Effects of exercise training on urinary tract function after spinal cord injury

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Abstract

Spinal cord injury (SCI) causes dramatic changes in quality of life including coping with bladder dysfunction which requires repeated daily and nightly catheterizations. Our lab has recently demonstrated in a rat SCI model that repetitive sensory information generated through task-specific stepping and/or loading can improve non-locomotor functions, including bladder function. To target potential underlying mechanisms, the current study included a forelimb-only exercise group to ascertain whether improvements may be attributed to general activity effects that impacts target organ-neural interactions or to plasticity of the lumbosacral circuitry that receive convergent somato-visceral inputs. Male Wistar rats received a T9 contusion injury and were randomly assigned into three groups two weeks post-injury – quadrupedal locomotion, forelimb exercise, or a non-trained group. Throughout the study (including pre-injury), all animals were placed in metabolic cages once a week for 24 hours to monitor water intake and urine output. Following the 10-week period of daily one-hour treadmill training, awake cystometry data were collected and bladder and kidney tissue harvested for analysis. Metabolic cage frequency-volume measurements of voiding and cystometry reveal an impact of exercise training on multiple SCI induced impairments related to various aspects of urinary tract function. Improvements with both quadrupedal and forelimb trained groups implicate underlying mechanisms beyond repetitive sensory information from the hindlimbs driving spinal network excitability of the lumbosacral urogenital neural circuitry. Furthermore, the impact of exercise training on the upper urinary tract (kidney) underscores the health benefit of activity-based training on the entire urinary system within the SCI population.
Introduction

Improving bladder deficits is among the areas of highest priority following spinal cord injury (SCI) as urinary tract impairment has an enormous impact on quality of life (2, 3, 26). Life-long urologic care is required for SCI individuals, yet most efforts treat symptoms but do not improve intrinsic function (68, 80). Bladder management requires intermittent catheterization throughout the day/night to avoid incontinence, bladder over-distention (which can create high pressure and reflux to the kidneys), inflammation, infections, and autonomic dysreflexia.

Despite bladder dysfunction being a high priority for SCI individuals, the focus of health care professionals is on rehabilitation aimed at optimizing mobility and the remaining musculoskeletal function. Locomotor training (LT) has emerged as a safe and effective therapy for post-SCI motor deficits with many benefits (cardiovascular function, strength, mobility) (9, 23, 43, 52, 84). Recent animal studies, however, have shown that LT post-SCI also improves bladder function (46, 90), a finding consistent with a few reports from human SCI studies (42, 48, 75). For example, a recent study from our lab has shown, using a spinal contusion model in adult male rats, functional gains of lower urinary tract function as assessed with terminal urodynamic measures after 12 weeks of daily LT for a period of 60 minutes per day (90). The interaction of lower limb musculature with the bladder and its sphincter has been observed sporadically over the years, as far back as 1933, in both humans and animal studies (20, 53, 74). Flexor and extensor reflexes can be modulated by the state of bladder filling and voiding in normal humans and those with CNS damage (64). In humans with spasticity, the general pattern is that detrusor contractions precede limb
flexor spasms (69). This vesicosomatic relationship involving lumbosacral reflex circuitries could contribute to the enhancement of bladder function with LT.

The multi system functional gains with task-specific training have generated multiple novel hypotheses regarding potential underlying mechanisms. With respect to the improvements in bladder function with 60 minutes/day of stepping on a treadmill using body weight support and manual facilitation in a natural position (90), the current study was designed to expand upon our initial findings to include: (1) non-weight bearing stepping with a forelimbs only exercise group; (2) collection of weekly metabolic cage data to monitor SCI-induced persistent polyuria (over-production of urine); and (3) further tissue assessments that include the structural integrity of the bladder wall and the impact of training on the upper urinary tract (kidneys).

The bladder wall itself has been shown to be more compliant after spinal transection and the extracellular matrix components largely determine its mechanical properties (36). Collagen and elastin, two major connective tissue proteins, provide tensile strength and elasticity and are implicated as being directly responsible for the mechanical changes of the bladder wall of SCI rats (83), so their quantification will give a more accurate picture of potential bladder composition remodeling.

