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Comparison of spinal cord contusion and transection: functional and histological changes in the rat urinary bladder

Benjamin N. Breyer[1*], Thomas M. Fandel[1*], Amjad Alwaal[1], E. Charles Osterberg[1], Alan W. Shindel[2], Guiting Lin[1], Emil A. Tanagho[1], Tom F. Lue[1#]

[1]Department of Urology, University of California, San Francisco, CA, USA
[2]Department of Urology, University of California at Davis, CA, USA

*These authors contributed equally to this work.

#Corresponding author:
400 Parnassus Ave
Campus Box 0738
San Francisco, CA 94143
Phone: (415) 476-2105
Fax: (415) 476-8849
E-mail: tom.lue@ucsf.edu

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Abstract:

Objective: To compare the effect of complete transection (tSCI) and contusion injury (cSCI) on bladder function and bladder wall structure in rats.

Materials and Methods: 30 female Sprague-Dawley rats were randomly divided into three equal

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groups: uninjured controls, cSCI, and tSCI. The cSCI group underwent spinal cord contusion, while the tSCI group underwent complete spinal cord transection. 24-hour metabolic cage measurement and conscious cystometry were performed at 6 weeks post-injury.

Results: Conscious cystometry analysis showed that cSCI and tSCI groups had significantly larger bladder capacities than the control group. The cSCI group had significantly more non-voiding detrusor contractions than the tSCI group. Both injury groups displayed more non-voiding contractions compared to the control group. Mean threshold pressure was significantly higher in the tSCI group than in control and cSCI groups. The number of voids in the tSCI group was less compared to the control group. Metabolic cage analysis showed that the tSCI group had larger maximum voiding volume as compared to control and cSCI. VAChT/smooth muscle immunoreactivity was higher in control than in cSCI or tSCI rats. The area of calcitonin gene-related peptide (CGRP) staining was lower in tSCI as compared to control or cSCI.

Conclusions: Spinal cord transection and contusion produce different bladder phenotypes in rat models of SCI. Functional data suggest that the tSCI group has obstructive high-pressure voiding pattern, while the cSCI group has more uninhibited detrusor contractions.

Introduction:

The estimated incidence of traumatic spinal cord injury (SCI) in the United States was 40 cases per 1 million population in 2015, with incidence rates remaining relatively stable over the last two decades [1]. SCI is a catastrophic event that often leads to serious sequelae, including lower urinary tract dysfunction [2]. Urinary tract infection with urosepsis, and decreased renal function in the course of hydronephrosis are among the most life-threatening complications of neurogenic bladder dysfunction [3, 4]. While pharmacologic and surgical interventions have reduced morbidity and mortality in the spinal cord-injured patient population [5], lower urinary tract management remains a challenging aspect of caring for such patients.

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Further improvements in medical and surgical therapies for SCI patients will require a clearer understanding of the pathophysiology of SCI and how it may affect the bladder and urethra. Animal studies have been critical in improving our understanding of lower urinary tract physiology after SCI. Two principal modalities have been used to produce spinal cord lesions in rats: complete transection (tSCI) and contusion (cSCI) by blunt injury using weights [6-8]. Spinal cord transection is the most utilized method to study SCI as it yields a highly reproducible and complete injury model [7]. However, most patients suffer from an incomplete, contusion type of SCI [9], which makes cSCI a clinically more relevant model and highlights the importance of studying cSCI and comparing its effects on the lower urinary tract to tSCI.

The aim of this current study is to better understand anatomical and functional differences in the lower urinary tract of animals with SCI produced by contusion and transection. The anatomic changes in the bladder following SCI will be evaluated with tissue harvesting and immunohistochemistry using neurotransmitters commonly implicated in bladder contractility e.g. acetylcholine uptake into pre- synaptic vesicles [21, 22]. The functional changes we will be evaluated with metabolic cage analysis and conscious cystometry. Our hypothesis is that tSCI produces a more severe injury associated with higher voiding pressures secondary to the traumatic transecting injury compared to cSCI.

Materials and Methods:

Animals

Thirty female, 3-month old Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 270-350 g were used. They were housed 1 per cage and given a 12 h light-dark cycle, with food and water provided ad libitum. The experimental protocol was approved by the University of California San Francisco Institutional Animal Care and Use Committee.

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SCI

Rat were randomly divided into three groups of 10 each; uninjured controls, cSCI and tSCI. SCI was performed with the rats under 2% isoflurane anesthesia. Isothermia was maintained at 37°C by placing the animal on a heating pad. The rat was placed in prone position with the limbs spread apart. The surgical plane was at the 8th vertebra, roughly corresponding to the 8th thoracic spinal segment. Through a dorsal midline incision, a T8 laminectomy was performed. In the tSCI group, the spinal cord was completely transected using a surgical blade. Transection was complete when the dorsal surface of the vertebral body was visualized after retraction of both ends of the spinal cord. Complete transection was ensured by scraping the cavity with a small hook, and then a sterile piece of Gelfoam was placed between the severed ends of the spinal cord. In the cSCI group, contusion was produced using the weight-drop device (cSCI group) described by Noble and Wrathall [10]. In brief, a metal rod with a plastic impounder at the bottom (3 mm diameter at the tip) was attached to a micromanipulator. The impounder was free to move upward but not downward. The rod was lowered onto the exposed dura until contact with the spinal cord caused the impounder to move precisely 3 mm up. A 10 g weight was dropped from a height of 50 mm onto the impounder delivering a standardized force to the spinal cord to generate a moderate injury severity. The dura mater was inspected visually after contusion to confirm that it had not been lacerated; no lacerations occurred in the cSCI group. In all SCI rats the wound was closed in two layers. Control rats did not undergo laminectomy to minimize the risk of inadvertent SCI in this population.

