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Reduced colonic smooth muscle cholinergic responsiveness is associated with impaired bowel motility after chronic experimental high-level spinal cord injury☆

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ABSTRACT

The mechanisms underlying bowel dysfunction after high-level spinal cord injury (SCI) are poorly understood. However, impaired supraspinal sympathetic and parasympathetic control is likely a major contributing factor. Disruption of the descending autonomic pathways traversing the spinal cord was achieved by a T3 complete spinal cord transection, and colonic function was examined in vivo and ex vivo four weeks post-injury. Total gastrointestinal transit time (TGTT) was reduced and contractility of the proximal and distal colon was impaired due to reduced M_3 receptor sensitivity. These data describe a clinically relevant model of bowel dysfunction after SCI.

1. Introduction

Neurogenic bowel is an autonomic dysfunction resulting from spinal cord injury (SCI), and 98% of individuals report associated bowel problems such as fecal incontinence, constipation and abdominal pain that adversely impacts their quality of life (Anderson, 2004; Krassioukov et al., 2010; Burns et al., 2015). Clinical studies report that patients with high-level SCI present with decreased colonic motility, particularly in the distal colon and rectum, which relates to difficulty evacuating (Korsten et al., 2007).

The autonomic nervous system (ANS) plays a major role in regulating colon function. Parasympathetic activity promotes colonic smooth muscle contractility and motility, primarily through the M_2 and M_3 receptor subtypes (Uchiyama and Chess-Williams, 2004), while sympathetic activity slows motility by relaxing the colonic wall (Lynch et al., 2001). The loss of appropriate colonic function after chronic SCI likely results from alterations in the ANS, as a SCI disrupts descending spinal pathways. In addition, ANS influence over the enteric nervous system (ENS) is disrupted, which is important as the ENS has an important role in regulating bowel peristaltic activity and propulsion of bowel contents.

The ENS is an integrative neuronal network consisting of two ganglionated plexuses (myenteric and submucosal) that is composed of neurons and enteric glial cells and which controls the activity of the smooth muscle of the gut, mucosal secretion and blood flow. The ENS controls gut motility and secretion via local reflexes that are triggered by local distension of the intestinal wall, distortion of the mucosa, and the contents in the lumen. This neuronal regulation of GI function is due

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to the specific neuromediators synthesized and released by functionally defined enteric neurons. The ENS works in concert with CNS reflexes and command centres, and with neural pathways that pass through sympathetic ganglia to control digestive function. There is an exchange of information between the ENS and CNS, as well as between the ENS and sympathetic prevertebral ganglia (Fajardo et al., 2003; Furness et al., 2014; Furness, 2008). Cortical centres that govern voluntary control of defecation provide inputs that either inhibit or enhance excitability of neurons in the brainstem through which autonomic pathways to the distal colon and rectum are activated. These neurons project to the spinal defecation centre in the intermediolateral column (IML) at S1 level, which in turn connects with intrinsic reflex pathways of the ENS, via the pelvic ganglia. Afferent (sensory) neurons that detect pressure and mucosal irritation in the colon contribute to urge, and neurons that sense pressure in the abdominal cavity enhance defecation. These connect to second order neurons that make local connections in the spinal cord and provide sensory information to the pons and cortex (Callaghan et al., 2018). Although far more complicated and mediated by complex interactions within the ENS, peripheral and CNS (including both somatic and autonomic components) most likely contribute to peristaltic/anti-peristaltic function. Peristaltic disturbances of the upper gastrointestinal tract and in the descending colon affect motility after chronic SCI (Fajardo et al., 2003; Qualls-Creekmore et al., 2010; Fealey et al., 1984; Stinneford et al., 1993). Parasympathetic input to the proximal colon is mediated by the vagus nerve while the pelvic nerve that originates from the sacral spinal cord mediates parasympathetic input to distal colon (Callaghan et al., 2018; Hermann et al., 1998; Holmes et al., 1997). In high-thoracic or cervical chronic SCI (which is the most common level of clinical SCI), the proximal colon has intact parasympathetic but not sympathetic supraspinal control, while the distal colon loses all supraspinal autonomic control (Phillips and Krassioukov, 2015). Thus, changes in autonomic function likely underpin colonic control and bowel dysfunction after SCI (Hou and Rabchevsky, 2014).