Long-standing detrusor sphincter dyssynergia with chronic SCI, even with careful bladder management, may lead to vesicoureteral reflux due to high bladder pressures from over-distention and complications that include kidney infections, pyelonephritis, and hydronephrosis (4, 13, 59). Bladder infections from multiple daily catheterizations may also spread to the kidney. Chronic kidney disease is highly prevalent in the SCI population, with complications that include decreased glomerular filtration rate and renal plasma flow (30,
60, 73). For the current study, the expression of two proteins indicative of tissue turnover in
the kidneys, Transforming growth factor-beta (TGFβ, a fibrogenic growth factor implicated
in the pathogenesis of renal scarring) and Cluster of Differentiation Molecule 11b (CD11b,
an adhesion molecule which promotes cell-cell adhesion between leucocytes and
leucocyte-endothelial cells in inflammation), were assessed in order to determine if
improved bladder function with LT could also reduce the kidney’s susceptibility to post-SCI
complications. TGFβ promotes fibrogenesis, cell apoptosis and tissue healing and
suppresses excess cellular proliferation, differentiation and immunity (66). Although
increased expression of TGFβ is considered a valuable marker in determining fibrosis with
kidney disease (1, 11, 38, 94, 95), exacerbation of the immune response or autoimmunity
has been reported in association with the absence or decreased expression of TGFβ (37)
and the immune system is known to be impacted in persons with SCI (61). Also, damage
to kidney tissue in ischemic reperfusion injury can be mitigated by blocking CD11b (71) as
it contributes to epithelial injury, inflammation and fibrosis (32).

Methods

All experimental procedures were conducted according to NIH guidelines and
protocols were approved by the Institutional Animal Use and Care Committee at the
University Of Louisville School Of Medicine. A total of 55 adult male Wistar rats (Harlan
Sprague Dawley, Inc, Indianapolis, IN), weighing initially approximately 250 grams, were
individually housed in an animal room with a 12-hour light and dark cycle. They had ad
libitum access to water and food (Laboratory Rodent Diet).
Spinal Cord Injuries The Infinite Horizon (IH) impactor device (Precision Systems and Instrumentation, LLC; Fairfax Station, VA) was used to make a clinically relevant contusion injury (225 kilodyne) at the T9 spinal level of 48 rats (7 additional rats served as sham surgical controls). This impact produces a moderate to severe incomplete SCI. Procedures for SCI followed our previously published protocols (41, 49, 50, 91). Briefly, animals were anesthetized with a mixture of Ketamine (80mg/kg) and Xylazine (10 mg/kg) (i.p.) and 0.1 ml supplemental doses were given i.p. as needed to maintain a deep surgical level of anesthesia. Vital sign monitoring included heart rate with a rodent stethoscope, respiratory rate and ventilator status with a small animal oximeter (Starr Life Sciences Corp.), body temperature with a rectal thermistor, and anesthetic depth with corneal, palpebral, pedal and pinna reflexes as well as tail pinch.

For the spinal contusion, the cord was exposed at the T9 level via removal of the overlying vertebral lamina and the IH impactor force-driven device (76) was used to make the contusions (no dwell time). After the injury, a piece of thrombin-soaked Gelfoam was placed into the vertebral defect, the surrounding musculature and subcutaneous tissue sutured in layers with 4-0 monofilament, and the skin closed with Michel clips (removed 7 days post-op). All rats were injected subcutaneously with 0.5ml of dual penicillin (Penicillin G coupled with Procaine, PenJect ®, The Butler Company, Columbus, OH) as a general prophylactic and recovered in a temperature-controlled environment. In the days immediately following SCI, all animals were also given subcutaneous injections of ketoprofen (Ketofen, 2.5mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) for analgesia (twice a day for 2 days) and 5mg/kg gentamicin (GentaFuse®, Butler Schein, Dublin, OH) to prevent bladder infections (once per day for 5 days).
The urinary bladder was initially emptied by manual crede every 8 hours until the micturition reflex occurred automatically (spontaneously triggered by contact with cage bedding or otherwise without caretaker assistance), 6–10 days after injury (29, 51).

Residual urine was collected and measured three times over a 24-hour period for every rat on the days immediately post-injury and recorded in the animals’ surgery recovery log. Only the maximum single daily urine volume (typically the a.m. volume after lights on – i.e., after the active phase during which more water tends to be consumed) was used for analysis as this value is most reflective of the largest bladder capacity. Note that the amounts obtained at a 3-4 day time point are reflective of the initial peak bladder dysfunction immediately post-SCI with higher volumes indicative of a more severe injury (91). These data along with a two week time-point score of overground locomotion using the 21 point Basso-Beattie-Bresnahan (BBB) open field locomotor test (6) (as previously described in detail (90, 91)) were set aside for end-of-study post-hoc analysis to check for pre-training group differences in terms of lesion severity. Training was initiated 14 days post-injury after animals were randomly divided into three study groups (as described below). This delayed time frame was chosen because (1) rehabilitation efforts initiated too early after SCI have been shown to be detrimental as they can exacerbate secondary injury cascades (79) and (2) two weeks is beyond the period of SCI induced plasticity of sacral parasympathetic bladder reflex pathways (18, 19, 86) which can occur up to 10 days (29, 51).