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Post-operative care and behavioral assessment of SCI

Perioperative enrofloxacine (5 mg/kg) and 10 ml saline were injected intraperitoneally twice daily for 10 days in all animals. Since animal locomotion was impaired, intraperitoneal injection of saline was substituted until after spinal shock. In SCI rats, the bladder was emptied manually by Crede's maneuver twice daily and the urine volume was weighed on a digital scale (Utah Medical Products, Midvale, UT). Motor recovery after spinal cord contusion was assessed by the Basso, Beattie, and Bresnahan locomotor rating scale (BBB) at day 1, 3, 7, and weekly thereafter until 42 days after SCI. [11] All animals were observed in an open field to evaluate locomotor recovery of the hind limbs. Complete paralysis was scored as 0, increasing joint movements without weight support scored 1 to 8, abnormal locomotion with weight bearing scored 9 to 20, and normal movement was scored 21. Serum creatinine measurements were obtained before injury and 6 weeks after by blood drawn from the tail vein.

Metabolic cage assessment

Animals were acclimatized to the metabolic cage on two consecutive days for 60 minutes each day after which rats were placed in a metabolic cage for 24 hours to record voiding patterns 6 weeks after injury. Urine output was measured on an electronic scale (Utah Medical Products, Midvale, UT) connected to a microcomputer for the recording of micturition frequency, duration, and volume. Data were recorded and stored using LabView 6.0 software (National Instruments, Austin, TX). The following micturition parameters were evaluated: 1) H_20 consumption per 24-hour period normalized by weight; 2) total urine excreted normalized by weight; 3) number of voids per 24-hour period; 4) number of voids during daylight; 5) number of voids during night; 6) mean volume per void; and 7) void during cycle with the maximum volume.

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Conscious cystometry

Awake cystometrograms were performed in all three groups at 6 weeks. Under 2% isoflurane anesthesia, a transvesical catheter was inserted into the rat bladder 2-3 days before cystometry. The tip of a polyethylene catheter (PE-90, Clay-Adams, Parsipanny, NJ) was heated to create a collar. After a lower abdomen midline incision, the catheter tip was implanted at the bladder dome and a preset purse string suture was closed. After suturing, the bladder was filled to the leak point to ensure that no leakage occurred at the implantation site. The catheter was passed through the abdominal wall muscle and tunneled subcutaneously to emerge at the dorsum of the neck. The abdomen was closed in two layers. For cystometry, the animal was restrained in a custom-made tunnel attached to a metabolic cage grid (Braintree Scientific, Braintree, MA). The bladder was filled with normal saline at a rate of 0.1 ml/min using an infusion pump (KD Scientific, Holliston, MA). The PE-90 catheter was connected to a pressure transducer (Utah Medical Products, Midvale, UT). Intravesical pressure changes were recorded at a rate of 10 samples per second using a computer with LabView 6.0 software (National Instruments, Austin, TX). After expressing and emptying the bladder, several micturition cycles were recorded in each animal without emptying the bladder in-between. To assess the uninhibited bladder contractions, the first 30 minutes of filling were observed (upon emptying of the bladder in SCI animals and after the first micturition in control animals). Involuntary contractions were defined as intravesical pressure increases of $\geq$ 15 cm H_2O not associated with voiding. The following parameters were used to evaluate all animals over the 30 to 70 minute time period: baseline bladder pressure, threshold volume, maximum voiding pressure, and duration of contraction. Measurements were taken from a series of 4 voiding cycles and averaged over a 30-minute time period for data collection. The baseline pressure was defined as the lowest pressure between 2 voids. The threshold pressure was defined as the bladder pressure that resulted in the first drop of

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urine at the urethral meatus. Maximum pressure or peak pressure was defined as the highest bladder pressure of the voiding cycle. The number of detrusor contractions greater than 15 cm H20 from baseline pressure that did not result in voiding was recorded. Residual bladder urine was measured by holding the end of the catheter below body level after the last micturition and weighing the resultant urine output. Following cystometry, the animals were euthanized and the bladder was harvested for histological evaluation.

Immunohistochemistry

Three random sections of the bladder neck, bladder dome and lateral bladder walls were chosen from areas of maximal diameter tissue thickness from all animals (n = 10 animals / group, 3 groups). The 90 tissue samples (3 x 30) were fixed in cold 2% formaldehyde and 0.002% saturated picric acid in 0.1 M phosphate buffer, pH 8.0, for 4 hrs followed by overnight immersion in buffer containing 30% sucrose. The specimens were then embedded in OCT Compound (Sakura Finetek USA, Torrance, CA) and stored at –70 °C until use. Fixed frozen tissue specimens were cut in a longitudinal fashion at 5 microns, mounted onto SuperFrost-Plus charged slides (Fisher Scientific, Pittsburgh, PA) and air dried for 5 min. The slides were then placed in 0.3% H_2O_2/methanol for 10 min, washed twice in PBS for 5 min and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 min at room temperature. After draining this solution from the tissue section, the slides were incubated overnight at 4°C with primary antibodies of Vesicular acetylcholine transporter (VAChT), Calcitonin Gene-Related Peptide (CGRP) and smooth muscle. Control tissue sections were similarly prepared except no primary antibody was added. Staining of the tissue was performed with the Elite ABC kit (Vector Labs, Burlingame, CA), followed by hematoxylin counterstain. For image analysis, five randomly selected fields from each group per tissue were photographed and recorded using the Retiga Q Image digital still camera and

