Effects of intravesical Botulinum toxin-A on bladder dysfunction and autonomic dysreflexia after spinal cord injury: Role of CGRP primary afferents and NGF

by

« Mohamed Soliman Elkelini »

A thesis submitted in conformity with the requirements for the degree of « MSC-Medical Science »

« Institute of Medical Science »
University of Toronto

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Abstract

Spinal cord injury (SCI) remains a significant cause for morbidity and mortality in North America. Bladder dysfunction following SCI is very common and could lead to severe complications including renal failure and autonomic dysreflexia (AD). AD involves life threatening episodes of hypertension in patients with SCI above T6 level. Current management protocols for AD are symptomatic and usually ineffective. Botulinum toxin-A (BTX-A), has been successfully used recently in SCI patients because it reduces the detrusor contractility via inhibiting acetylcholine release from efferent nerve endings. Recent evidence, however, suggests a sensory involvement via modulation of sensory neuropeptides, neurotransmitters, and receptors. It is still, however, unclear whether BTX-A can affect putative spinal neurons involved in AD. In this study we demonstrated that intravesical BTX-A treatment has blocked AD in rats with T4-SCI, and also provided a novel mechanism for the control of autonomic dysreflexia via a minimally invasive treatment modality.
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Sincerely,

Mohamed Elkelini, MD
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Chapter 1

1 Introduction

Spinal cord injury (SCI) is a significant cause for morbidity and mortality in developed nations with a global annual incidence of 15 to 40 cases per 1 million individuals [1]. This incidence translates into approximately 10,000 to 12,000 SCI cases in North America each year. With improvements in medical and nursing protocols, SCI patients are afforded increased rates of survival and life expectancy [2]. In fact, the number of SCI survivors is dramatically more widespread than previously believed. According to a recent study, an estimated 5,596,000 Americans (1 in 50) are living with paralysis [3]. About 1,275,000 people attribute their paralysis to SCI.

Bladder dysfunction and autonomic dysreflexia (AD) are a common outcome following cervical and high thoracic spinal cord injury (SCI). Bladder dysfunction after SCI is characterized by a brief spinal shock phase, followed by return of bladder activity after 2-12 weeks, which includes detrusor overactivity (DO) and detrusor-sphincter-dyssynergia (DSD), leading to a high bladder pressure and impaired renal integrity. Also, bladder distension may cause initiation of AD, which involves life-threatening episodes of paroxysmal hypertension and bradycardia [4].

AD and DO after SCI develop in a time-dependant manner, suggesting that neuroplasticity contributes significantly to both conditions. Following SCI, the bladder afferent pathways are reformed inducing C-fibre afferents containing the neuropeptide calcitonin-gene-related-peptide (CGRP). CGRP primary afferents have been used as a marker for sprouting after SCI and also linked to the development of AD and DO [5-6].

Nerve growth factor (NGF) is a member of neurotrophins family and an important regulator of neural survival, development, function, and plasticity. NGF plays a major role in the enlargement of the afferent arbour following SCI [7]. Furthermore, intrathecally delivered anti-NGF has reduced AD in rats after SCI [8]. These findings, therefore, suggest that NGF and its receptors in the bladder and spinal cord may offer potential targets for new therapies to control AD and DO following SCI.
Current management protocols for bladder dysfunction with SCI include anticholinergics, alpha-blockers, and intermittent catheterization. Nevertheless, most patients are not satisfied because the treatment is either ineffective or they cannot tolerate the adverse effects of these medication [9]. Botulinum A Toxin (BTX-A), has been successfully used recently in SCI patients because it reduces the detrusor contractility via inhibiting acetylcholine release from efferent nerve endings. BTX-A is delivered by injections into the bladder, and its effect lasts for 6-12 months [10]. More recently however, there is increasing evidence that BTX-A may also affect sensory nerve fibres and afferent signaling mechanisms [10]. Furthermore, BTX-A has reduced NGF levels in the bladder tissue of patients with neurogenic detrusor overactivity [11]. It’s still however unknown, if intravesical BTX-A administration may affect primary afferent fibres sprouting and putative spinal neurons involved in DH and AD following SCI and hence may block autonomic dysreflexia and detrusor overactivity.

1.1 Pathophysiology of spinal cord injury

The spinal cord is an intricate meshwork of nerve fibres extending from the brain stem to the lumbosacral spinal column. The human spinal cord has 31 pairs of spinal nerves that project out from ventral roots of the spinal cord as preganglionic fibres. These preganglionic fibres synapse with postganglionic neurons located within target organs such as skin, muscles, limbs and other parts of the peripheral nervous system to permit the passage of ascending and descending nerve impulses between the brain and the rest of our body [12].

It is currently widely accepted that acute SCI is a two-step process involving primary and secondary mechanisms. The primary mechanism involves the initial mechanical injury due to local deformation and energy transformation, whereas the secondary mechanism encompasses a cascade of biochemical and cellular processes that are initiated by the primary process and may cause ongoing cellular damage and even cell death [13].
1.1.1 Primary mechanisms

The primary mechanism of acute SCI is a combination of both the initial impact and the subsequent persisting compression. This is common in injuries such as burst fractures and rupture discs. Ongoing compression does not always present especially in cases where ligamentous injuries dislocates and then spontaneously reduces. Also, missile injuries can produce a variety of clinical scenarios including laceration and compression [14].

1.1.2 Secondary mechanisms

Following the primary injury a cascade of secondary auto-destructive events is generated to yield a positive feedback loop of amplified pathology and functional deficit. This process of secondary injury is thought to persist years after the SCI. Post-SCI secondary injurious events include spinal cord ischemia-reperfusion, lipid peroxidation and membrane decomposition, glutamate-mediated excitotoxicity and ionic imbalance, generation of free radicals and reactive oxygen species, and extensive inflammation [13]. Secondary injury cascades also involve the activation and up-regulation of pro-inflammatory cytokines, proteases, toxic metabolites and neurotransmitters that cause rostral-caudal expansion of the lesion epicentre [15].

Secondary injury events following SCI negatively impact autonomic function [16]. Loss of urination control occurs as a result of uncoordinated efforts between the external urethral sphincter via somatic cortical control and smooth muscles lining the bladder via autonomic control [17]. Furthermore, injuries above T6 produce autonomic dysreflexia, which is characterized by an arterial pressor effect in response to stimuli such as bladder and rectal distension. These stimuli produce an exaggerated sympathetic response that leads to very high levels of blood pressure and bradycardia via vagal stimulation [18].

1.2 Autonomic dysreflexia following spinal cord injury

The autonomic dysreflexia reaction is seen in spinalized patients with a level of lesion above the sixth thoracic segment. The symptomatic triad of AD comprises high blood pressure, bradycardia and hot flushes with occasional headaches. All these symptoms
are related to a dysfunctional autonomic nervous system, which plays a major role in blood pressure and heart rate control [19]. Major splanchnic outflow from the sympathetic system arises from T5 to L2 under the inhibitory action of supraspinal centre. Spinal cord injury seriously disturbs blood pressure control that normally depends upon supraspinal regulation of sympathetic preganglionic neurons. Spinal reflexes have minimal contribution to cardiovascular control; in spinal cord injury, however, these reflexes dominated blood pressure control and the unchecked activity of spinal reflexes leads to autonomic dysreflexia. Furthermore, dysreflexia occurs after injury at, or above the 6th thoracic spinal segment, because injury at this level leaves the sympathetic control off the extensive abdominal circulation amenable to unrestrained spinal reflexes [20] (Figure 1.1).