Treadmill Training  SCI rats were randomly divided into 3 groups of 16 each two weeks post-injury; two groups that followed a step-training regimen over a treadmill belt assisted
by a body weight support system (Exer-3R treadmill from Columbus Instruments) (90, 91) and a non-trained control group with no body support. All groups were harnessed with a cloth rodent vest (Robomedica Inc., Mission Viejo, CA) with spring scales clipped onto both ends of the vest for weight support as needed. The quadrupedal-trained group (n=16; SCI+QT) stepped on the treadmill with all four limbs, bearing weight on the hindlimbs with manual facilitation below the level of injury (90), with the spring scales self-adjusted to allow for quadrupedal stepping and complete paw placement. The forelimb-only trained group (n=16; SCI+FT) had its hindlimbs slightly elevated from the surface of the treadmill using the spring scale (non-weight bearing; similar to arm crank exercise in human SCI) so that it only stepped with its forelimbs. The non-trained control group (n=16; SCI) was also harnessed on the treadmill for the equivalent one hour time frame but was not stepped. Seven additional rats, used as non-injured controls (surgical shams – spinal cord exposed as the other groups but no contusion), were handled weekly but remained in their home cages for the duration of the study.

Given the time necessary for the daily step training of each rat, the current study was done in four groups of 12 rats each over a period of about two years. Each set of 12 comprised 4 SCI+QT, 4 SCI+FT and 4 SCI only control rats. In a given hour session, one SCI+QT and one SCI+FT rat were on the 15 inch wide treadmill belt with one non-trained SCI rat in a harness next to it. Thus, four hours of training was done in total per day, with 15 minute breaks every hour between groups. Each set of 12 rats took part in the study for a total of approximately 14 weeks (acclimation period, pre-injury testing, SCI, ten weeks of training/testing starting two weeks post-SCI, awake cystometry at the end of training immediately prior to sacrifice and tissue removal). A time-line is provided in Figure 1.
Prior to SCI and post-SCI/pre-training, all rats were exposed to the treadmill environment for at least two half-hour sessions. Training was initiated on schedule at 14 days after injury for all rats given that there were no signs of infection or other complications arising from the surgery (a rare occurrence). The SCI+QT and SCI+FT groups of animals were at first placed on the treadmill in the harness vest for 20 minutes beginning at a slow speed. The session time was gradually increased from 20 minutes to the target of 58 minutes over the course of the first 7 training days as described previously (90). All rats on the treadmill were monitored by an experienced investigator at all times. However, to help provide adequate afferent feedback related to stepping for the SCI+QT group (similar procedure done with locomotor training in humans), the experimenter manually assisted for plantar paw placements with full toe extension and no ankle rotation. Independent stepping was encouraged when the rats had achieved better co-ordination, stability and absence of hind limb dragging. Although sessions are terminated early for any animals showing signs of stress (for example, diarrhea, porphyrin stains in eyes or irregular breathing pattern) or a session skipped if any abrasions from training were observed on the paw or skin (until healed, as noxious input can interfere with spinal learning (39)), there were no such instances in the current study.

Voiding Behavior Voiding behavior was assessed weekly with a six station Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS). The CLAMS unit and corresponding software (Oxymax for Windows version 4.83) was used to collect 24-hour urination data, food and water intake for all groups of rats. Animals were placed in cages once a week throughout the experiment (including pre-injury, the two weeks post-
SCI, and throughout the 10-week training period) for twenty-four hour data collection periods with food and water *ad libitum*. Following each 24-hour testing session, each metabolic cage was disassembled, cleaned, and reassembled for the next session.

*End of Study Awake Cystometry*  Filling cystometry (non-stop transvesical) experiments were conducted in conscious rats (see (92, 93) for justification) after approximately 10 weeks of training (or equivalent time frame) for all groups. For catheter placement (47, 54, 63), a 1.5cm midline abdominal incision was made under brief gas anesthesia (2% isoflurane). The catheter (PE-60 tubing), with previously heated tip to form a collar of about 2 mm from the end, was inserted into the bladder through the dome. The tubing was secured to the bladder with a purse string suture (4-0 Ethilon), exteriorized, and the abdominal muscles and skin closed with wound clips. All animals were returned to their home cages and closely monitored for the brief recovery period for signs of discomfort or stress. No animal in any group (including spinally intact shams) showed signs of stress or discomfort (e.g., irregular respiratory patterns, vocalization to handling, porphyrin staining around the eyes), and thus no analgesics were administered. Two hours after recovery from implantation, the animal was placed in the harness vest used for training to restrict movement, and the exteriorized catheter was connected to an infusion pump and pressure transducer for saline infusion at a rate of 0.25 ml/min using standard protocols (63, 92, 96). Once the voiding cycles were consistent (at least five consecutive voiding events with consistent time intervals in between), five consecutive cycles were recorded using a Neuralynx High Density Electrophysiology System (Lynx-8 amplifier from Neuralynx Inc., Bozeman, MT). Various parameters, including baseline pressure, maximum amplitude of
contraction, and contraction time, were retrieved for each of the 5 contractions and averaged for each animal as described previously (63, 90). Note that it is possible for bladder filling to indirectly induce some pressure on the abdomen and thus potentially some discomfort around the incision site, particularly in the non-injured sham group.