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ACT-1 software (Nikon Instruments Inc., Melville, NY). Quantification of VAChT and CCRP was recorded as percent area per area smooth muscle and absolute number of pixels per high power field, respectively.

Statistical analysis

A Wilcoxon rank-sum test was used to analyze differences in all variables with the exception of whether or not voiding dysfunction was present, when a Chi-square test was used. All data are presented as the mean value +/- the SEM unless otherwise indicated. Graphs were prepared using the statistical program Graphpad Prism 4.0. A p-value of less than 0.05 was considered statistically significant.

Results

SCI

No animals died prior to time of planned sacrifice. At 1 day after injury, BBB scores in the cSCI group indicated almost total hind limb paralysis and areflexia (Figure 1). The BBB scores steadily improved and plateaued by 2 weeks. By 6 weeks after injury, the cSCI animals were able to perform weight-bearing locomotion and noticeable coordination of front and hind limbs, indicating that supraspinal neural pathways were intact. Gross inspection of all groups verified that the tSCI had the most profound hindlimb deficit. These animals regained some function and plateaued at 2 weeks as well, however they were unable to perform weight-bearing locomtion and did not demonstrate coordination of front and hind limbs. Overground locomotion with full weight bearing requires supraspinal input [12]. BBB testing was not applied to the tSCI as the locomotion rating scale has only been validated in contusion animals [11].

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Metabolic Cage Assessment

Serum creatinine measurements were taken at baseline and at 6 week follow-up. There were no significant differences between groups (Table 1). Each group underwent 24 hour metabolic cage testing prior to cystometry and transvesical catheter placement (see Table 1). The transection group weighed significantly less (p <0.02) than the control animals and the mean change in weight from week 0 to week 6 was also significantly less in the transection group versus the control group (p<0.05). This potentially reflects a decreased muscle mass due to paralysis (there was food placed on the bottom of the cage so that the animal had no problem to reach it).The volume of H_20 consumed and urine excreted was not statistically different between groups before and after normalization for rat weight. The groups had similar rates of day and night voiding and overall number of voids. The transection group had significaintly higher maximum void volume (p<0.05) (see Table 1).

Conscious cystometry

Analysis of conscious cystometry data showed that both cSCI (1.0±0.05 ml) and tSCI groups (0.9±0.03 ml) had significantly larger bladder capacities than the control group (0.5±0.02 ml) (p=0.01 and 0.04, respectively) (Figure 2a). The tSCI also had a larger residual urine volume after conscious cystometery and metabolic cage than the cSCI and control (p<0.05) (Figure 2b and 2c respectively).

Number of voids in the tSCI group (1.9 ± 0.07) was significantly lower compared to the control group (3.2 ± 0.15) (p<0.03) (Figure 2d). Analysis of metabolic cage data showed that the tSCI group had significantly larger maximum voiding volume (2.5±0.09 ml) as compared to control (1.7±0.04 ml) and cSCI (1.9±0.05 ml) (p<0.001). Both injury groups displayed significantly more uninhibited detrusor contractions than the control group (0.3±0.05) (p <0.01 for each). The cSCI group had significantly more detrusor contractions (3.8±0.39) that

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did not result in urination than the tSCI group (2.2±0.22) ($p < 0.01$). Mean threshold pressure was significantly higher in the tSCI group (44.3±0.59 cmH2O) than in the control (28.8 ± 0.39 cmH2O) or cSCI group (29.4 ± 8.1 cmH2O) ($p<0.001$) (Figure 3a). Differences in awake cytometry tracings shows increased detrusor activity in the cSCI and tSCI groups compared to the control group however the tSCI group had less overactivity versus the cSCI group. (Figures 3b, 3c, 3d)

Immunohistochemistry

VAChT/smooth muscle immunoreactivity was significantly higher in control (1.49± 0.50%) than in cSCI (0.97±0.40%) or tSCI (0.65±0.23%) ($p<0.05$) (Figure 4a). The area of CGRP staining was significantly lower in tSCI (1176±488 pixels/HPF) as compared to control (3814±1202 pixels/HPF) or cSCI (3490±1448 pixels/HPF) ($p<0.01$) (Figure 4b).

Discussion:

The lower urinary tract has two main functions: storage and elimination of urine. The micturition reflex is mediated by a bulbospinal pathway passing through the pontine micturition center (Barrington's nucleus) in the rostral brainstem [13]. Voiding requires the integration of autonomic and somatic pathways within the lumbosacral cord [14]. Disruption of the pathways between the pontine micturition center and the sacral spinal cord in rats has served as a model for human SCI for many years.

