in an extended position. Trained and untrained groups had similar BBB scores, and no significant differences were found in either the PSI or RI. Taken together, these data show that training with a load progression did not improve overground locomotion, plantar paw placement, forelimb-hindlimb coordination or the characteristics of joint movement during stepping. The fact that the terminal PSI of the trained group was only 30 suggests that training with a load progression was less effective at maintaining the capacity for plantar stepping than training for the assessed task, stepping with 50-60% body weight support in 5cm of water.

Training vs Maintenance

As mentioned earlier, Heng and DeLeon (2009) recently showed that body-weight supported treadmill training of adult rats with moderate thoracic contusion injuries resulted in a reduced number of paw drags and an improvement in step cycle trajectories when trained and untrained groups were compared on the treadmill. Interestingly, they found that the number of paw drags exhibited by the untrained group increased significantly from week 6 post-injury to week 14, whereas the trained group exhibited a similar small number of paw drags at the two time points examined. Thus, the authors suggest that with respect to this parameter, partial weight-supported treadmill training maintained the quality of stepping present at 6 weeks out to 14 weeks, rather than bringing about frank improvements. Subsequently, Courtine and colleagues (Courtine et al., 2009) published an in-depth look at a combination of body-weight supported treadmill training, epidural stimulation and serotonin receptor activation in adult rats with complete thoracic transections. One of the many striking findings of this study was the fact that this combination of treatments allowed fully transected rats to generate near normal hindlimb stepping on the treadmill at 1 week post-injury. Over time, training with this combination of treatments allowed the normal kinematic pattern to be maintained while the percentage of weight support that was necessary decreased such that at 9 weeks post-injury most animals could step beautifully on the treadmill with no need for weight support. These findings, taken together with those of Heng and DeLeon and of the present paper, suggest that the spinal cord below the level of either a thoracic transection or contusion injury, has the capacity to generate hindlimb steps with near-normal gait and kinematic characteristics at acute (within 1 week) and sub-acute time points, but that this capacity is masked by the lack of ability to generate weight support. We would speculate that contusion injuries, sparing some descending input including reticulospinal serotonergic fibers, requires enhanced afferent input to facilitate a stepping pattern, while full transections appear to require both serotonergic replacement and exogenous afferent input (epidural stimulation) to facilitate a stepping pattern. Enhanced afferent input was provided by the treadmill in the Heng and DeLeon (2009) study and by the lego™ pool bottom in the present study. Thus, the capacity of the spinal cord below the level of injury to generate a normal stepping pattern is not diminished acutely, post-injury, but the ability to generate weight support is greatly reduced. The logical conclusion, therefore, is that locomotor training at acute and sub-acute time points is successful if it improves the capacity to generate weight support, but chronically (as with the majority of clinical situations), both pattern generation and weight support must be addressed.

An additional component to consider, that was not systematically studied in the present experiments, is trunk stability (posture). During overground stepping at sub-acute and chronic time points, abilities to plantar place and to generate a normal stepping pattern may be hampered by inappropriate posture and trunk instability. The buoyancy provided by 5cm of water, as utilized in the present experiments, provides natural stabilization, dynamically, and may lead to independent improvements in trunk stability over the training period. If step training in shallow water did provide improvements in trunk stability, these did not appear to translate to improvements in overground stepping.

Conclusion

In these studies, animals trained to step in shallow water maintained that ability out to 9 weeks post injury, but showed no improvements in overground stepping compared to untrained controls. The reverse was also true. Untrained animals showed improvements in overground locomotion, observed as “normal” functional recovery, that were not accompanied by improvements in stepping in shallow water. Thus, these data support the concept of spontaneous functional recovery resulting from the in-cage activity of animals over the first few weeks post-injury, providing a ceiling of recovery that swim, shallow water step or treadmill training does not improve upon (Fouad et al.,2000; Smith et al., 2006; Magnuson et al., 2009; Heng and DeLeon, 2009). Finally, our data supports the idea that the capabilities for weight-support and generating a stepping pattern are, at least in part, functionally and perhaps anatomically distinct and that a capability for weight-support is a necessary prerequisite for the expression of a functional stepping pattern following spinal cord injury.