Tissue Removal  Following the cystometry recordings, rats were overdosed with a mixture the Ketamine/Xylazine mixture and immediately perfused with heparinized saline followed with a solution of 30% RNAlater (Ambion, Grand Island, NY) in a 1mg/ml phosphate buffer solution for tissue retrieval (spinal cord lesion site, kidney and bladder tissue). The bladder was removed, blotted dry and weighed. The left kidney was also removed and along with the bladder placed in a 100% solution of RNAlater for 24 hours at -20°C and then flash frozen in liquid nitrogen and stored at -80°C. As described below, bladder tissue was used for measuring connective tissue proteins that provide tensile strength and elasticity (elastin and collagen) using ELISA. Western blots were done to assess the expression of two proteins in the kidney whose presence are indicative of tissue stress or damage (TGFβ and CD11b). Spinal cord tissue containing the lesion area (T8-T10) was also removed and immersed in 4% paraformaldehyde for at least 48 hours, followed by 30% sucrose/phosphate buffer solution with 1% sodium azide for at least 24 hours and until the tissue was cut transversely.

After the fourth and final set of 12 rats was completed, all collected tissues (coded to maintain blindness of experimenter to group identity) were processed together (see procedures for ELISA and Western Blots below). After all experiments were complete, the data were decoded and separated into groups (sham, SCI, SCI+QT, SCI+FT) and analyzed
using SigmaStat. One-way analysis of variance (ANOVA) or one way repeated measures ANOVA with a significance level of $p < 0.05$ was used followed by the Holm-Sidak method for pairwise multiple comparisons according to previously published protocols (90, 91).

**ELISA for elastin and collagen**  The amount of collagen and elastin present in bladder tissue was quantified using a collagen assay and elastin assay (Biocolor, Northern Ireland, UK). For collagen analysis, frozen bladder tissue (kept at -80°C) was thawed and placed in an acid-pepsin solution overnight at 4°C to make the collagen soluble. A 100µl sample containing the tissue or three reference standards were added to a 1.5 ml microcentrifuge tube and 1 ml of Sircol Dye reagent was added to each tube. Following 30 minutes on a shaker, tubes were centrifuged for 10 minutes and then drained leaving the collagen at the bottom of each tube. An acid-salt wash was then applied to remove any remaining dye. A 250µl solution of alkali reagent was then added to each tube and vortexed for 5 minutes. 200µl of standard, blanks, and samples were then pipetted into individual wells of a 96 well plate which was then read at 555nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) to determine the tissue collagen content.

For elastin analysis, bladder tissue was placed in 0.25M oxalic acid and heated to 100°C for one hour. This was done twice for each sample. A 100µl volume of sample, standard or blank was added to a 1.5 ml microcentrifuge tube followed by 100µl of elastin precipitating reagent. After 15 minutes, tubes were centrifuged and drained leaving the α-elastin at the bottom of each tube. A 1 ml volume of dye was then added to each tube and placed on a shaker for 90 minutes. Tubes were then centrifuged and drained again. Then 250µl of dye dissociation reagent was added to each tube and vortexed. Each tube was
then re-vortexed 10 minutes later. An amount of 200µl from each tube was then placed in a 96 well plate which was read at 513nm using the Spectramax Plus microplate reader to determine the tissue elastin content.