We tested the hypothesis that tSCI would lead to greater voiding dysfunction than cSCI. In our study tSCI animals had generally more severe bladder dysfunction. This was evident immediately after injury as the tSCI group retained more urine and took longer than cSCI to develop reflexive voiding. Interestingly, the cSCI group manifested more evidence of bladder overactivity than the tSCI group; the cSCI group had nearly twice as many non-

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voiding detrusor contractions >15 cm H_20 than the tSCI group. Residual intact bulbospinal pathways in the cSCI group likely produced this detrusor excitability. In tSCI animals, complete disruption of bulbospinal pathways and reliance solely on reflexive spinal voiding led to much more stable bladder pressures between voids.

The forceful detrusor contractions against a closed external urethral sphincter (EUS) in both injury groups (more prominent in the cSCI group) resemble the human condition of detrustor sphincter dyssynergia (DSD). In a rat model, it has been demonstrated that phasic burst activity of the EUS promotes efficient voiding [15-17]. Phasic EUS activity consists of bursts of contraction followed by urethral relaxation, which allows urine to pass. When a tonic or spastic EUS pattern is induced pharmacologically, profound decreases in voiding efficiency are observed [18]. Similar to Leung et al [17], injured rats expressed more urine volume at 6 weeks reflecting larger bladder capacity with voiding by overflow, with tSCI rats expressing significantly more urine volume with less frequency.

Pikov and Wrathall performed a study where progressively larger weights were dropped on the spinal cord to induce progressively worse spinal cord contusions. [19] They demonstrated that more severely injured rats retained a higher residual urine volume and required a longer recovery period before resumption of spontaneous micturition. Twenty percent white matter sparing at the injury epicenter was sufficient for complete recovery of detrusor-EUS coordination by 8 weeks after SCI. It was concluded that sparing of supraspinal projections to areas in the lumbosacral spinal cord likely resulted in recovery of EUS-detrusor coordination, as well as sprouting and or synaptic plasticity of spared serotonegeric fibers during recovery [19].

Pikov et al. studied the urodynamic effects of T8 contusion injury (n=7) compared to complete transection (n=3) in anesthetized rats and found that urodynamic parameters were essentially similar at 1 and 2 weeks after injury, with the exception that DSD was less

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common in the contusion group. Preservation of some bulbospinal projections was the postulated mechanism for improved lower urinary tract synergy in the contusion animals [20]. This important work provided interesting data but given that the experiment was conducted under anesthesia the applicability to conscious voiding is unclear.

In another study of cSCI versus tSCI, Leung et al. demonstrated that animals with incomplete SCI (contusion) generally had quicker return to reflex voiding than animals that had complete SCI (transection) [17]. Cystometry was performed at 2 and 6 weeks after injury. Animals in the cSCI group had detrusor contractions and voiding pressures similar to those of control rats, while tSCI rats demonstrated slower reflexive bladder development in terms of longer contraction duration and higher voiding pressure. Despite the overall trend some rats that underwent tSCI had partial recovery of voiding function. The most important factor in restoration of near normal voiding behavior in both groups of rats appeared to be resumption of phasic (rather than tonic) activity in the external urethral sphincter. Interestingly, in our experiment at 6 weeks post-injury a number of tSCI animals voiding patterns appeared more normal than their cSCI counterparts. The tSCi animals experienced on average half the detrusor overactivity and had similar amount of residual urine after cystometry.

In order to help understand the pathophysiology of these functional changes we performed tissue harvesting and immunohistochemistry. Acetylcholine is an important neurotransmitter in voiding. VAChT is a membrane protein that is involved in acetylcholine uptake into pre- synaptic vesicles [21, 22]. VAChT immunoreactivity is a surrogate to the functional neuronal recovery. Takahara et al. performed conscious cystometry and subsequent VAChT staining on rats with a tSCI at T9 at days 3, 7, 14, and 28 after SCI [22]. Downregulation of VAChT was seen in the pelvic ganglion and spinal cord (L1, L2, L6, S1)

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after injury and the intensity of VAChT terminals recovery paralleled bladder function recovery.

In our study, VAChT immunoreactivity was highest in bladders from the control group and lowest in bladders from the tSCI. The reduction in VAChT straining in the tSCI is indicative of decreased neuronal input in these bladders. It is unclear if the level of VAChT immunoreactivity had plateaued at 6 weeks or if it would have continued to improve (or worsen) in the SCI groups. CGRP is considered a marker for afferent nerve fibers, being present in type C and A-delta fibers [23-25]. While both injury models result in decrease of efferent nerve fibers to the urinary bladder, our data demonstrate that only the tSCI group shows a significant reduction in afferent nerve fibers, suggesting that tSCI may lead to impairment of the spinal reflex circuit, while cSCI preserves the spinal reflex circuit. This preservation is likely due to sprouting and unmasking of type C fibers as a result of cSCI, as previously been demonstrated in SCI models [26].

A strength of the present study comes from the use of conscious cystometry. In 1999 Yoshiyama et al. confirmed the feasibility of awake cystometry in SCI injury animals[27]. Awake cystometry eliminates anesthetic effect as a potential confounder of urodynamic results. Many prior studies of voiding function in SCI rats were conducted under urethane anesthesia, which is known to alter the micturition reflex[28]. SCI may actually increase the sensitivity of the micturition reflex to urethane and when withdrawn can greatly enhance response. Significant changes in bladder functionality may occur with even light doses of anesthetic agents [17]. This finding calls into question the accuracy of existing urodynamic data obtained in anesthetized animals.