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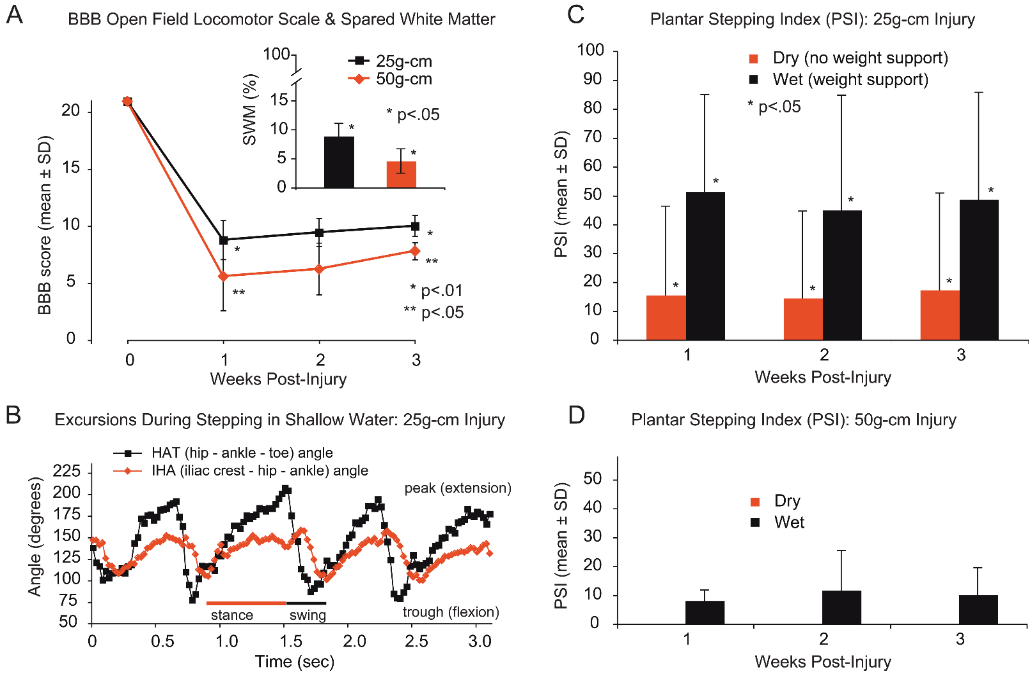

Figure 1.

The BBB scores over time for 25 and 50g-cm injured animals are shown in A. Each outcome measure increased significantly from week 1 to week 3 (ANOVA and post-hoc t-test; *, p<. 01; **, p<.05). The inset shows the mean spared white matter (SWM; cross sectional area) at the injury epicenter, which was significantly different for the two groups (Independent t-test; *, p<.05). B. The angular excursion of the IHA (iliac crest – hip – ankle) and HAT (hip – ankle – toe) angles over time are shown for 3 steps taken by a representative 25g-cm injured rat at week 1 with the weight support of 5cm of water. C shows the mean PSI ± SD (Plantar Stepping Index) for 25g-cm injured animals at weeks 1, 2 and 3 when assessed walking in a dry tank (Dry) and with the weight support of 5cm of water (Wet). Animals achieved significantly higher PSIs when walking in 5cm of water as compared to a dry tank at each time point tested (Independent t-test; *, p<.05). D. Shown is the mean PSI ± SD for 50g-cm injured animals at weeks 1, 2 and 3 assessed in both the dry and wet (5cm of water) conditions. The PSI indicates that these animals could achieve a few plantar hindlimb steps in 5cm of water but could not when walking in a dry tank.