1.2.1 Causes of autonomic dysreflexia

Afferent stimulation below the lesion in SCI patients provokes AD. Bladder distension is the most common cause followed by rectal distension. Other causes include pressure ulcers, burns and ingrown toe nail. Distension of the bladder may occur during cystoscopy, urodynamics, and also precipitated by urinary tract infections, kidney stones, and bladder percussion [21].

1.2.2 Symptoms and signs

The presenting symptoms of the AD reaction are diverse and include pounding headache, paresthesias, shivering, flushing and sweating of the head (above the lesion), nasal obstruction, desire to void, anxiety, malaise and nausea. There may also be a feeling of dullness in the head and blurring of vision is not uncommon. Severe headache, usually of occipital, bitemporal and bifrontal location is noted in more than half of the patients [20]. Systolic and diastolic blood pressure increases are usually sudden and severe, frequently associated with bradycardia. Blood pressure of 20-40 mmHg above baseline may be a sign of AD. However, systolic blood pressure above 300 mmHg and diastolic blood pressure above 220 mmHg have been reported [20].
1.2.3 Pathophysiology of autonomic dysreflexia

Mechanisms for autonomic dysreflexia that have been considered include upregulation of vascular catecholamine receptors, increased neural release of catecholamines, decreased presynaptic reuptake of catecholamines, loss of the baroreceptor reflex, altered glutamatergic control of spinal neurons and loss of tonic bulbospinal inhibitory input to spinal neurons [19]. Furthermore, vasoconstriction of the vascular bed especially the splanchnic bed is believed to play an important role in the development of AD. A lesion level above the sixth thoracic segment involves an interruption of the connection between the brain and the splanchnic vascular bed, entailing an inability to dilate the vascular bed by central command when needed. Animal research has also shown an increase in renal sympathetic activity during induced dysreflexia, indicating an active vasoconstriction in the kidney. In contrast, Electrophysiological recordings have been obtained from leg vasoconstrictor nerves of cord-injured subjects to determine the magnitude of spinal sympathetic reflexes [22]. Only small sympathetic reflexes were recorded in these people during episodes of dysreflexia. However, sympathetic nerves of the leg are not those that cause the hypertension. Instead constriction of the abdominal visceral beds is crucial and, accordingly, discharge of vasomotor sympathetic nerves innervating these beds would be a better indicator of the sympathetic reflexes contributing to dysreflexia [22].

1.2.4 Autonomic dysreflexia develop in time-dependant manner

Experimental studies in rats have shown that, soon after SCI, bulbospinal pathways are damaged and this disrupts the control of sympathetic preganglionic neurons. This renders the neurons less receptive to any excitatory input, which explains why there is no AD during the shock phase [23]. After 30 days, there has been a significant recovery in the preganglionic fibres and reorganization of synaptic input with the return of the excitatory sympathetic responses. Another study showed that primary afferent sprouting of myelinated and unmyelinated fibres occurred in response to spinal cord injury [6]. Afferent sprouting, however, did not reach the autonomic or motor neurons but may have caused hyperreflexia by increasing inputs to the neurons [6]. The combination of lost descending pathways to the sympathetic preganglionic neurons and formation of
new synaptic inputs is thought to play an important role in the development of exaggerated reflex responses and abnormal cardiovascular control.

Figure 1.1

Figure 1.1 Mechanisms and manifestations of autonomic dysreflexia.
1.3 Bladder dysfunction following spinal cord injury

Bladder dysfunction is very common and problematic for SCI patients. Common symptoms of impaired bladder emptying include inability to void, incomplete emptying, lower abdominal discomfort, dribbling and recurrent urinary tract infections [24-25]. Moreover, because bladder dysfunction may cause severe urinary retention, urinary tract infection, or even chronic renal failure, it has become one of the main causes of death in patients with SCI [26]. These symptoms impact SCI patients’ quality of life and require a life long management plan.

1.3.1 Anatomy and function of the lower urinary tract

Bladder function is two-fold; to store urine at low pressure, and to voluntarily eliminate urine. Regulation of these bladder functions requires an intricate control involving the spinal cord and brain. Basic understanding of this complex neuronal control over the bladder is important to understand the reasons for changes in bladder behaviour following SCI. Bladder dysfunction after SCI is characterized by a brief spinal shock phase followed by return of bladder activity after 2-12 weeks, which includes detrusor overactivity (DO) and detrusor sphincter dyssynergia (DSD), leading to a high bladder pressure and impaired renal integrity [4].

The urinary bladder and urethral sphincter act in reciprocal fashion. During urine storage, the bladder outlet is closed; and the bladder smooth muscle is quiescent, allowing intravesical pressure to remain low over a wide range of bladder volumes [27]. Sensory input is conveyed to the spinal cord via pelvic and hypogastric nerves. The afferent fibres carry impulses from tension receptors and nociceptors in the bladder wall to neurons in the dorsal horn of the spinal cord. These afferents include myelinated (Aδ fibre) or unmyelinated C fibre axons [28]. Also, bladder afferent impulses activate a sacral to thoracolumbar intersegmental spinal reflex pathway, which triggers sympathetic firing to the bladder, and subsequently inhibits bladder activity and contracts the bladder outlet. Furthermore, Pudendal motoneurons are also activated by the bladder impulses, to induce a contraction of the striated sphincter muscle [29].
When bladder volume reaches the micturation threshold, afferent activity originating from the bladder mechanoreceptors trigger the micturation reflexes. These reflexes include activation of the parasympathetic pathways and inhibition of the sympathetic and somatic pathways, which leads to contraction of the bladder and simultaneous relaxation of the outlet [30]. Bladder afferents in the pelvic nerve synapse on neurons in the sacral spinal cord, which then send their axons rostrally to the micturation center in the dorsolateral pons. This centre acts as an on-off switch, activated by impulses from the bladder mechanoreceptors and also receives inhibitory and excitatory inputs from the brain. Descending input from the micturation centre directly inhibits the pudendal motoneurons and subsequently the activity in the pudendal efferent pathway to the striated urethral muscles is suppressed to reduce outlet resistance [31].

During voluntary micturition, the initial event is a reduction of intraurethral pressure, which reflects a relaxation of the pelvic floor and the periurethral striated muscles, followed by a detrusor muscle contraction and an opening of the bladder neck. Reflex inhibition of the smooth and striated urethral sphincter also occurs during micturation [32]. In the human bladder smooth muscle, only two muscarinic receptor (M2 and M3) subtypes have been indentified. Even though the M2 receptor is the most dominant subtype in the bladder, the contraction of the bladder is mainly mediated by the M3 receptors [33]. Voiding reflexes are mediated by myelinated (Aδ fibre) bladder afferents, which activate a supraspinal micturation reflex in the brain stem. Unmyelinated (C fibre) bladder afferents are also present but they are in a quiescent state and do not respond to bladder distension [27] (Figure 1.2).

1.3.2 Effects of spinal cord injury on voiding

The effects of SCI on the lower urinary tract depend on the level, duration and completeness of the cord lesion. According to the level, it may give the picture of an upper motor neuron lesion (UMNL) or lower motor neuron lesion (LMNL). UMNL forms the majority of patients with SCI, and it initially leads to a phase of spinal shock, which is followed by a recovery phase during which neurologic changes emerge. In the shock phase, the bladder becomes flaccid and areflexic, however the activity of the external
sphincter rapidly recovers after SCI [4]. Thus urinary retention develops and patients may need intermittent or continuous catheterization in order to eliminate urine.