Western Blots for TGFβ and CD11b Expression of the proteins TGFβ and CD11b were analyzed with Western Blots following our previously published protocols (40). The left kidney was sectioned (to include both cortex and medulla in the sample) on an ice tray and homogenized in ice cold lysis buffer [50mM Tris HCL (pH 8.0), 200mM NaCl, 50mM NaF, 0.3% TritonX100, 1M DTT, 1M Benzamidine, 100mM Na-orthovanadate, 100mM PMSF] and protease inhibitor (Halt protease inhibitor single use cocktail Prod# 78425, Thermo Scientific). For protein assay, the Biorad Protein assay reagent was used. Protein estimation was done in a photometer at 590 nm absorbance. Samples were loaded with 4X loading buffer (dye) by adjusting the proportion according to the value derived from the estimation. Gels were run in mini protean gel tanks (Bio-Rad mini protean tetra system) in 1.5 mm pre-cast gels (mini protean TGX gels, Bio-Rad #456-1083) using 100V for 1.5 hours. The running buffer was 1X Tris-glycine- SDS buffer. Protein ladder (Precision plus protein standards, dual color Bio-Rad #161-0374) was used for the band level detection. Protein was transferred overnight at 30 amp on nitrocellulose membrane (Bio-Rad #162-0115) in the transfer buffer (1X Tris-glycine-methanol-SDS buffer). Membranes were then blocked in 3% non-fat dry milk and a TBST solution was applied overnight and washed the next day with TBST. The membranes were then cut at the level of 75 kDa, the upper portion was incubated in Rabbit anti-CD11b (Abcam #75476) and the lower in Rabbit anti-TGFβ (Cell signaling #3711). The membranes were incubated overnight in primary
antibody at 1:1000 dilution (diluted in TBST and 3% non-fat dry milk solution) on a mechanical shaker in a cold room. The following day, membranes were incubated in HRP conjugated anti-rabbit secondary antibody (cell signaling #7074) for one hour at room temperature after washing in TBST buffer three times for 5 minutes each on a mechanical shaker. The membranes were treated with HRP antibody detection reagent (HyGlo chemiluminescent) and exposed to autoradiography film (HyBlot autoradiography film, Denville Scientific). B-actin (Cell Signaling #4967S) was used as a loading control. After exposure for principal proteins, the membranes were stripped and re-probed with rabbit anti B-actin antibody at 1:1000 dilution.

All the samples were run at-least three times and the bands from all the tests were analyzed by inverse densitometry using ImageJ 1.47 (ver. 1.47, NIH) according to our previous protocols (40). Each individual value was normalized by subtracting the background and dividing the value with corresponding B-actin values (minus background). The values obtained from the ImageJ (1.47v, NIH) program were then analyzed for the various group comparisons. For the calculation, final raw values for the proteins were obtained after subtracting the background and dividing the result with the loading control. Each value obtained from the calculation for SCI and training groups was normalized with the mean value of shams (i.e., sham controls were set to a value of 1). Statistical analysis included one-way ANOVA with Tukey HSD post hoc t-tests. All values were expressed with ± standard deviation (SD). A p value of ≤.05 was considered statistically significant.

**Histology** The lesion site tissue was sectioned on a cryostat (Leica CM 1850) at 30-µm-thickness and stained with Luxol fast blue and cresyl violet (Kluver-Barrera method) per
established protocols (41, 90). Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and a Nikon E400 microscope were used to obtain measurements for quantification of the lesion epicenter (based on total lesion area) as previously described (41, 91). The % white matter sparing was determined by dividing the white matter remaining at the epicenter by the average area of white matter present in more intact sections. The intact area of white matter for a given region is estimated by averaging measures from two sections 2mm rostral and two 2mm caudal to the epicenter. To compare white matter sparing with bladder function, a multiple regression analysis was performed.

**Results**

A total of 46 male rats were randomly divided into 3 groups (SCI, SCI+QT, SCI+FT) at two weeks post-injury (two of the initial 48 died due to complications following the injury). Post-hoc analysis of the group data for contusion parameters (kilodyne force and impactor displacement), four-day urine volume and two-week BBB locomotor score reveals no significant differences with respect to the injury itself and functional outcomes between the groups prior to the initiation of training (Figure 2).

Data from the weekly metabolic cage CLAMS system was generated by the Oxymax computer software and saved for post-hoc analysis. To avoid potential week by week variability, 24-hour data obtained from two separate time points were averaged for analysis, including two baseline measures pre-injury, each of the two weeks measures post-injury/pre-training, two mid-way training time-points (weeks 3 and 4) and the last two training week time-points (weeks 8 and 9 – week 10 [12 post-SCI] was terminal
assessment week). The data for total 24-hour urine volume, average volume per void, total number of voids in 24 hours, and total 24-hour water intake are presented in Figure 3. A representative example of 24-hour micturition cycle for an SCI and an SCI+QT rat at four different time points (prior to injury, after injury, mid-training time-point, late in training time-point) is provided in Figure 4. Note that the SCI-induced increase in production of urine after injury was not due to an increase in water intake (compare with pre-injury control values for all groups in Figure 3), as we have shown previously (91). The CLAMS data, when considered in their entirety, suggest that the higher volume per void for the SCI control group after the 9 week training period could be a compensation for the higher 24-hour production of urine (polyuria) for that group. The terminal awake cystometry data (Figure 5) is consistent with these findings, as the maximum amplitude of contraction was significantly higher for only the SCI non-trained group relative to shams – i.e., a higher void volume requiring a larger bladder contraction to empty (#, Figure 5). Note, however, that the trained SCI animals as a group were also significantly different from the non-trained SCI animals (**, p<0.01) but individually, only the SCI+FT group was significantly different (*, p<0.01). No other differences were found between groups relative to shams (intercontraction interval and peak pressure, not shown). No differences were found in bladder weight and the ratio of elastin-to-collagen between SCI groups, although bladder weight was significantly higher for all groups at 12 weeks post-SCI (p<0.05; Figure 6).