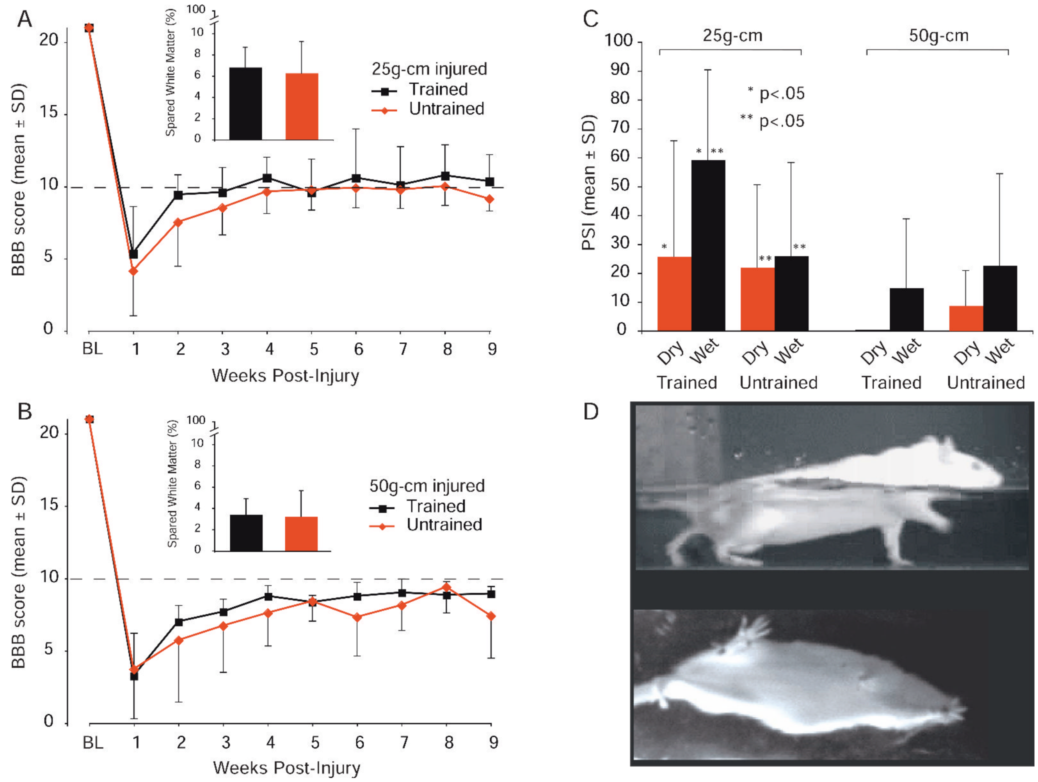

Figure 2.

The results from the phase 2 experiment are shown in A and B as the BBB scores over time for 25 (A) and 50g-cm (B) injured groups that received training in shallow water (black squares) or remained untrained (red diamonds). Training had no significant effect on BBB scores (repeated measures ANOVA). Insets show that the mean spared white matter (cross sectional area) at the injury epicenter was not different for the trained and untrained groups for each injury severity. C shows that 25g-cm injured animals that were trained in shallow water achieved a significantly higher mean PSI when stepping in shallow water than when stepping in a dry tank (Independent t-test; *, $p<.05$) and a significantly higher mean PSI than the untrained group stepping in a dry tank or in shallow water (Independent t-test; **, $p<.05$). In contrast, training had no influence on the mean PSI of the trained and untrained 50g-cm injured animals. Still images taken from sagittal and ventral video of 25g-cm injured animals stepping in shallow water are shown in D to illustrate the limb positions during plantar stepping from both aspects (means ± SD).

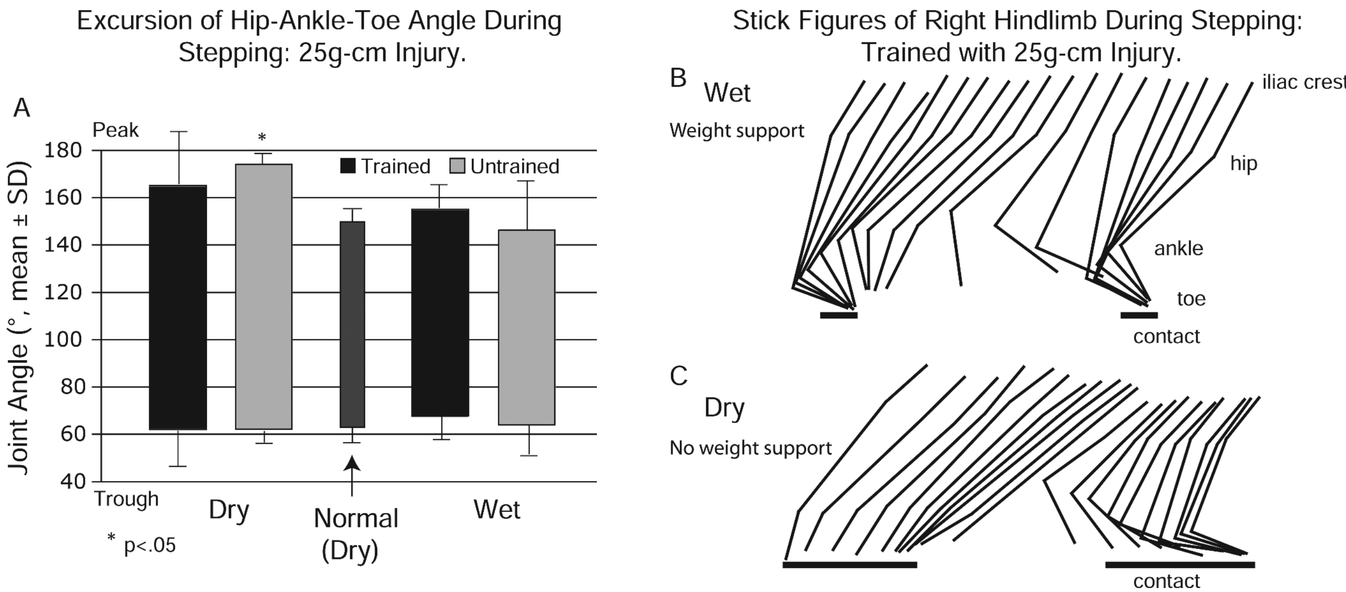

Figure 3.