Following the shock phase, voiding reflexes start to re-appear after 2-12 weeks, which are involuntary reflex bladder contractions. These reflexes generate low vesical pressure initially, but over time, bladder contractions become more powerful and produce involuntary contractions. Because of the loss of coordinated action between the bladder and urethral sphincter (DSD), bladder is usually partially emptied and postvoiding residual volume increases over time. Bladder overactivity and DSD lead to high intravesical pressure, with or without vesical-ureteral reflux leading to impairment of renal functions. In the LMNL type of SCI, the bladder and its outlet become flaccid; hence, bladder capacity and compliance are increased [27].

Injuries above T6 produce autonomic dysreflexia, which is characterized by an arterial pressor effect in response to stimuli such as bladder, and rectal distension. These stimuli produce an exaggerated sympathetic response leading to very high levels of blood pressure, and bradycardia via vagal stimulation. This could be life threatening, and may cause cerebral hemorrhage or seizures [34].

In summary, bladder dysfunction following SCI include four major problems: (1) inadequate or excessive detrusor function, (2) inadequate or excessive sphincter function, (3) dyssynergy between detrusor and sphincter actions and (4) impaired ability to sense the bladder. Treatment modalities are therefore based on controlling bladder dysfunction and may change to fit patients' lifestyle.
Figure 1.2

Figure 1.2 Voiding (A) and Storage (B) reflexes of the urinary bladder
1.4 Role of C fibres in the pathogenesis of autonomic dysreflexia and detrusor overactivity

Normally, the micturition reflex is mediated by a long-latency supraspinal reflex pathway, and is activated by myelinated Aδ-fibre bladder afferents. However, following SCI, the afferent limb of the micturation reflex is remodelled to consist of unmyelinated C-fibre afferents [4]. In spinalized cats, subcutaneously administered capsaicin (C-fibre neurotoxin), completely blocked reflex bladder contractions induced by bladder distension, whereas capsaicin showed no inhibitory effects on reflex bladder contractions in spinal intact cats [35]. Clinically, capsaicin treatment also inhibited bladder overactivity and autonomic dysreflexia via its action on C fibres [36] (Figure 1.3).

Further evidence of a reorganization of C-fibre mediated reflex pathways in subjects with spinal cord injury was obtained in studies of the cold stimulation-evoked voiding reflex. This reflex is normally present during infancy but disappears later in life. In cat, a bladder-to-bladder cooling reflex (C-fibre afferent) seems to be responsible for cold-induced bladder reflexes [37]. Moreover, in rats, cold stimulation induced detrusor-sphincter-dyssynergia and that capsaicin treatment prevented it [38].

Another unique feature of these fibres is their ability to store and release certain neuropeptides in their nerve terminals. For instance, neuropeptides including calcitonin gene-related peptide (CGRP) are synthesized in the capsaicin-sensitive primary afferents and have been implicated in the development of detrusor overactivity and autonomic dysreflexia. CGRP, in particular, was found to be exclusively present in their nerve terminals at bladder level [39].

In the dorsal horn, the arbours of small-diameter primary afferent neurons can enlarge greatly in rats and mice after SCI potentially leading to increased reflex excitation of preganglionic neurons, via interneuronal pathways. For instance, after SCT, the 2-week time course for the increased arbour size correlates with the gradual increase in the magnitude of autonomic dysreflexia [6, 40]. This afferent arbour remains enlarged at 1 month after cord injury, a time when autonomic dysreflexia is well developed in rats [23].
Furthermore, CGRP-primary afferents sprouted in L6/S1 spinal cord and has been also linked to the emergence of bladder activity after spinal cord transection in rats [5].

**Figure 1.3**

*Figure 1.3 Role of C-fiber afferent pathways in the pathogenesis of detrusor overactivity and autonomic dysreflexia*
1.5 Role of NGF in the pathogenesis of autonomic dysreflexia and detrusor overactivity

Nerve growth factor (NGF) is a growth factor that acts directly on neurons to support their growth, differentiation and survival [41]. NGF was discovered in the 1950s as a key player in target-mediated regulation of peripheral innervations, particularly, sympathetic and sensory neurons. During nervous system development, NGF is released by the target tissue, taken up in responsive neurons and transported retrogradely to the cell body where it exerts its trophic/differentiative effects. In addition, NGF expression responds to numerous stimuli and is markedly upregulated in tissues after injury [41]. Following SCI, NGF has positive and negative roles; for instance, NGF promotes regeneration of sensory axons into spinal tracts after dorsal root injury with restoration of thermal sensory function. In contrast, NGF may have deleterious effects such as causing apoptotic death of white matter oligodendrocytes which leads to demyelination conduction deficits [42].

1.5.1 NGF and bladder dysfunction

NGF has been involved in detrusor overactivity via its effect on C fibre primary afferents [42]. Chronic administration of NGF into the bladder of rats has also induced bladder hyperactivity and increased the firing frequency of dissociated bladder afferent neurons [43]. Furthermore, NGF content has increased in the bladder after spinal cord injury [42]. In addition, a recent study has found that increased NGF in the spinal cord after spinal cord injury is also responsible for inducing hyperexcitability of C-fibre bladder afferent pathways, and that intrathecal application of NGF antibodies, which neutralized NGF in the spinal cord, suppressed detrusor overactivity and detrusor-sphincter-dyssynergia in spinal cord injured rats [44].

1.5.2 NGF and autonomic dysreflexia

Recent studies have shown that NGF causes enlargement of the afferent arbour in spinalized rats, and intrathecal anti-NGF completely blocks the sprouting of the small-diameter afferent fibres in the dorsal horn. When autonomic dysreflexia was assessed in those rats, arterial pressure increases induced by colonic stimulation were significantly reduced [8, 45]. In a different study, in which intrathecal anti-NGF was
given for 2 weeks, autonomic dysreflexia was reduced in rats by 30% [46]. Further studies demonstrated that NGF is significantly increased at the site of the spinal cord injury, and also named the cells producing NGF at the injury site [7]. Together, these evidences suggest a strong relationship between NGF and changes in the afferent arbour that can contribute to autonomic dysreflexia. Moreover, the effectiveness of anti-NGF might be clinically important in controlling the development of autonomic dysreflexia.

1.6 Botulinum toxin-A

Botulinum neurotoxin type A (BTX-A) is one of the botulinum toxins family, and widely known as the most potent biologic toxin known to man. BTX-A operates by inhibiting acetylcholine release at the presynaptic neuromuscular junction, and hence producing paralysis of the muscle [47-48] (Figure 1.4). Based on this mechanism of action, BTX-A was introduced as a treatment modality for several urological problems including urge-incontinence caused by neurogenic bladder overactivity [49]. In the bladder, BTX-A is injected intravesically at multiple sites, and in a long-term follow-up study, BTX-A responses lasted for 4-14 months [50]. BTX-A injections have increased bladder capacity, and bladder compliance as well as induced changes in detrusor function with decreases in detrusor pressures during bladder filling and voiding [49]. These symptoms and urodynamics changes are quite remarkable and hence warranted further explanation.