Expression of the proteins TGFβ and CD11b were analyzed with Western blots using the left kidneys from shams and the three SCI groups. For TGFβ, two different bands were detected; one protein band located at 25 kDa and the other around 50kDa. These bands represent mature TGFβ and TGFβ precursors (known to produce a band between
45-65 kDa), respectively, per the manufacturer’s datasheet. The 25 kDa bands (mature) of TGFβ were analyzed and a comparison between the expressions in sham versus SCI (non-trained) animals revealed a significant decrease in kidney TGFβ levels following chronic SCI (Figure 7B). For the SCI+QT and SCI+FT trained groups, the protein levels were not significantly different from shams (p>0.05). In contrast, the kidney CD11b levels (location around 160 kDa per manufacturer’s datasheet) increased in density at the 12 week post-injury time-point (SCI) and remained significantly higher, relative to shams, in the SCI+QT/SCI+FT training groups (Figure 7C).

Histological analysis of white matter sparing at the lesion epicenter as well as estimates of the total lesion volume revealed no significant differences between the three groups of SCI rats. A summary of the mean data are presented in Figure 8. Total lesion volume ranges for the three groups were 9.1-15.6 mm³ (SCI), 9.6-14.7 mm³ (SCI+QT), and 9.0-14.9 mm³ (SCI+FT). Epicenter percent white matter sparing ranges for the three groups were 4.5-32.4% (SCI), 6.1–42.2% (SCI+QT), and 5.9-44.1% (SCI+FT). To further explore the possibility of relationships between injury severity and bladder function, a multiple regression analysis was done and found no relationship (p>0.05) between epicenter white matter sparing and bladder outcomes (including voided volume) within each training group and that the training effect is independent of lesion variability.

Discussion

The results of the present study demonstrate an impact of exercise training on multiple post-SCI induced impairments related to various aspects of urinary tract function using metabolic cage frequency-volume measurements of voiding and awake cystometry.
Improvements with both the SCI+QT as well as the SCI+FT groups implicate underlying mechanisms beyond repetitive sensory information from the hindlimbs driving spinal network excitability of the lumbosacral urogenital neural circuitry. Furthermore, preliminary evidence for the potential impact of exercise training on the upper urinary tract in addition to the lower urinary tract underscores overall health benefits of activity-based training on the entire urinary system within the SCI population.

SCI-Induced Polyuria  Metabolic cage data indicate that following SCI, mean 24-hour urine volume and the average volume per void increased in all groups of animals, a finding consistent with our previous study demonstrating SCI-induced polyuria for a wide range of spinal lesion severities (mild to severe) (91). After 10 weeks of activity-based training, total urine volume and average volume per void were significantly lower for both the SCI+QT and SCI+FT groups relative to the SCI group, although the trained group values were still significantly above pre-injury baseline. The further increase in average void volume at the later time-points could be reflecting better efficiency (i.e., lower residual volumes) which would be consistent with the significantly higher maximum contraction amplitudes for the SCI rats relative to the sham animals as revealed with terminal cystometry. Note that the metabolic cage procedure that was done weekly does not give a measure of residual volume. Future studies would need to involve a time course for awake cystometry with a chronically implanted bladder catheter to further address these novel findings. The benefits regarding the duration of intense physical activity with 60 consecutive minutes of training (current and previous data on locomotor and non-locomotor systems including the bladder (90)) but not 30 minutes of stepping as we have shown previously for SCI-induced polyuria...
(91) is consistent with a study on locomotion using a rat spinal transection model showing dependence of functional recovery on the number of repetitions of the weight bearing stepping activity (15).