The graph shown in A compares the mean peak (extension), trough (flexion) and excursion of the hip-ankle-toe angle for trained and untrained 25g-cm injured animals, in both dry and wet (5cm of water) conditions, with uninjured baseline measures (normal, dry) at 9 weeks post-injury after 8 weeks of training (all are means ± SD). No group differences were detected except for the untrained group that had a significantly higher mean peak (extension) in the dry tank compared to baseline (normal, dry; Independent t-test; *, $p<.05$). The stick figures in B and C illustrate the limb and joint movements during stepping of a representative trained 25g-cm injured animal in shallow water (B) and in a dry tank (C). In shallow water the point representing the toe remains fixed during stance. In contrast, when walking in a dry tank this animal exhibited dragging of the hindlimb in both extended and flexed states as illustrated by the movement of the point representing the toe relative to the bottom of the tank (contact).

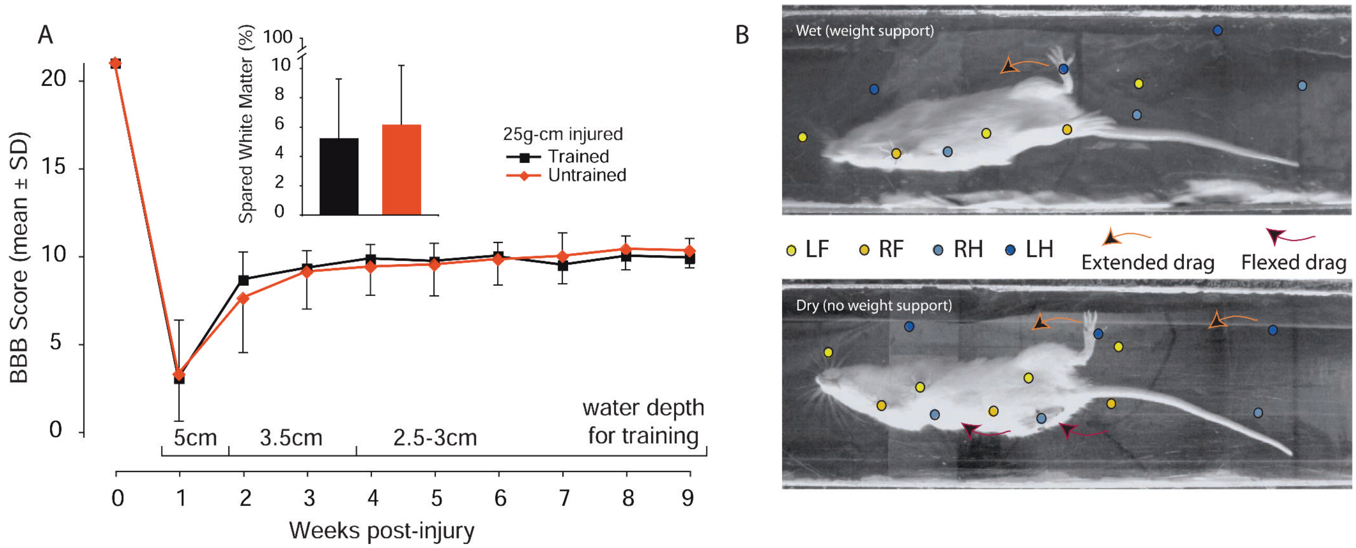

Figure 4.

In A, the mean BBB scores (± SD) of trained and untrained 25g-cm injured animals are shown over time relative to the depth of water utilized during training sessions occurring the week of the assessment. No group differences were found at any time point. The inset shows the spared white matter at the injury epicenter for the two experimental groups, which were not different. In B, still images taken from digital videos of the ventral view of the same trained 25g-cm injured animal show the placement of fore and hindpaws during stepping in shallow water (top) and in a dry tank (bottom). Orange arrows designate dragging of the hindlimb with the ankle in an extended position (dorsal contact) and red arrows designate dragging of the hindlimb with the ankle in a flexed position (plantar contact).