To date, there is limited preclinical data assessing the mechanisms underlying bowel dysfunction after high-thoracic SCI. A study using a T10 SCI-injury animal model reports reduced contractile responsiveness to cholinergic stimulation and of bowel function in acute SCI (Joo et al., 2011). A very recently published study reports that reduced colonic contraction and impairment of the colonic neuromuscular interface occurs as the result of T3-contusion, and that SCI leads to diminished colonic motility (White and Holmes, 2018). The findings of the latter study stress the importance of an improved understanding of colonic impairment after high thoracic SCI.

The objective of this study was to use a preclinical model of high-thoracic SCI to evaluate bowel function in vivo (e.g., transit time), and ex vivo (e.g., ascending and descending cholinergic sensitivity). We hypothesized that high-thoracic complete spinal cord transection leads to reduced colonic motility and transit time, which is associated with decreased colonic cholinergic sensitivity, and a compensatory overexpression of colonic cholinergic receptors. We expected these changes to occur mostly in the distal colon.

2. Material and methods

All protocols and procedures performed were conducted in strict accordance with the Canadian Council for Animal Care and approved by the Animal Care Committee of the University of British Columbia. Male Wistar rats (n = 7/group; Harlan Laboratories, Indianapolis, USA) weighing 220–250 g were housed in a temperature-controlled environment with minimal exposure to any stress.

Complete spinal cord transection surgery at T3 segment, and follow up care, were performed as previously described (Phillips et al., 2016). We selected a T3 complete spinal cord transection as this model results in a clinically-relevant level of injury, while also completely disconnecting supraspinal influences over autonomic circuits below the injury. All animals survived after surgery for both SCI and sham-SCI groups. Colonic motility was assessed using: A) Total number of fecal pellets: and B) Total gastrointestinal transit time (TGTT). To measure total number of fecal pellets, each animal was housed individually with fresh bedding for 6 h during the dark cycle with no access to food or water. The number of pellets expelled at the end of this period was used as an estimate of colonic motility (Li et al., 2006). We did not measure chow intake while measuring fecal pellets, although animals were fed with fresh food once a day and their body weights checked daily over the one month period of testing, with no signs of hyperphagia being observed (Qualls-Creekmore et al., 2010). To measure TGTT, after animals were briefly anaesthetized with isoflurane (< 60 s), a solution of carmine red (500 μL; 6%; Sigma-Aldrich) suspended in 0.5% methylcellulose (Sigma-Aldrich) was administered by gavage through a 21-gauge round-tip feeding needle. We limited isoflurane exposure to this short duration so as to not impact TGTT, as there is evidence that six minutes of isoflurane anaesthesia slows gastric motility (Torjman et al., 2005). The time at which gavage took place was recorded as T0. After gavage, animals were placed in single cages with clean bedding and fecal pellets were personally monitored at 10-min intervals for the presence of carmine red, and TGTT was recorded as the interval between T0 and the time of first observing carmine red in a pellet (Li et al., 2011). Both tests were performed with a staggered day, before (baseline) and four weeks post-SCI.

For isolation of gut segments to use in contractility studies, the gut was exposed through an abdominal midline incision. Two longitudinal segments (1 cm) of muscle strips for each of the proximal and distal colon were dissected from each animal. Tissue segments were mounted in a chamber on a wire myograph (DMT 620M, Danish Myotechnology, Denmark) for measuring isometric force. Chambers were filled with 5 mL of Krebs Solution that was saturated with 95% O_2 and 5% CO_2 and maintained at pH 7.4 and 37 °C. Tissue was equilibrated for 1 h, with the Krebs solution replaced at 15 to 20-min intervals, and the resting tension was gradually increased to an optimal value (5.5 mN) according to preliminary studies. After equilibration, colonic tissues were challenged with 80 mM potassium chloride (KCl) to ensure tissue viability before creating concentration-response curves to additions of half-log increments (10^{-9}–10^{-4} M) acetylcholine (ACh), Force was normalized relative to the maximal contraction induced by 80 mM KCl, and maximum force of contraction (E_{max}) and half-maximal effective concentrations of ACh (EC_{50}) were determined in each group. In addition, after a 30-min washout period, colonic muscle strips were incubated with 4-DAMP (10^{-4} M) for 5 min to inhibit M_3 muscarinic receptors, and an ACh concentration-response curve determined. Responses to ACh were calculated as the percent increase in force relative to maximum contraction to ACh without antagonist (% contraction). The area under the curve (AUC) after 4-DAMP was subtracted from the total AUC without 4-DAMP to estimate the role of M_3 receptors in contraction. Furthermore, the AUC after 4-DAMP was considered to estimate the role of M_3 receptors in colonic contraction. We found that the ex-vivo wire-myography was a suitable technique to evaluate the response of the proximal and distal colonic segments to cholinergic agents as it has been shown by previous studies (Capeto et al., 2015; Tugay et al., 2003; Tugay et al., 2001; Joo et al., 2011).