1.6.1 Effects of BTX-A on bladder afferent pathways

Experimental research has provided evidence of sensory involvement via modulation of sensory neuropeptides, neurotransmitters, and receptors. For instance, BTX-A inhibited substance P release in cultured rat dorsal root ganglion (DRG) cells [51]. In another animal model, BTX-A blocked the release of glutamate and affected the release of substance P [52]. Furthermore, BTX-A reduced the release of ATP, a neurotransmitter involved in generation of detrusor overactivity, in rats' bladder [53]. ATP receptor, is almost exclusively expressed in sensory neurons, and plays a role in pain sensation. During inflammation, P2X3 is activated by ATP to generate stronger painful sensation [54]. Moreover, TRPV1 receptor (transient receptor potential channel, vanilloid family
member 1) is thought to mediate painful sensation from the bladder. BTX-A administration blocked TRPV1 receptor which reduced nociception and hyperalgesia [55].

1.6.2 Effects of BTX-A on CGRP

CGRP, a neuropeptide, is present in sensory axons and released in response to painful stimulation and acts as an inflammatory mediator. CGRP also plays an important role in the pathogenesis of bladder overactivity [56]. An increase in CGRP concentrations in the bladders of women with overactive bladder was also reported. Furthermore, BTX-A is believed to reduce CGRP release at afferent nerve terminals. This hypothesis is supported by data from animal models of bladder pain in which BTX-A significantly reduced pain responses and inhibited CGRP release from afferent nerve terminals [57-58].

1.6.3 Effects of BTX-A on NGF

NGF has been involved in the pathogenesis of bladder overactivity. In particular, increased NGF in the bladder was reported to produce bladder overactivity. Intravesical BTX-A injection, however, lowered NGF content in the bladder tissue of patients with neurogenic detrusor overactivity [11]. Further evidence came from recent studies that found that Intravesical BTX-A injection decreased urinary NGF levels in patients with interstitial cystitis/bladder pain syndrome [59], and detrusor overactivity [60].
Figure 1.4 Botulinum toxin-A blocks release of ACh at nerve terminals
1.7 Project rationale

Spinal cord injury (SCI) is a significant cause for morbidity and mortality in developed nations. With improvements in medical and nursing protocols, SCI patients are expected to live longer and they demand a better quality of life. Autonomic dysfunction, including bladder dysfunction and autonomic dysreflexia are very common following cervical and high thoracic SCI and associated with severe complications such as renal failure, seizures, and death. Current management protocols for bladder dysfunction with SCI are either ineffective or associated with severe adverse effects. BTX-A has been successfully used recently in SCI patients to inhibit bladder contractility via inhibiting acetylcholine release at nerve terminals. However, recent reports suggest a sensory role to explain BTX-A mechanism of action. It’s still however unknown, if BTX-A administration in the bladder has the ability to block autonomic dysreflexia.

Therefore, the objectives of this thesis were:

1) To develop an animal model to assess both detrusor overactivity and autonomic dysreflexia

Several experiments have used the rat as an animal model to examine the pathologic and pharmacologic features associated with SCI. In autonomic dysreflexia, particularly, colon and bladder distension were used to trigger the dysreflexia response. Although these models provided valuable knowledge, they did not reflect clinical situation as they used standard volumes and pressures to dilate the bladder. In our model, we distended the bladder via cystometry to trigger the dysreflexia response which provides a consistent and clinically relevant assessment tool.

2) To assess the significance of BTX-A intravesical administration in blocking autonomic dysreflexia response

   a) To determine the effects of BTX-A intravesical administration on blood pressure and heart rate in conscious rats.

   b) To investigate whether CGRP and NGF is associated with BTX-A control of autonomic dysreflexia and bladder dysfunction following SCI.
Chapter 2

2 Materials and Methods

Female Sprague-Dawley rats (n = 44) with body weight of 200-250g were used in this study. These rats were stratified into 3 groups with (12-16) rats per group: Sham group, spinal cord injury (SCI) only group, spinal cord injury with BTX-A treatment group. Each group was further subdivided into two subgroups: a) Autonomic dysreflexia assessment via CMG (6-8 rats per group), b) CGRP/NGF assessment via RIA/ELISA (6-8 rats per group) (Table 2.1). Animals were maintained for 3 weeks after SCI surgery (Figure 2.1).

Table 2.1

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>AD via CMG</th>
<th>CGRP/NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-control</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>SCT</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SCT+BTX-A</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1 Number of animals per group
2.1 Spinal cord injury procedure

2.1.1 Rationale

Complete spinal transection in the suprasacral segments results in bladder overactivity. Also, lesions above T6 spinal cord segment produce autonomic dyreflexia.

2.1.2 Methodology

All protocols for these experiments were approved by the University Health Network, University of Toronto, Animal Care Committee in accordance with the policies established in the Guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. Spinal cord transection was performed in spinal cord injury only group and spinal cord injury with BTX-A treatment group [61]. In the sham control group, however, only laminectomy was performed with intact spinal cord. Under general anesthesia using a combination of xylazine (5mg/kg) and ketamine (50 mg/kg), a midline incision was made through the skin overlying the upper part of the thoracic part of the spinal column and the 4th intervertebral space was identified using
anatomical landmarks. After performing a limited laminectomy to the 4\textsuperscript{th} and 5\textsuperscript{th} vertebrae to expose the spinal cord, using a sharp micro-scissor, a complete transaction of the spinal cord was performed under direct visual control then aided by an operating stereomicroscope (Spencer, American Optical Company, NY, USA). To ensure a complete transection of the spinal cord, the tip of 16-G needle was passed several times around the inner surface of the exposed vertebra. (Figure 2.2; 2.3)
Figure 2.2 SCT procedure

The rat’s body temperature was monitored and maintained during and after the procedure using a heating pad controlled by an electronic rectal body core thermometer. Supplementary subcutaneous lactated Ringer’s solution (20 mL/kg body weight) was administered during transection and in the first 2 days afterward, and perioperative antibiotics in the form of Clavamox (amoxixillin/clavulanic acid) were added to water for 5 days postoperatively. The rats were kept in low height cages for easy access to food and water in a 24-25 C warm room. The rats’ bladders were evacuated by manual expression following the spinal cord transection 3 times per day.
Figure 2.3 SCT procedure (a) laminectomy of T4-5 vertebrae, (b) complete transection of the spinal cord at T4 segment (black arrow)
2.1.2.1 The care of rats’ urinary bladder following SCI

Urinary tract infection is a common complication after SCI; therefore bladder evacuation was extended to cover the atonic phase of the urinary bladder which usually lasts up to 3 weeks after SCI. In addition, any change in the rats’ behaviour or urine color warranted a more careful physical examination of the animal and performing Culture and Sensitivity testing. Once the urinary tract infection was suspected, a broad spectrum antibiotic (Clavamox) was started, and manual evacuation of the bladder was resumed. In severe cases however, IV hydration, and intermittent catheterization was necessary to control the urinary tract infection.

2.2 Intravesical instillation of Botulinum toxin-A (BTX-A)

2.2.1 Rationale

Intravesical instillation of BTX-A suppresses detrusor contractility in overactive bladder, and is proposed to block autonomic dysreflexia following spinal cord injury.