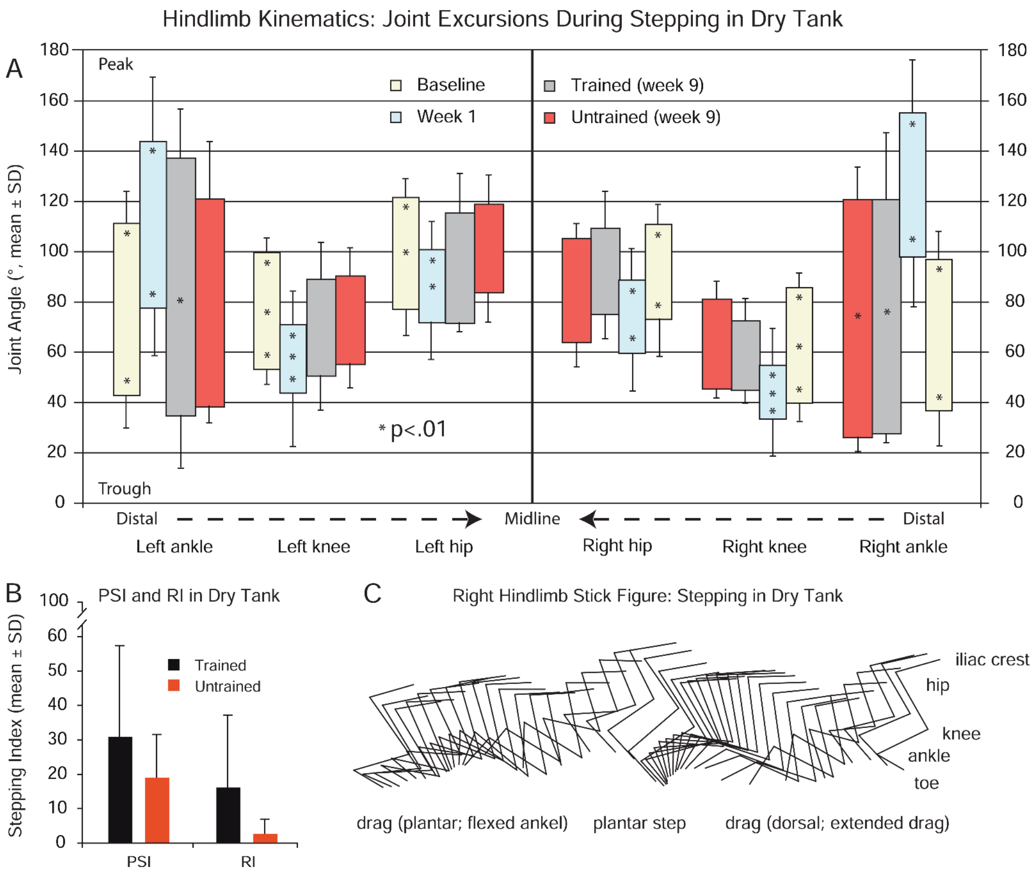

Figure 5.

Shown in A are the means ± SD for the peak (extension), trough (flexion) and excursion of the hip, knee and ankle of trained and untrained 25g-cm injured animals, pre-injury (baseline), at week 1 post-injury (pre-retraining) and at week 9, after 8 weeks of training or cage rest (untrained). Asterisks (*) indicate significant differences from baseline for maximum joint extension (peak, top of bar), maximum joint flexion (trough, bottom of bar) and total joint excursion (peak - trough, middle of bar). These data were analyzed using repeated-measures ANOVA followed by a Tukey's post-hoc t-test. With the exception of ankle excursion, no significant differences were found for trained or untrained groups compared to baseline measures or for the trained group compared to the untrained group. B. The mean PSI and RI (± SD) for walking in a dry tank were not statistically different for trained compared to untrained animals. The stick figures shown in C represent three common occurrences observed during stepping in a dry tank: 1. Plantar drag (when the plantar surface of the paw is visible to the ventral camera and is in contact with the surface but is dragged along the surface without providing significant weight support). This is not defined as a plantar step for the PSI. 2. Plantar step (where the plantar surface of the paw is clearly visible to the camera and does not shift position until swing is initiated). This kind of step is associated with weight support as reflected by the hip height and would be defined as a plantar step for the PSI. 3. A limb drag with the ankle extended (the plantar surface of the paw is not visible to the ventral camera and the hip height is low). This would not be defined as a plantar step for the PSI.

Table 1

Experimental Design

	Injury	n	Training	Duration
Phase 1	25 g-cm	9	none	wks 1, 2 & 3
	50 g-cm	7	none	wks 1, 2 & 3
Phase 2	25 g-cm	6	5cm water	9 weeks
	25 g-cm	4	none	9 weeks
	50 g-cm	6	5cm water	9 weeks
	50 g-cm	5	none	9 weeks
Phase 3	25 g-cm	9	5 to 3cm water	9 weeks
	25 g-cm	8	none	9 weeks