Colonic motility tests and concentration–response curves for ACh were analyzed using a two-way repeated measures ANOVA followed by the post hoc Tukey test. In our study the following factors were tested using 2-way ANOVA: Increasing concentration of ACh (factor 1) by group (factor 2), either in sham or T3-SCI animal groups. A nonlinear regression (curve fit) of all concentration-response curves was used calculate $logEC_{50}$ and $E_{max,}$ and statistical analyses carried out using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Differences in Emax and EC50 between groups for each of the proximal and distal colons were assessed using independent *t*-tests. Difference between means was considered statistically significant when $p < 0.05$. Data are shown as mean ± standard error mean (SEM).

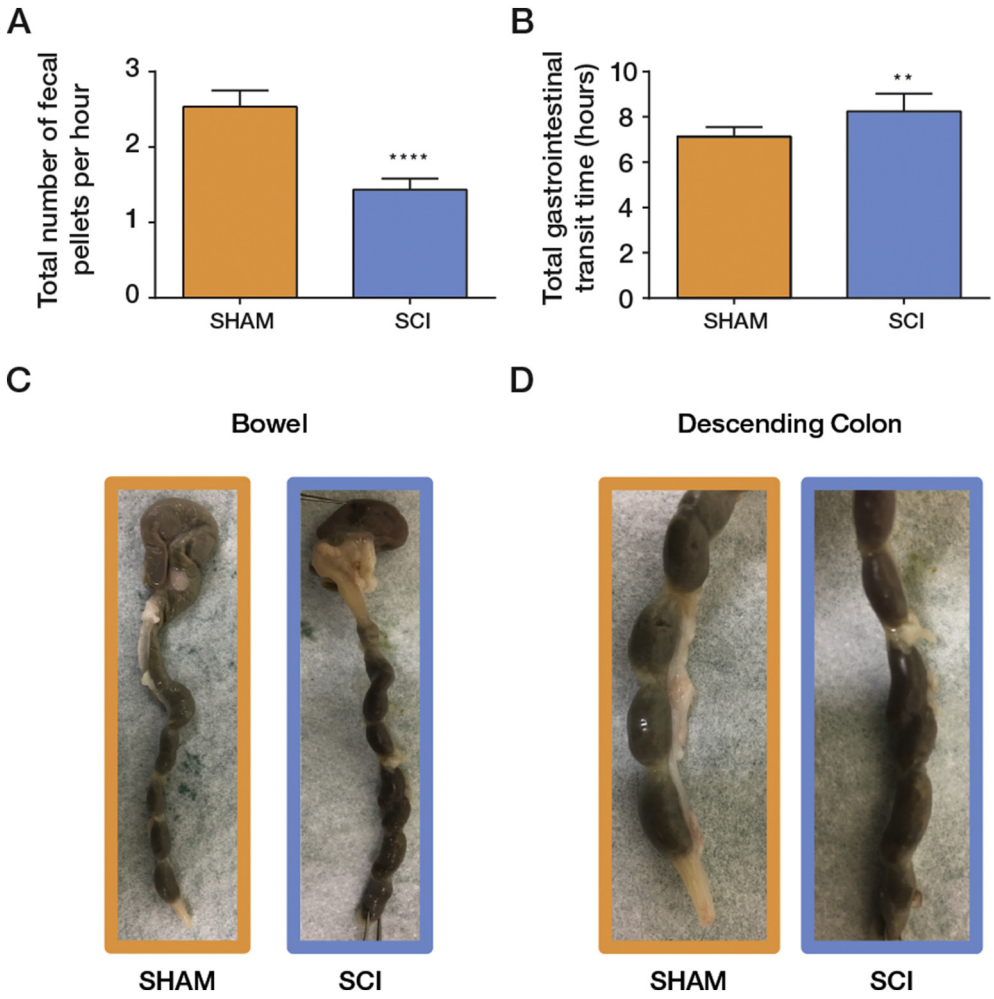

Fig. 1. Severe bowel impaction and impaired bowel motility after high-thoracic SCI. A) Total number of fecal pellets produced over six-hour period was significantly reduced after T3-SCI ($p < 0.0001$). **B)** Total gastrointestinal transit time was significantly prolonged after T3-SCI ($p < 0.01$). **(C&D)** Bowel impaction after SCI in the descending colon.

3. Results

The total number of fecal pellets was reduced 44% ($p < 0.0001$, Fig. 1A) and TGTT was prolonged by 20% ($p < 0.01$; Fig. 1B) after T3-SCI. Fecal impaction was evident in the distal colon of T3-SCI animals (Fig. 1D).

The isolated colonic smooth muscle response maximum contraction to cholinergic stimulation (E_{max}) and sensitivity in response to ACh (EC_{50}) were significantly reduced in the proximal colon after T3-SCI (Fig. 2A–C; $p < 0.05$). Similarly, the Ach E_{max} were reduced at all concentrations in the distal colon in T3-SCI (Fig. 2D–F). Specifically, the distal colon E_{max} was impaired by 48% compared to sham ($p < 0.0001$), while the proximal colon was reduced by only 10% ($p < 0.0004$). Sensitivity (EC_{50}) of ACh to KCl was unchanged between sham-injured and T3-SCI both in the distal ($p = 0.9952$) and proximal colon ($p = 0.22$).