2.2.2 Methodology

Under general anesthesia using a combination of xylazine (5mg/kg) and ketamine (50 mg/kg), a PE-50 tubing (Clay-Adams, Parsippany, New Jersey) was inserted into the bladder through the urethra. The bladder was emptied of urine and slowly filled with BTX-A (1 ml, 20 U/ml in saline) (Allergan, Irvine, California) and left indwelling for 30 minutes. Rats were allowed to recover from anaesthesia and freely accessed food and water. 48 hours later, these rats either had suprapubic bladder catheter implantation for CMG (autonomic dysreflexia assessment group) or sacrificed for DRG retrieval (RIA/ELISA group). We chose 48 hours as an optimal point to measure BTX-A’s effect, given prior experiments demonstrating the onset of BTX-A effect on rat bladder tissue [62-63]. Furthermore, BTX-A administration was conducted in a biological safety cabinet according to safety precautions guidelines set by the Toronto Western Research Institute.
2.3   Suprapubic Catheter Implantation and autonomic dysreflexia assessment during Cystometrogram (CMG)

2.3.1   Rationale

The study of bladder function during the filling CMG is a standard method to record changes in the bladder pressure in conscious rats. Furthermore, bladder distension induced by CMG produces autonomic dysreflexia in rats.

2.3.2   Methodology

While under general anaesthesia using a combination of xylazine (5mg/kg) and ketamine (50 mg/kg), a silicon tube was implanted into the bladder via laparotomy. The procedure of implantation was based on the method described previously [61]. Briefly, the peritoneal cavity was opened through a midline incision at the lower abdomen. The bladder was exposed and a small opening (2 mm) was made at the bladder dome. Then a sterile silicon tube (OD 1.65mm) was inserted into the bladder. The tube was tunnelled subcutaneously to emerge from the skin of the back. Rats were allowed to recover from anaesthesia and had to be conscious and freely moving before conducting CMG (Figure 2.4).

Rats were then moved to a restrainer for a baseline blood pressure and heart rate assessment. The restrainer also has a tail cuff (7/16 inches) (IITC Life Science®, CA, USA) with sensors attached that have the ability to detect rats' blood pressure via the tail photoelectrically. The optimal ambient temperature for blood pressure reading was set at 30° C; therefore the restrained rats were placed in a warming chamber set at 30° C. IITC Life Science®, rats' blood pressure monitoring system was used in this study. This system includes automated sphygmomanometer, an amplifier, scanner, computer interface, and a computer with IITC software to analyze the records.
Figure 2.4

**Figure 2.4 Suprapubic tube implantation for CMG**

Rats were allowed to acclimatize to their new environment until for at least 30 minutes, and were judged to be ready by the absence of visible motion and slowed breathing patterns. Blood pressure measurements were initiated through the computer interface and recorded with the help of IITC BpMon software. Briefly, the software inflated a tail sensing cuff to 150 mmHg and pulse was detected with a built-in photoelectric sensor. To ensure that the blood pressure was stable and consistent, 3 reading were taken and the average values were calculated.

Autonomic dysreflexia assessment was induced by bladder distension via CMG. In CMG, bladder was filled with sterile saline at a rate of 0.2ml/min using an infusion pump.
(Model 2620, Harvard Apparatus). At least four micturition cycles were monitored in each rat. Bladder pressures were recorded by Grass Polygraph (Model 7D). Immediately before maximum voiding pressure (which could be predicted after few micturition cycles), blood pressure measurements were initiated and recorded. Again, 3 blood pressure and heart rate measurements were taken and the average values were calculated (Figure 2.5).

Figure 2.5

Figure 2.5 IITC Life Science blood pressure monitoring system
2.4 CGRP Radioimmunoassay Quantification

2.4.1 Rationale

The expression of neuropeptides such as CGRP in the dorsal root ganglia (DRG) correlates linearly with the activity of the C-afferent sensory fibers.

2.4.2 Methodology

Following euthanasia, the vertebral column was removed and laminectomy to all vertebrae to expose the spinal cord was performed. Dorsal root ganglia (DRG) from T4, L5 and L6 segments were quickly retrieved on a dissection tray using ultrafine forceps and an operating stereomicroscope (Spencer, American Optical Company, NY, USA). DRGs were immediately flash frozen in liquid nitrogen and stored at -80°C (Figure 2.6).

Figure 2.6
Figure 2.6 Dorsal root ganglia retrieval (a) vertebral laminectomy, (b) T4 DRG (black arrow)
CGRP extraction was conducted based on a previous report [64]. Peptides were extracted from DRGs with an acid buffer containing 2M acetic acid, 11.4mM HCl, 1mM disodium EDTA, 1mM dithiothreitol, and 4% protease inhibitor cocktail (Sigma) to prevent degradation. Tissues were boiled in 500μL extraction buffer for 15min, sonicated on ice for 20s and centrifuged at 3900g in 4°C for 15min. The supernatant was collected in a fresh Eppendorf tube and total protein content was determined with a BCA assay (Pierce) for standardisation. Remaining supernatant was stored at -80°C. Prior to the assay, samples were lyophilized for 24h and reconstituted with different volumes of RIA buffer (Phoenix Pharmaceuticals) to achieve identical total protein content in each sample.

Equal volumes of standardised samples were transferred to 13x75mm tubes in which the remainder of the radioimmunoassay procedure was carried out. A commercial rat CGRP radioimmunoassay kit (Phoenix Pharmaceuticals, Mountain View, CA) was used. Primary antibodies were added to each sample and the contents were vortexed and incubated at 4°C for 24h. Tracer solution containing 125I-labelled CGRP was added to each sample and contents were vortexed and incubated 4°C for 24h. Secondary antibody was added to each sample and contents were incubated at room temperature for 90min. Samples were centrifuged at 1700g in 4°C for 20min. Supernatant was aspirated and a gamma counter was used to assess radioactivity of the pellet.

All samples were assayed in duplicate at two different dilutions; the standard peptides are assayed in triplicate. Least squares regression analysis was used to construct the standard curves and to calculate the peptide content of each assay sample. Calculations were performed according to Phoenix Pharmaceuticals and with the help of a standard curve, sample CGRP concentrations were derived (Figure 2.7).
2.5 NGF Immunoassay

2.5.1 Rationale

The expression of NGF responds to numerous stimuli and is markedly upregulated in tissues after spinal cord injury. NGF contributes to the pathogenesis of bladder overactivity and autonomic dysreflexia.
2.5.2 Methodology

NGF extraction was based on a previous report [65]. Peptides were extracted from DRGs with a lysis buffer containing 137mM NaCl, 20mM Tris HCl pH 8.0, 1% NP40, 10% glycerol and 4% protease inhibitor cocktail (Sigma) to prevent degradation. Tissues were placed in 500 µL lysis buffer, sonicated on ice for 20s and centrifuged at 1500g in 4 °C for 20min. The supernatant was collected in fresh Eppendorf tubes and total protein content was determined with a BCA assay (Pierce) for standardisation. Unequal volumes of samples were diluted to 1:5 in DPBS to achieve identical total protein content in each sample. Equal volumes of standardised samples were transferred to 13x75mm tubes in which the remainder of the ELISA procedure was carried out. A commercial rat NGF ELISA kit (Promega Madison, WI) was used.