Our data adds to the work of Leung et al. on lower urinary tract function in two types of SCI. It is readily apparent from both studies that tSCI produces a very different functional phenotype compared to cSCI. This phenotype consists of the tSCI group having a larger

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bladder capacity, larger maximal voided volume and higher threshold pressures meanwhile the cSCI group has more uninhibited detrusor contractions. These differences must be kept in mind when interpreting existing data on SCI and in new investigations. While both spinal transection and contusion occur in human trauma, contusions are a more common mechanism of SCI [9]. Due to the heterogeneity of spinal contusions in humans, it is difficult to extrapolate our experimental findings to the clinical setting. Nevertheless, we demonstrate a distinct bladder phenotype from the two differing mechanisms of injury. This is consistent with clinical scenarios whereby patients experience some detrusor overactivity with spinal contusions and an atonic bladder with spinal transection. Given that there are functional differences between these two modalities, future studies should focus on contusion so as to be more readily applicable to human patients.

Limitations of our study include a lack of histological or other assessment of the spinal cord lesion. Longer duration of follow-up may have yielded more interesting results with respect to functional recovery in the lower urinary tract. Given that the implanted catheter cannot be easily removed without creating scarring and that catheters implanted long-term pose a substantial risk of bladder infections, awake cystometry was performed as a terminal procedure and could not be repeated over time in one animal. Lastly, SCI contusion based models are heterogeneous by virtue of an intact spinal cord however we standardized our technique of contusion. Nevertheless, we chose a contusion injury model because it is clinically relevant to traumatic SCI. Extrapolation of these data to humans is limited.

These data have important prognostic implications for humans with SCI and serve as an important step in improving our understanding of the complex interplay between the nervous system and lower urinary tract. Further research is required to understand more clearly the mechanisms of bladder function after SCI. An improved understanding of lower

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urinary tract physiology in the setting of SCI will indubitably enhance the urological management of SCI patients.

Conclusions:

Spinal cord transection and contusion produce different bladder phenotypes in rat models of spinal cord injury. Functional data suggest that the tSCI group with its larger bladder capacity, larger maximal void and higher threshold pressure with a high pressure-voiding pattern. The cSCI group, on the other hand, has more uninhibited detrusor contractions. This likely arose from intact spinal transmission to and from the supraspinal micturation centers. The preserved supraspinal input in the cSCI group may explain the improved VAChT and CGRP neuron preservation seen in the bladder wall muscle of this group.

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Figure Legends:

Figure 1. Locomotor recovery after injury with the Basso, Beattie, and Bresnahan locomotor (BBB) scores in the cSCI group indicated almost total hind limb paralysis and areflexia

Figure 2

a. Voided volume during awake cystometry at 6 weeks for control, contusion, and transection groups
b. Residual urine after awake cystometry studies at 6 weeks for control, contusion, and transection groups
c. Residual urine after metabolic cage studies at 6 weeks for control, contusion, and transection groups
d. Mean number of voids per a 20-minute period during awake cystometry at 6 weeks for control, contusion, and transection groups

Figure 3. Awake cystometry outputs at 6 weeks

a. Mean bladder threshold pressures for all groups
b. Control group cystometry tracing
c. Contusion group cystometry tracing
d. Transection group cystometry tracing

Figure 4

a. Vesicular Acetylcholine Transporter Protein-staining in bladder tissue at 20 x magnification. Asterisks denotes areas of positivity.

b. Calcitonin Gene-related Peptide-staining in bladder tissue at 20 x magnification. Asterisks denotes areas of positivity.

Table 1. Serum Creatinine and 24-hour Metabolic Cage (+/- standard deviation) Data (*= $p < 0.05$)

	Control	Contusion	Transection
Weight (mg)	324.7+/-38.6	312+/-27.4	295.1+/-16.9*
Mean Change in Weight from 0 d to 6wk (mg)	37.6 mg +/- 8.3	26.7 +/- 14.7	0.1 +/- 13.7 *
Water consumed/mg	0.10+/0.002	0.09+/-0.004	0.10+/-0.003
Creatinine (study start)	0.63+/-0.005	0.68+/-0.004	0.71+/-0.004
Creatinine (6 weeks post)	0.76+/-0.005	0.75+/-0.008	0.75+/-0.005
Urine excreted/mg	0.05+/-0.001	0.04+/-0.003	0.05+/-0.001
Number voids/24hr	19.6+/-0.48	14.0+/-0.64	13.4+/-0.59
Number voids/daytime	8.0+/-0.17	6.7+/-0.29	6.1+/-0.31
Number voids/nighttime	11.6+/-0.39	7.3+/-0.37	7.3+/-0.29
Mean voided volume (mL)	0.8+/-0.02	1.0+/-0.05	1.3+/-0.06
Max voided volume (mL)	1.7+/-0.04	1.9+/-0.05	2.5+/-0.09*