Incubation of the proximal colon with 4-DAMP decreased the AUC by 41% (sham) and 48% (T3-SCI) (Fig. 2A–C, both $p > 0.05$), suggesting that both M_3 and M_2 receptor function was similar after SCI in the proximal colon. In the distal colon, incubation with 4-DAMP reduced the AUC by only 26% in sham-injured animals, but by > 60% after T3-SCI (Fig. 2D–F, $p < 0.0001$). The remaining AUC after 4-DAMP was not different between groups for either the proximal or distal colon, suggesting that reduced colonic smooth muscle cholinergic responsiveness was due to a loss of M_3 receptor sensitivity after T3-SCI. It may be important to select anticholinergics that do not inhibit M_3 receptor function in this population to alleviate this concern.

4. Discussion

We describe the preclinical model of bowel dysfunction after high-thoracic chronic SCI. Although several previous studies described effects of the SCI on bowel function in rodents, the unique aspect of our study was that it utilized a combined evaluation of “in vivo” functional gut outcomes with examination of “ex vitro” physiological changes in gut function in a T3 spinal cord injury model. Bowel dysfunction was characterized by delayed transit time that was accompanied by impaired colonic smooth muscle contraction in both the proximal and distal segments. Impaired colonic smooth muscle contraction was associated with impaired M_3, but not M_2, receptor sensitivity. These findings lay the groundwork for future research into bowel dysfunction in a clinically-relevant SCI model.

Bowel dysfunction following high-thoracic SCI is accompanied by prolonged transit times in humans (Krogh et al., 2000). Increased colonic transit times in both paraplegic and tetraplegic SCI patients has been demonstrated using a variety technologies including scintigraphy, radioisotopes and recently with non-invasive SmartPills (Williams et al., 2012; Christensen et al., 2003; Keshavarzian et al., 1995; Rasmussen et al., 2013). Preclinical studies have replicated these findings (White and Holmes, 2018; Joo et al., 2011), but further investigation of the pro-motility cholinergic sensitivity of the colon is necessary.