Samples were acid-treated to increase the amount of detectable NGF and stored at -20 °C. Nunc MaxiSorp 96-well ELISA plates were coated with pAb in carbonate coating buffer and incubated at 4 °C for 24h. The plate was washed with TBST wash buffer, the wells were blocked with Block & Sample 1x Buffer (Promega, Madison, WI) and the plate was incubated at room temperature for 1h. The plate was washed, mAbs were added to the wells and the plate was incubated at 4 °C for 24h. The plate was washed, anti-rat IgG HRP conjugate was added to the wells and the plate was incubated at room temperature for 2.5h with shaking (220rpm). The plate was washed, TMB One solution was added and the plate incubated at room temperature for 10min with shaking (220rpm). The reaction was stopped with 1N HCl and absorbance was read on a plate reader at 450nm. Using a standard curve, sample NGF concentrations were derived. The sensitivity was 5 pg/well, and the intraassay and interassay variations were 5.5% and 6.5%, respectively (Figure 2.8).
2.6 Bladder histology

2.6.1 Rationale

Following SCI, neurogenic inflammation occurs due to axon release inflammatory mediators, such as substance P, neurokinin A and CGRP, into peripheral tissues, which exert direct vasoactive effects (vasodilatation and increased vasopermeability) with consequent tissue oedema.
2.6.2 Methodology

Bladders were removed, rinsed in Krebs’ solution and cut transversely at the mid-point of the bladder body. Tissue was mounted in cryostat embedding medium, frozen in liquid nitrogen, and then stored in -80°C. Bladders were sectioned using a cryostat with a dry ice cooled blade at a cabinet temperature of -20°C. Transverse sections (7µ) of bladder body were stained with Ehrlich’s haematoxylin and eosin, and images captured using a light microscope with a digital camera.

2.7 Statistical analysis

Independent t-test and one-way / single-factor analysis of variance (ANOVA) were used to analyze the data. Significant ANOVA values were subsequently subjected to post-hoc comparisons of individual means using the Bonferroni method. During multiple comparisons, alpha values were corrected by Bonferroni correction method using SPSS 17.0. P values less than 0.05 were considered significant.
Chapter 3

3 Results

3.1 SCI procedure

Complete transection (SCT) of the spinal cord at T4 segment resulted in total flaccid paralysis of the lower limbs accompanied by bladder areflexia. We also observed that rats started to regain bladder contractility at approximately 10 days following surgery as evidenced by smaller evacuated volumes of urine during regular daily bladder squeezing. Furthermore, a small improvement in locomotor function was noted by day 14 in most rats as they were able to move their hips and knees’ joints. We, however, did not appreciate any weight bearing ability in rats during the 3 weeks study period. Mortality rate ranged from 0% to 20% among study groups (Control group: no mortality, SCT group: 20%, SCT+BTX-A: 5%). Main reasons for death were shock following spinal cord transection, and acute renal failure triggered by dehydration and urinary tract infection.

3.2 Pressor and heart rate responses caused by bladder distension/CMG in rats after SCT

3.2.1 CMG results

Visual inspection of the bladder during the surgical procedure to insert the transvesical catheter revealed that all female SCT rats had distended and hypertrophied bladders (Figure 3.1). We were not able, however, to distinguish gross morphological differences between SCT rats with and without BTX-A treatment. During CMGs, the sham control group had a smooth filling phase, with no detrusor activity. (Figure 3.2) In contrast, CMGs of the SCT rats showed a filling phase with uninhibited contractions (8.0±0.7/ micturation cycle) that reached 31.24± 4.7 cm H2O. Furthermore, BTX-A treatment significantly reduced the number of uninhibited contractions (3.0±0.4/ micturation cycle) (P = 0.0001) that reached 24.8±6.3 cm H2O. These pressure waves, termed uninhibited contractions, were not accompanied by release of fluid from the urethra and were defined as intravesical pressure waves (greater than 8 cmH2O) before voiding.
Figure 3.1 Bladder hypertrophy 3 weeks after SCT
Figure 3.2 Cystometrogram of (a) normal rat, (b) SCT rat, (c) SCT+BTX-A rat
Large amplitude voiding contractions occurred in all study groups; however, the voiding parameters were markedly different. The resting pressure, lowest pressure during the micturation cycle, was significantly higher in the SCT group (8.25±0.85 cm H2O) than the control group (2.5±0.28 cm H2O) (P=0.04). However, we found no significant difference in resting pressure between SCI rats with and without BTX-A treatment. Furthermore, the maximum voiding pressure was significantly higher in the SCT group (54.25±2.1 cm H2O) than the control group (33.3±2.4 cm H2O) (P = 0.0001); whereas, BTX-A treatment group was significantly lower (41.00±1.2 cm H2O) than the SCT group (54.25±2.1 cm H2O) (P = 0.003). (Table 3.1)

**Table 3.1**

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>Resting Pressure (cmH2O)</th>
<th>Max. Voiding Pressure (cmH2O)</th>
<th>No. of uninhibited contractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>2.5±0.28</td>
<td>33.3±2.4</td>
<td>0</td>
</tr>
<tr>
<td>SCT (8)</td>
<td>8.25±0.85</td>
<td>54.25±2.1</td>
<td>8.0±0.7</td>
</tr>
<tr>
<td>SCT+BTX-A (8)</td>
<td>8.75±1.7</td>
<td>41.00±1.2*</td>
<td>3.0±0.4**</td>
</tr>
</tbody>
</table>

*P=0.003 **P=0.0001

Table 2 Cystometrogram data from control, SCT, SCT+BTX-A groups

### 3.2.2 Blood pressure/heart rate monitoring

Three weeks following SCT procedure, mean arterial pressure in the SCT (90.66±1.4 mmHg), and SCT+BTX-A (93.25±6.7 mmHg) groups were not different from the sham control group (94.25±5.0 mmHg). In contrast, heart rate in the SCT group (520.0±27.1 mmHg) was significantly higher than the sham control group (417.75±11.5 bpm) (P = 0.006), while the heart rate in the BTX-A treatment group was not significantly different from the sham control group.
In the sham control rats, bladder distension via CMG caused a non-significant change in mean arterial pressure and heart rate (Figure 3.3). In contrast, bladder distension 3 weeks after SCT increased the mean arterial pressure by (35.67±5.13 mmHg) (P = 0.001); (Figure 3.4) whereas, in the BTX-A treatment group the mean arterial pressure was only increased by (14.5±2.0 mmHg) (P = 0.011) (Figure 3.5). Moreover, bladder distension 3 weeks after SCT decreased the heart rate by (104±47.88 bpm) (P = 0.01) while, in the BTX-A treatment group the heart rate was only decreased by (28.75±9.1 bpm) (P = 0.024).

Table 3.2

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>Baseline</th>
<th>CMG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAP (mm/Hg)</td>
<td>HR (bpm)</td>
</tr>
<tr>
<td>Control (6)</td>
<td>94.25±5.0</td>
<td>417.75±11.5</td>
</tr>
<tr>
<td>SCT (8)</td>
<td>90.66±1.4</td>
<td>520.0±27.1</td>
</tr>
<tr>
<td>SCT+BTX-A (8)</td>
<td>93.25±6.7</td>
<td>458.25±11.98</td>
</tr>
</tbody>
</table>

*P=0.001 **P=0.01

Table 3 MAP and HR data at baseline and CMG from control, SCT, SCT+BTX-A groups.
Figure 3.3 Arterial pressure and heart rate response to bladder distension via CMG in control rat (a) baseline, (b) during CMG
Figure 3.4 Arterial pressure and heart rate in SCT rats (a) baseline, (b) during CMG
Figure 3.5 Arterial pressure and heart rate in SCT after BTX-A treatments (a) baseline, (b) during CMG
Figure 3.6 AP(a) and HR(b) change in response to CMG

Figure 3.6 AP(a) and HR(b) change in response to CMG
3.3 CGRP levels in DRG neurons

Three weeks after SCT, CGRP concentration was significantly higher in T4-DRG (59.00± 5.29 fmol/ml) than the sham control group (31.0± 2.64 fmol/ml) (P = 0.001); whereas, BTX-A treatment in rats with SCT has significantly reduced CGRP concentration in T4-DRG (21.0±1.0 fmol/ml) than SCT study group (P = 0.001). Furthermore, BTX-A significantly lowered CGRP concentration in L5-DRG (24.33 ± 10.78 fmol/ml) than SCT study group (50.0 ± 7.9 fmol/ml) (P = 0.03). In addition, CGRP levels in L6-DRG did not significantly vary among study groups.