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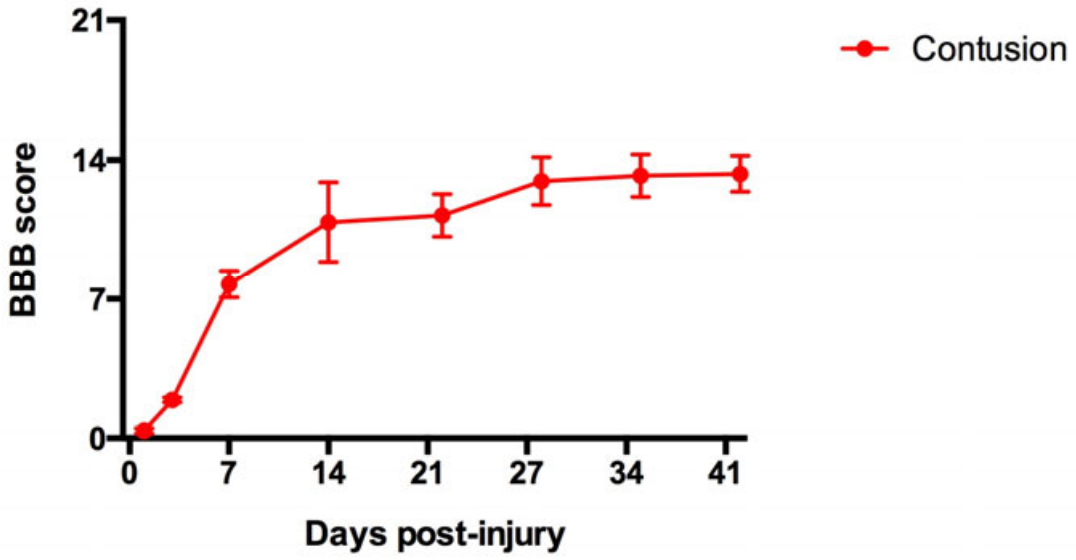

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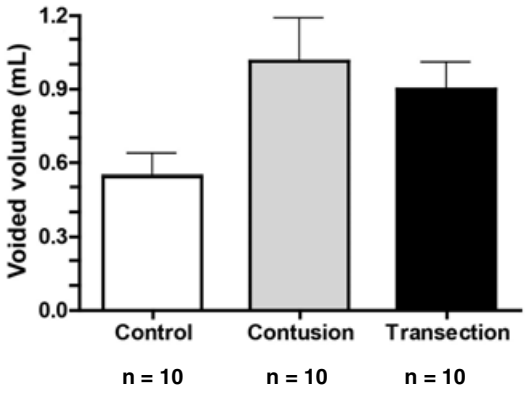

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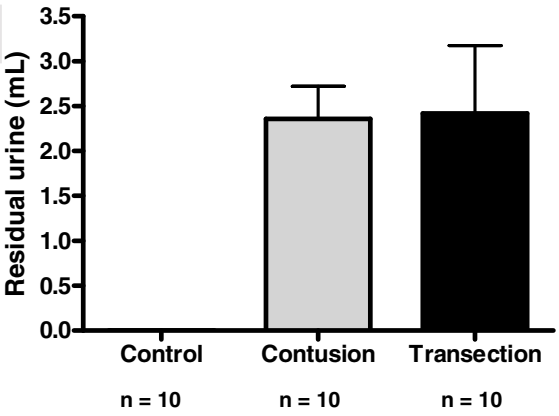

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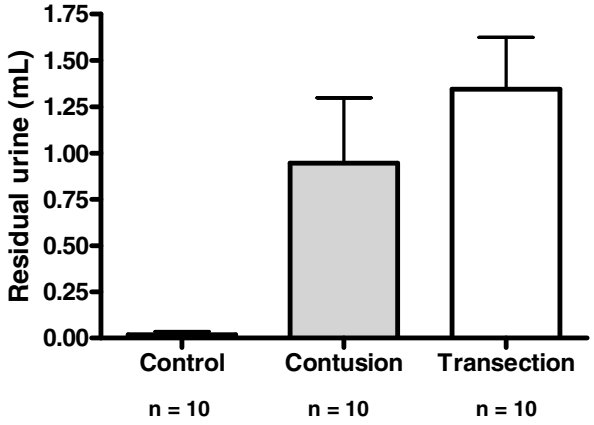

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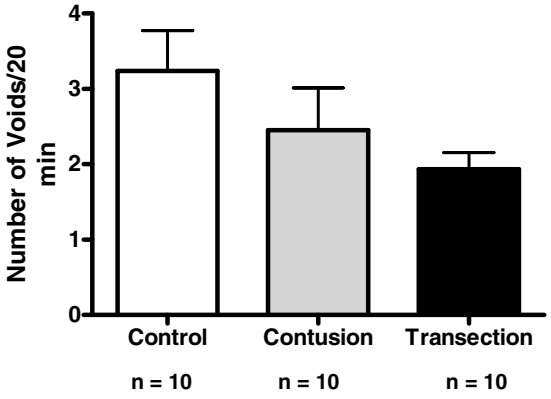

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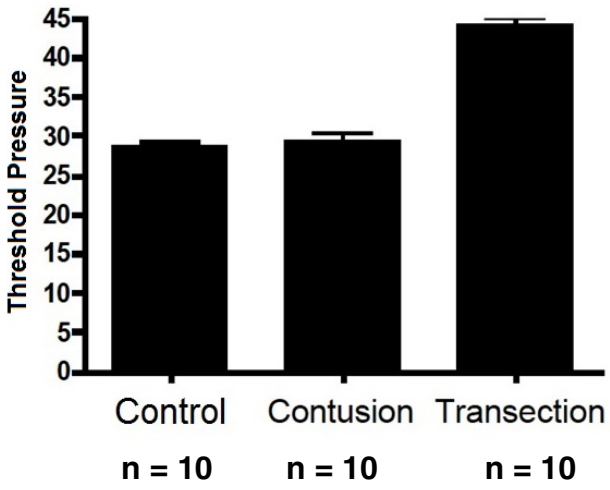