Potential mechanisms for improving SCI-induced bladder dysfunction with activity-based training include general exercise effects that impact target organ-neural interactions or potential plasticity of the spinal bladder reflex circuitry (such as changes in the properties of afferent neurons in the bladder receptors and/or cell bodies and/or terminals within superficial laminae of the dorsal horn). For example, one likely contributor to the observed SCI-induced polyuria and subsequent alterations in bladder function is hyponatremia (a decrease in the level of serum sodium) which can often occur due to high water content in the blood (55). Hyponatremia is often associated with hyposmolality (a decrease in blood osmolality). One of the easiest ways for the body to increase the level of sodium and increase plasma osmolality is to increase water excretion through urination. Several studies have shown that hyponatremia develops following SCI (10, 33, 70). The trigger that the body uses to increase water excretion in response to hyponatremia and/or hyposmolality is to decrease the release of antidiuretic hormone (ADH; also known as vasopressin) from the posterior pituitary gland (neurohypophysis). When plasma ADH levels are low, the body excretes more water. Importantly, a number of studies involving able-bodied human subjects indicate that exercise can increase plasma ADH levels (45, 87, 88). Given that exercise and LT are rehabilitative therapies that many SCI individuals receive, there is potential for improvement in urologic function as long as sufficient amounts are provided (recall that our animal data indicate improvements with 60 but not 30 minutes (91) per day of intense training). Our initial data with human SCI research participants (at
least two years post-injury) indicate significant improvements in bladder function (as assessed with cystometry) following 80 sessions of LT or LT plus stand training for 60 minutes per day five days per week (48). Note that if polyuria following SCI is triggered by hyponatremia and/or hyposmolality, then there should be a decrease in the plasma ADH levels post-SCI. Recent data from our lab using male rats with a T9 contusion indicate that serum ADH levels decrease significantly two weeks after severe SCI (unpublished observations), which is consistent with our metabolic cage data showing significantly elevated total 24-hour urine volume at two weeks (Figure 3). Further studies are in progress. Anecdotally, SCI individuals often report limiting fluid intake to decrease the number of catheterizations over a 24-hour period. However, this lack of fluid intake can lead to other metabolic consequences (77). Although polyuria is common after SCI (56), few studies have investigated the mechanisms underlying this condition.

An additional potential mechanism for the observed changes in urologic function with LT could involve somato-pudendal reflex interactions such as the flexor and extensor reflex circuitry interactions with external urethral sphincter reflexes as described previously by Tai, Roppolo and de Groat (81). Motor and autonomic output of the spinal cord is driven in large part by afferent input and local or propriospinal circuitry emphasized after SCI conditions (7, 12, 17, 34, 44) which creates the potential for interactions and the triggering of some plasticity within the lumbosacral circuitry to the bladder. For example, multiple sensory inputs from the periphery during locomotion, particularly limb loading (28) and stepping rate (31) provide information to these networks to improve stepping (16, 21, 22, 24, 25). These interactions, however, would not explain the improvement observed with the forelimb exercise group of rats receiving 60 minutes of daily training. It is conceivable
however, that intense repetitive forelimb exercise with sufficient spinal network excitability via residual supra-spinal input induces a net improvement in functional reorganization of the lumbosacral neural circuitry (14, 58, 67, 78). In individuals with an incomplete spinal cord injury, there is some facilitation of the lower extremity muscles in those subjects when walking with reciprocal arm swing versus without (walking while holding on to parallel bars) (8, 85). Specifically, the presence of some residual intact long propriospinal inter-enlargement pathways that mediate inter-limb coordination for locomotor function in the rat (72) could induce adaptive changes to neural networks within the lumbosacral spinal cord such as those controlling the bladder. However, given the severity of injury (22.0 ± 2.7% white matter sparing at the epicenter for the forelimb-trained SCI group) and the fact that there was little or no hindlimb activity in the FT group during training and no air-stepping, the probability for the occurrence of such interactions is likely low. Also note that the animals with the most spared white matter did not have the best recovery of bladder function. Thus, although load-bearing on the hindlimb has been shown to be of critical importance for stepping (82), other systemic factors may be vital contributors for improving bladder function with task-specific training based rehabilitation.

Kidney Findings The results from the present study demonstrate that after a clinically relevant spinal contusion injury, there is a significantly lower level of TGFβ expression in the kidney (NT group) relative to shams. Since TGFβ controls T-cell activation and abolition of TGFβ causes gradual infiltration of leucocytes into multiple organs (37, 62, 65), our finding may indicate the presence of an altered immune response in the kidneys during the chronic phase after SCI. In addition to lower TGFβ levels, there was a corresponding rise of CD11b expression in the kidney after chronic SCI (SCI group relative to shams). TGFβ has
a known inhibitory effect on CD11b expression (5) which may explain the post-SCI increase in CD11b activity. Note that activation of macrophages which play a role in both injury and repair in kidney tissue (27) is required for the synthesis of TGFβ, so a rise in CD11b expression may also indicate activation of endogenous macrophages for tissue homeostasis.

In both SCI training groups, the level of TGFβ expression was not significantly different from the levels in surgical sham animals, indicating the possibility of immune homeostasis and maintenance of renal health (normalized glomerular filtration rate and kidney function) and decreased susceptibility to infection, which would impact quality of life for the SCI population. Exercise has been shown to reverse negative immune alterations (35) including after SCI (61) but the time since injury and the ideal starting point of training as well as the duration and intensity (57, 89) needs further consideration to optimize the most effective strategy not just for urinary tract function but functional recovery in general.