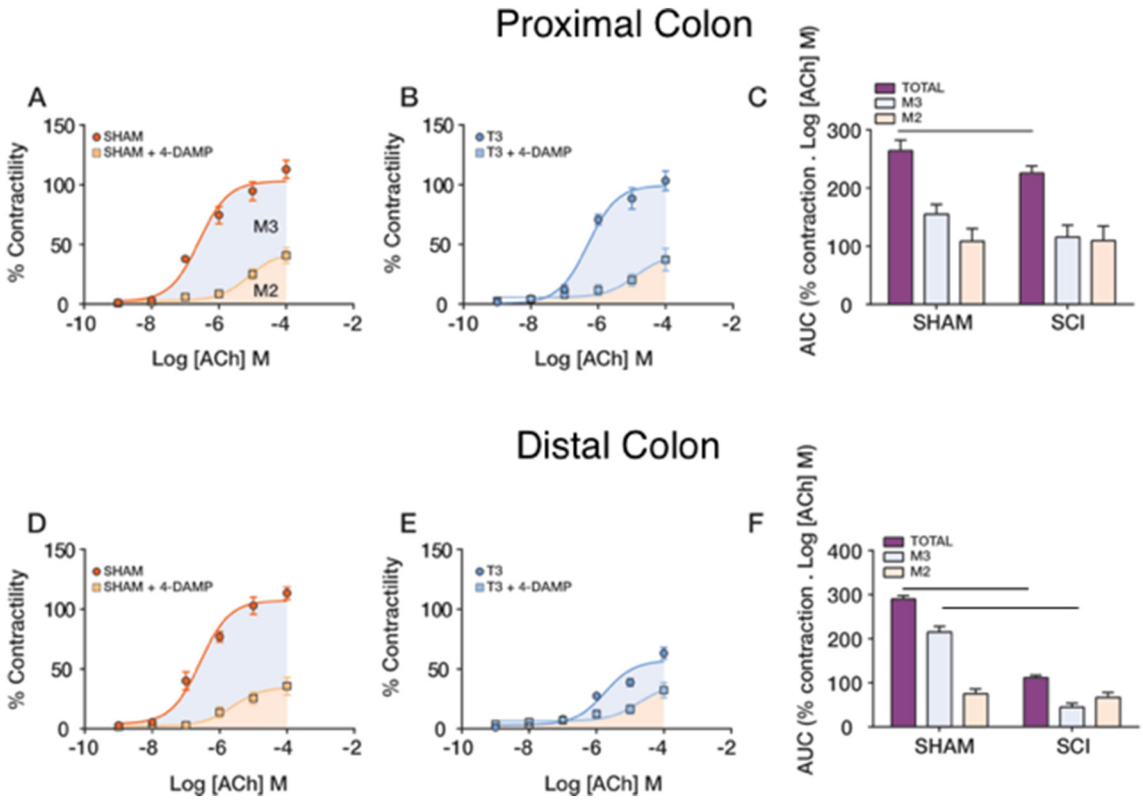

Fig. 2. Reduced colonic smooth muscle cholinergic responsiveness mediated by M_3 receptors after high thoracic SCI. A-C) Peak contraction and sensitivity in response to Ach were moderately impaired after T3-SCI, however there was no difference in the role of M_3 or M_2 receptors. E_{max} sham = 104.8% ± 3.9% vs. T3-SCI 93.8% ± 3.2%; $p < 0.0001$: $LogEC_{50}$ sham = -6.6 ± 0.1T3-SCI vs. -6.3 ± 0.1; $p < 0.0147$. **D-F)** Contractile response to Ach was drastically impaired after T3-SCI, due to the loss of M_3 receptor responsiveness. E_{max} sham = 107.3% ± 3.8%, vs. T3-SCI = 55.5% ± 3.1%; $p < 0.0001$: $LogEC_{50}$ sham = -5.8 ± 0.1 vs. T3-SCI = -6.5 ± 0.1; $p < 0.0001$ AUC, area under the curve.