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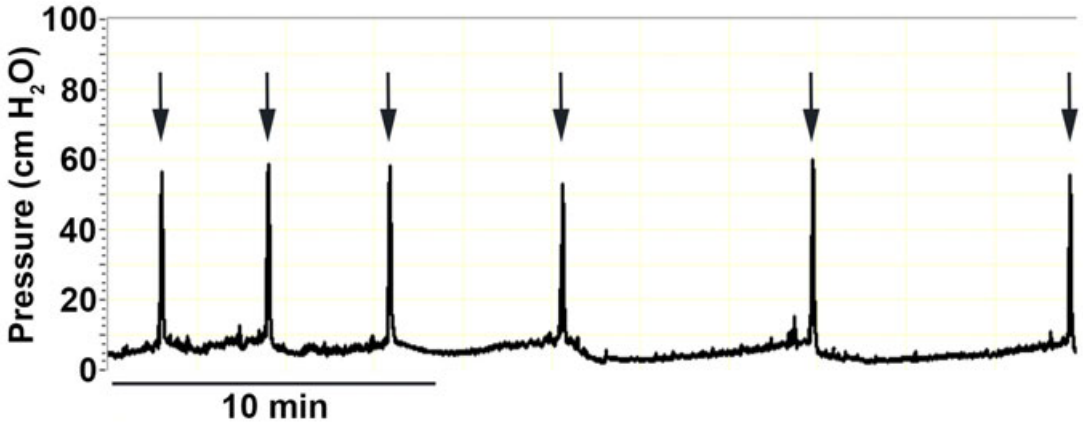

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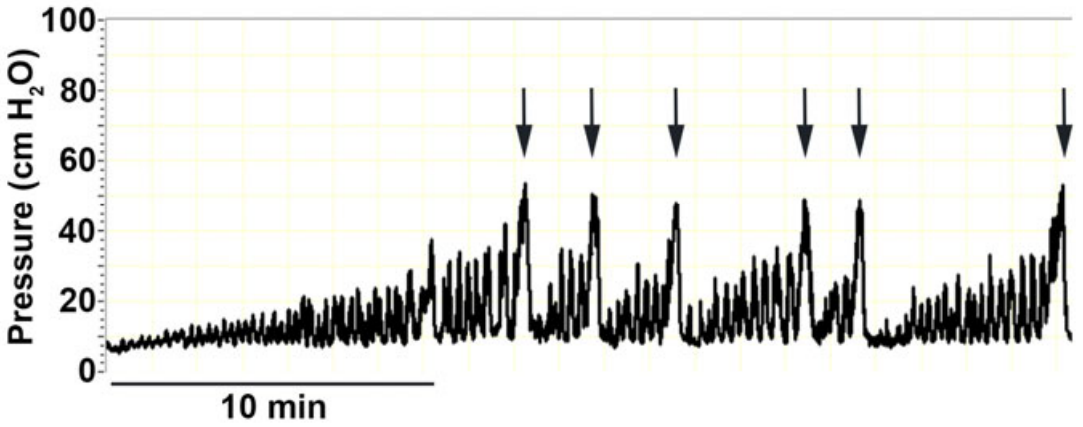

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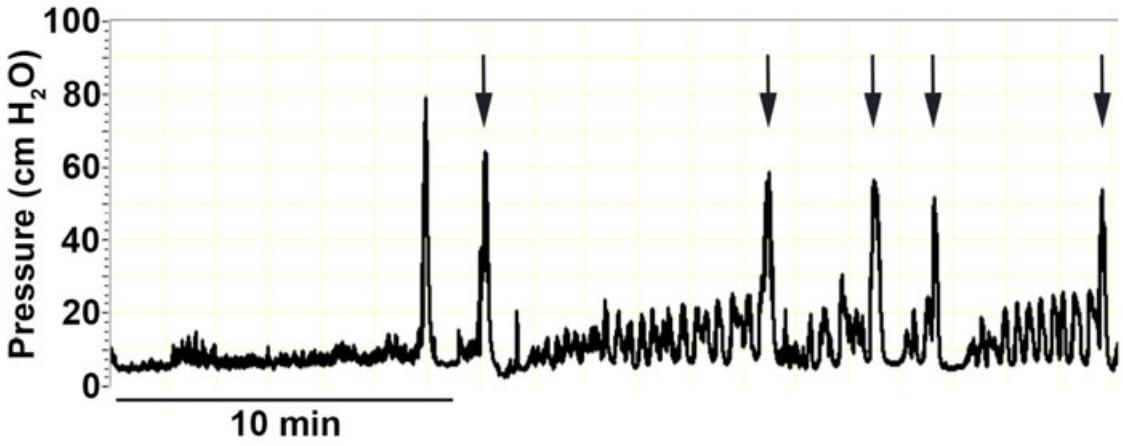