**Perspectives and Significance**

Bladder dysfunction after SCI is rarely studied in experimental animals, yet is overwhelmingly the most significant concern for those suffering from SCI, and urological complications results in significant morbidity and mortality. Importantly, even small improvements in bladder function can have a tremendous impact on these individuals’ continual health and quality of life. Results from this animal study provide some initial clues about potential underlying mechanisms regarding our findings on the effects of activity-dependent plasticity induced by LT after chronic SCI on non-locomotor systems (i.e., bladder function).
Conclusions  Our studies to date indicate that activity based training can influence urologic outcomes which is of great importance to persons with SCI. The positive benefits of exercise on bladder function post-SCI are likely indirect. These novel findings suggest that physical activity after SCI could translate to significant quality of life gains.

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**Figure 1.** Timeline. Experimental procedures and treadmill training are indicated relative to the time when the SCI was made (Day 0).

**Figure 2.** Pre-training group data. Post-hoc analysis of the computer generated IH impactor parameters (A – force; B – displacement) indicate that there were no significant differences between the three groups of animals for extent of injury (as anticipated with randomization). The data in A and B are consistent with the lack of functional differences for bladder (C; maximum residual volume collected from each rat on Day 4) and overground locomotion (D) between the groups prior to the initiation of training, as it’s known that larger residual volumes and lower BBB are reflective of greater injury severities (91). SCI (non-trained); SCI+QT (quadrupedal trained); SCI+FT (forelimb-only trained). Standard error of the mean is shown.

**Figure 3.** SCI-induced Polyuria. Total urine volume increased significantly post-SCI (*p<0.05) but was significantly lower after 9 weeks of either quadrupedal training (QT) or forelimb-only training (FT) (SCI+QT and SCI+FT groups) but still significantly above baseline (#, p<0.05). The average volume per void was also significantly greater after SCI (*p<0.05), although post-training only the non-trained (NT) SCI group had a further increase in average void volume (#, p<0.05). There were no differences found in the total number of urine events or water intake, suggesting that the higher voiding volume for the NT group after training compensated for the higher 24-hour production of urine. Note that the
surgical sham group was not subjected to this time-consuming testing as each animal served as its own control (i.e., pre-SCI baseline). Standard error of the mean is shown.

**Figure 4.** 24-Hour Micturition Cycle. Representative total 24-hour urine events measured with the CLAMS system for two rats, one from the SCI control group (A) and one from the SCI+QT group (B). Each plotted line represents a different time point of the experiment; pre-injury baseline – filled circles; two weeks post-injury (pre-training) – open circles; 6 weeks post-injury (4 weeks post-training time point in B) – filled triangle; 10 weeks post-injury (8 weeks post-training time point in B) – open triangle. Each individual symbol represents a single urine event that is a cumulative total over the 2-hour period. The horizontal bar represents the 12-hour phase when the housing facility lights are off (active period). Note that the majority of urine events occur during the active phase.

**Figure 5.** Terminal awake cystometry. Raw recordings of fill void cycles from each group of animals. In A, five full fill/void cycles are shown for a QT animal. In B, a representative example of one fill/void cycle (note the scale bar) is provided for each group (different QT animal from 5A) and the maximum amplitude values are shown (mmHg). The group means of the averaged data is graphed in C. Significant group differences are shown relative to shams (#) and relative to non-trained SCI animals (*, **). Standard error of the mean is shown.

**Figure 6.** Bladder tissue analysis. Bladder weight but not the ratio of collagen to elastin differed significantly at the 12-week post-SCI time point relative to non-injured surgical
No differences were found between the trained and non-trained SCI groups. Standard error of the mean is shown.

**Figure 7.** Kidney TGFβ and CD11b levels. A. Representative examples of kidney TGFβ and CD11b expression levels. B. Both SCI+QT and SCI+FT groups had similar expression of TGFβ relative to surgical sham controls. Note that although the SCI trained group levels showed a trend toward sham, they were not significantly different from the non-trained SCI group. C. Expression of CD11b was significantly higher relative to shams for all SCI groups, regardless of training. * indicates significant difference (p<.05); bars are standard error of the mean.

**Figure 8.** Lesion Histology. No differences were found between the trained and non-trained groups of injured animals at the 12 week post-SCI time-point. Standard error of the mean is shown.
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<td>Spinal Cord Injury (SCI)</td>
<td>Recovery (REC)</td>
<td>Residual Urine Collection (RUC)</td>
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Epicenter White Matter Sparing (%)

SCI  SCI+QT  SCI+FT

Spared White Matter - Lesion Volume (mm³)

SCI  SCI+QT  SCI+FT