Figure 3.7

Figure 3.7 CGRP concentration in T4 segment dorsal root ganglia

*P=0.0001, **P=0.0001
Figure 3.8

![Graph showing L5-CGRP concentration in Sham control, SCT, and SCT + BTX-A groups with error bars. *P=0.03,]

Figure 3.8 CGRP concentration in L5 segment dorsal root ganglia

3.4 NGF levels in DRG neurons

Three weeks after SCT, NGF concentration was significantly higher in T4-DRG (557.66± 79.54 pg/ml) than the sham control group (105.50± 33.21 pg/ml) (P = 0.002); whereas, BTX-A treatment in rats with SCT has significantly reduced NGF concentration in T4-DRG (152.66±63.28 pg/ml) than SCT study group (P = 0.006). In
L5-DRG, SCT significantly increased NGF concentration (496.00± 77.96 pg/ml) than the sham control group (98.00± 29.22 pg/ml) (P = 0.002). BTX-A treatment in rats with SCT has significantly reduced NGF concentration in L5-DRG (257.33±33.23 pg/ml) than SCT study group (P = 0.048).

**Figure 3.9**

*Figure 3.9 Sandwish ELISA to assess NGF levels*
Figure 3.10 NGF concentration in T4 (a) and L5 (b) segments dorsal root ganglia
In addition, NGF levels in L6-DRG was significantly higher after SCT (357.25± 69.07 pg/ml) than the sham control group (124.25± 33.78 pg/ml) (P = 0.031); BTX-A treatment, however, showed a non-significant reduction of NGF levels in L6-DRG. Bladder NGF level was significantly higher after SCT (610.33± 143.20 pg/ml) than the sham control group (11.86± 1.97pg/ml) (P = 0.01); whereas, BTX-A treatment in rats with SCT has significantly reduced NGF concentration in the bladders (136.00±58.66 pg/ml) than SCT study group (P = 0.028).

**Figure 3.11**

![Figure 3.11 NGF concentration in L6 segments dorsal root ganglia](image-url)
3.5 Bladder morphology assessment

Three weeks following SCI, bladders were grossly distended compared to sham the control group. Few samples from each group were collected for basic morphological assessment. The thicknesses of the lamina propria of the SCT and BTX-A treatment groups did not significantly vary from those of sham control rats. However, there was also an increase in the thickness of the urothelium, and the bladder muscle layers.
There were also signs of inflammation in bladder sections of SCT and BTX-A treatment groups including hemorrhage, edema and leukocyte infiltration.

**Figure 3.13**
Figure 3.13 Bladder morphological assessment using H&E staining, (a) control, (b) SCT, (c) SCT + BTX-A
Chapter 4

4 Discussion

Spinal cord injury (SCI) remains a significant cause for morbidity and mortality in North America. As medical protocols have significantly improved, patients are expected to live longer, and hence quality of life became very important for SCI patients [66]. In a recent quality of life (QoL) survey involving 347 quadriplegic and 334 paraplegic participants, the highest priorities were given to bladder / bowel function, sexual function, restoration of normal sensation and elimination of chronic pain [67]. Bladder dysfunction, in particular, warrants special attention because it can lead to renal impairment and autonomic dysreflexia [26].

4.1 Development of animal model

Most experimental studies of spinal cord injury have centered on using the rat as an experimental model. Rats have several advantages over larger animals because they provide inexpensive and reliable method to characterize complex clinical problems. For instance, patients with SCI above T5 had pressor response and bradycardia following bladder distension; similar results were observed in rats with SCI suggesting that rats are appropriate experimental model for the human condition [23, 68]. Furthermore, in our laboratory, we characterized the lower urinary tract dysfunction following spinal cord transection at T8 in rats. We found that the signs of uncoordinated ineffective voiding after spinal cord transection in the rat resemble the clinical situation observed in patients following spinal cord injury [61].

To assess autonomic dysreflexia and detrusor overactivity, we developed a complete spinal cord transection at T4 segment animal model. We chose T4 segment for the complete transection given previous published clinical and experimental data reporting higher incidence of AD in lesions at T6 segment or above [6, 23, 69]; also, dysreflexia occurs because injury at T6 or above levels leaves the sympathetic control of the extensive abdominal circulation amenable to unrestrained spinal reflexes [19]. Furthermore, lesions rostral to spinal micturation centre (such as T4) are also known to produce neurogenic bladder signs including detrusor overactivity and DSD [4]. This
animal model was used not only to assess physiologic changes following SCI, but also to examine the changes in neurotropic factors that are known to accompany neural remodelling at the cellular level. Hence, this animal model could improve our understanding of the pathologic and pharmacologic features of neurogenic bladder dysfunction, and assess important clinical problems that could provide translational benefits to patients with neurogenic bladders and autonomic dysreflexia.

As previously mentioned, bladder distension is the most common cause to trigger autonomic dysreflexia symptoms [21]. Bladder distension usually occurs during urodynamics assessment which is an integral part of SCI management protocols [70]. Therefore, autonomic dysreflexia produced via bladder distension provides a consistent and clinically relevant assessment tool. In this study, we measured the changes in blood pressure and heart rate in T4-SCT rats during urodynamics. We also correlated these measurements to a standard urodynamics parameter (Maximum Voiding Pressure). Several experiments used standard bladder dilatation using a bladder or colon balloon [6, 23]. As bladder volumes vary among rats within the same study group, choosing distension value via predetermined bladder balloon volume doesn’t provide a consistent distension within the same study group [23]. In contrast, our method of dysreflexia assessment provides a consistent and clinically relevant assessment tool.

Animal studies have showed that on the first day after cord transection, the increase in arterial pressure (AP), in response to visceral stimulation, can be as great as 41 mmHg, but within the next few days, these responses become smaller, perhaps associated with degenerative changes in the spinal sympathetic preganglionic neurons caudal to the injury [23]. Then, in the ensuing weeks, the increases in AP during dysreflexia become larger, reaching values as high as 52 mmHg by 5 weeks after SCI. The large response on the first day likely reflects the full capacity of the spinal reflex, without descending inhibitory restraint [71], and before the secondary effects of cord injury has impacted greatly. After this time, the magnitude of the hypertension appears to become a function of many changes ongoing in the spinal cord and vasculature, some degenerative and others, plastic responses to the injury. These studies have also suggested that NGF is a culprit in the development of autonomic dysreflexia [6, 8]. In this study, we chose to assess AD and bladder dysfunction after 3 weeks of SCT because NGF levels in spinal
cord within a few segments of a cord injury site have been reported to increase to a peak at 1 week post injury, remaining increased for 4 weeks [72]. Hence, blocking NGF at 2-3 weeks provides the highest possibility of blocking the dysreflexia response.