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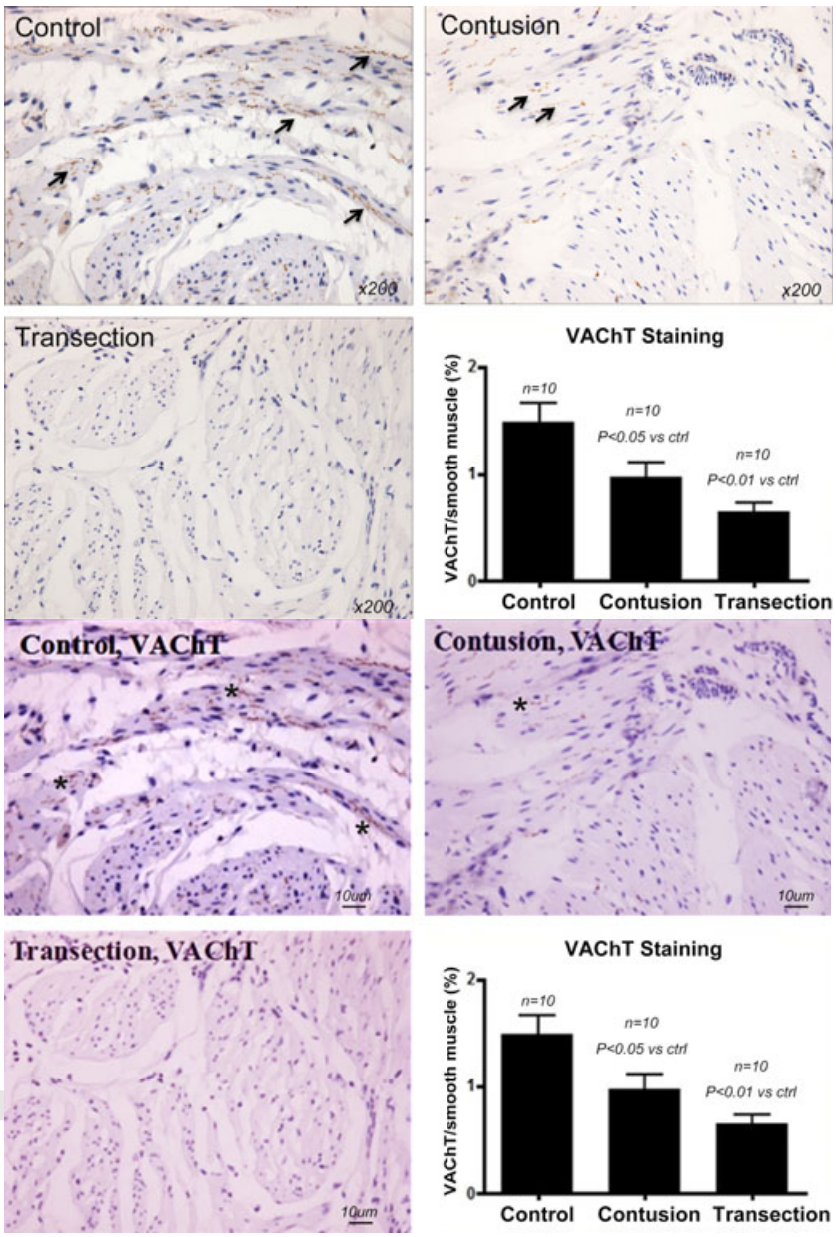

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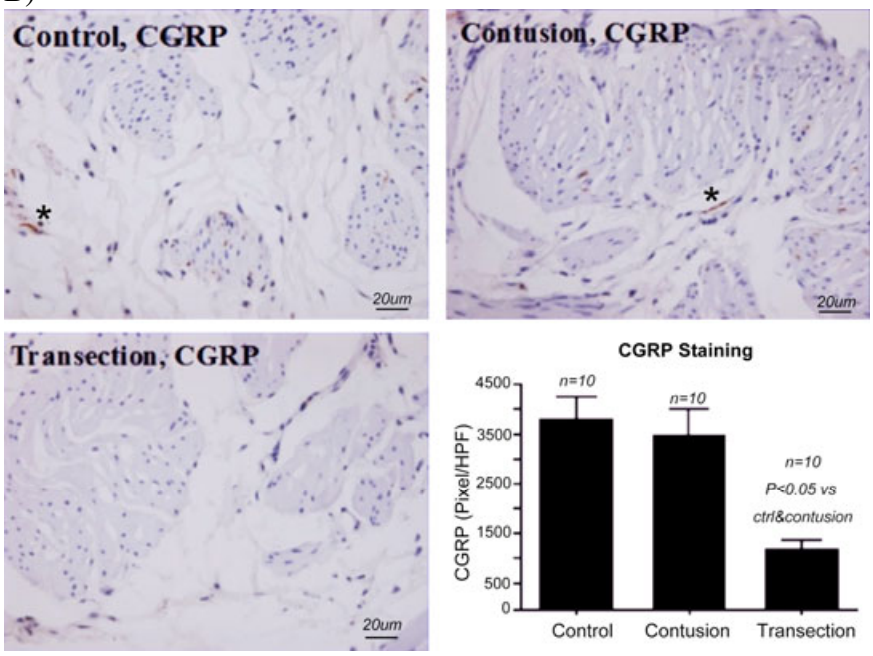

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