The SCI model used in our study accurately replicated this impaired colonic function by demonstrating prolonged TGTT and decreased number of fecal pellets at four weeks post-SCI, highlighting the rapid onset of bowel dysfunction. A recent study demonstrates reduced colonic motility in T3 contusion SCI animals, that was accompanied by morphological changes with reductions of spontaneous contractions of the proximal and distal colon that may be due to significant decreases in enteric neuron numbers within the colon and breakdown of the neuromuscular junction (White and Holmes, 2018). Smooth muscle contractility was reduced in response to cholinergic stimulation, predominantly in the distal colon, as opposed to the proximal colon. In T10-SCI animals, smooth muscle contractility of the proximal colon was increased while smooth muscle contractility of distal colon was decreased due to changes in the number or activity of muscarinic receptors in colonic smooth muscles, in particular M_2 and M_3 (Joo et al., 2011). The selective blockade of M_3 cholinergic receptors in our study suggests a loss of cholinergic sensitivity primarily through this receptor sub-type, likely resulting from a loss of parasympathetic control (Dale, 1914). Reduced smooth muscle contractility in the distal colon of T10-SCI animals was attributable to a decrease in the ACh response. Inhibition of M_3 receptors increased smooth muscle contractility of the distal colon (Joo et al., 2011). Bowel dysfunction has been studied using different techniques, including balloon manometer to record pressure changes (Holmes et al., 1997), or distension (Shafton et al., 2006). However, to our knowledge this is the first study to assess the cholinergic effects on proximal and distal colonic smooth muscle segments of chronic high-thoracic (T3) SCI-animals.

Smooth muscle contractility of the distal colon was more severely impaired compared to the proximal colon in terms of sensitivity and maximal contraction (Fig. 2). This finding demonstrates the clinical relevance of our model, as patients with SCI also experience greater impairment in motility in the descending as opposed to ascending colon after SCI (Faaborg et al., 2008; Emmanuel et al., 2009; Rasmussen et al., 2013; Hedsund et al., 2013). Greater dysfunction in the distal colon may be due to the loss of both parasympathetic and sympathetic regulation over this portion, whereas the proximal colon has preserved parasympathetic control. A previous study reported hypersensitivity of bowel smooth muscle cholinergic receptors following enteric denervation (i.e., both the sympathetic and parasympathetic inputs are lost) (Osinski and Bass, 1994). The loss of supraspinal control over autonomic circuits exerts differential effects on bowel cholinergic responses due to a T3-SCI, where spinal circuits below the level of injury that are involved in distal colon control are intact, but lacking supraspinal control (Phillips and Krassioukov, 2015). SCI at cervical or thoracic levels disrupts descending inputs that control the lumbosacral defecation centres. Disruption of inputs to neurons in the CNS can cause receptor hyperresponsivness (Callaghan et al., 2018; Ferens et al., 2011). In SCI, autonomic receptor expression and responsiveness are frequently altered below the level of injury in various tissues, and there is reorganization of autonomic circuits (Krenz et al., 1999; Krenz and Weaver, 1998). Hyperresponsiveness occurs in peripheral tissues where neural control has been disrupted by the SCI (Hou et al., 2008; Yeoh et al., 2004). Other pathways that are disrupted by SCI include those responsible for anorectal contractions, which are modulated by activation of autonomic circuits, and descending pathways originating from the caudal nucleus raphe obscurus project onto the ventral and lateral funiculi of the spinal cord, with arborizations in the thoracic intermediolateral cell column (Holmes et al., 2002; Holmes et al., 1997).

Our data confirms that both low SCI (initially reported by (Joo et al., 2011) as well as high-thoracic SCI (present study) are accompanied by an altered role for M_3 receptor regulation of intestinal contractility. Further research is necessary to better understand M_3

receptor mediated responses in the different colonic segments, e.g. in impaired colonic motility. Studies with a dual antagonist of M_3 and M_2 receptors would have strengthened our results. It is important to note that anticholinergic medications are frequently used for pain and bladder management after high-level SCI (Consortium for Spinal Cord Medicine, 2006; Moulin et al., 2007), and could further antagonize any residual preserved supraspinal control of bowel function. Additional studies are needed to explore the effects of anticholinergic agents such as such as neostigmine that do not target M_3 receptor subtypes (Korsten et al., 2005) in an effort to mitigate the exacerbation of bowel dysfunction after SCI.

These results of our translational study of bowel dysfunction after SCI shows that loss of smooth muscle contractility and muscarinic receptor alterations are likely mechanisms underlying impaired bowel function after SCI.

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Conflict(s)-of-interest/disclosure(s)

No competing financial interests exist.

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