4.2 Intravesical catheter implantation

The measurement of intravesical pressure by a catheter inserted through the dome of the bladder is a standard method. Several disadvantages are associated with this method include direct manipulation and potential irritation of the bladder wall and also a possible limitation of bladder movement during filling. Studies have found that one to three days after implantation of the catheter, the bladder is apparently irritated, however, during the first day after catheter implantation; this irritative change in bladder activity is not evident [73-74]. In this study, we inserted the catheter only few hours before the cystometrogram assessment to minimize the possibility of bladder irritation.

4.3 Dosage and intravesial instillation of BTX-A

Intravesical BTX-A instillation has been recently used in rats to inhibit bladder overactivity via its effects on afferent pathway. In one study, 20 units were given via intravesical instillation for 30 minutes in a cyclophosphamide induced cystitis animal model of chronic bladder inflammation [63]. Results of this study showed that intravesical BTX-A inhibits the afferent neural response without impairing efferent bladder function. Another study used 25 units BTX-A for one hour and showed similar effectiveness [57]. In a SCI-animal model, a study examined the effects of BTX-A with and without protamine sulphate (an agent used to enhance permeability of BTX-A delivery to the suburothelial tissues). In addition, they examined the effects of BTX-A on normal rats. Results of this study demonstrated that no significant changes in bladder contraction frequency in normal rats treated with BTX-A after protamine sulfate instillation or with BTX-A alone. In addition, authors concluded that protamine sulfate is not needed in SCI rats to achieve BTX-A penetration and efficacy [62].
4.4 Cystometrogram (CMG) and autonomic dysreflexia assessment

We conducted CMG in conscious rats which eliminated the effects of anaesthesia and markedly reduced its effect on voiding function in the SCI rats. Furthermore, measurements of blood pressure and heart rate were conducted using a non-invasive blood pressure monitoring system (IITC Life Science®, CA, USA) in conscious rats. According to the manufacturer, the photoelectric method of non-invasive blood pressure monitoring is very accurate and uses low temperature which reduces the stress of animals. This system is also the only system that has been validated to telemetry and direct blood pressure. Furthermore, rats were allowed to acclimate to the restrainers for at least one hour before testing to minimize stress during blood pressure measurements; hence improving the validity and reproducibility of the measurements.

One concern is whether BTX-A intravesical instillation has an effect on normal animals. Previous experiments have demonstrated that BTX-A is most effective in conditions of increased nerve activity [62], which supports the finding, that BTX-A is effective in SCI, but not normal in animals. In support of previous studies, our data showed that intravesical BTX-A inhibits bladder activity via sensory mechanisms. In this study, uninhibited contractions were noted as been reported in other experiments on conscious rats [75]. We also found that BTX-A administration has reduced the frequency of these contractions which supports previous published reports [57, 63]. Furthermore, in the BTX-A treatment group, maximum voiding pressure was significantly lower than the SCT group suggesting that local application of BTX-A has impaired afferent nerve-induced bladder hyperactivity.

In the spinal cord intact rats, we found that bladder distension via CMG did not produce any significant change in arterial pressure or heart rate. Whereas, in spinal cord injury rats, CMGs conducted 3 weeks after SCT caused a significant increase in arterial pressure accompanied with bradycardia. Recent data suggested that reorganization of the spinal pathways controlling sympathetic preganglionic neurons after the loss of supraspinal input to be the likely cause of the exaggerated sympathetic reflexes [76]. Our animal model, designed to mimic a common clinical manifestation of bladder
distension via CMG, also showed that when BTX-A was given intravesically the pressor
effects were significantly lowered in rats which was also associated with a decrease of
CGRP and NGF levels in dorsal root ganglia. These data support the concept that
afferent sprouting in the spinal cord dorsal horn is associated with the time-dependent
increase in hypertensive responses to sensory stimulation, and NGF plays an important
role in the primary afferent pathways sprouting.

4.5 Role of CGRP in the pathogenesis of detrusor hyperreflexia
and autonomic dysreflexia

CGRP, a neuropeptide, is present exclusively in the neuronal bodies of the C-afferent
fibres and also known to be inhibited by the neurotoxin capsaicin [77]. Hence, CGRP
content of neuronal bodies including the dorsal root ganglia can reflect the activity of the
C-afferent fibres. Furthermore, several studies have showed that the arbours of small-
diameter primary C-afferent neurons can enlarge greatly in rats and mice after SCI
potentially leading to increased reflex excitation of preganglionic neurons, via
interneuronal pathways. These afferent neurons are CGRP-immunoreactive, and
believed to mediate the spinal reflex pathways to the sympathetic preganglionic
neurons, and hence play a significant role in the pathogenesis of autonomic dysreflexia
[6, 40]. We demonstrated that intravesical BTX-A has reduced CGRP content in the
dorsal root ganglia at T4 and L5 spinal segments which may therefore, explain the
modulatory effect of BTX-A on the C-afferent fibres supplying the urinary bladder.

Experimental studies have shown that BTX-A blocks the release of CGRP from afferent
nerve terminals [57, 78]. However, the exact mechanism of BTX-A-mediated inhibition
of CGRP release is still unknown. Previous studies have also shown that BTX-A-
induced inhibition of sensory neurotransmitter release from bladder DRG neurons is
associated with a concentration-dependent cleavage of synaptosome-associated
protein (SNAP)-25 [79]. This suggests that the sensory-specific effect of BTX-A might
occur through inhibition of sensory neuropeptide vesicle release via a SNAP-mediated
mechanism. Furthermore, BTX-A mediated inhibition of CGRP receptor expression
remains possible, however, there is no available data regarding the effects of BTX-A on
CGRP receptor on sensory nerve endings and urothelial cells.
4.6 Role of NGF in the pathogenesis of detrusor hyperreflexia and autonomic dysreflexia

Nerve growth factor (NGF) is a growth factor known for regulation of sensory and sympathetic neuron growth. NGF concentrations are also very low in the normal spinal cord and increase at least 4-fold within a week after SCI. Furthermore, exogenous NGF causes trophic changes on sensory neurons after SCI suggesting that NGF plays a major role in the pathogenesis of autonomic dysreflexia. In the bladder, published reports found that NGF content is increased and contributes to the pathogenesis of bladder overactivity [59, 80-81]. In this study, we demonstrated that intravesial BTX-A treatment has reduced NGF content in the bladder, T4 and L5 dorsal root ganglia segments of SCI rats. Our findings also supports the clinical data that intravesical BTX-A injection lowers the NGF content in the bladder tissue and urine of patients with bladder overactivity [11, 60].

In one study, NGF content in dorsal root ganglia above and below the site of spinal cord injury was examined and the results confirmed that NGF levels in the injured rat spinal cord were significantly greater below the injury than rostral to the injury (T1 and 2) [7, 82]. This data supports our findings that the increase in NGF content at T4 segment (below the injury) is caused by the spinal cord injury, and the increased NGF content in dorsal root ganglia is directly involved in the secondary events following SCI. Another study identified the cells that produced NGF following SCI [7], and demonstrated that a variety of cells within the injured spinal cord including astrocytes and Schwann cells can produce NGF. These studies showed a strong relationship between NGF and changes in the afferent arbour that could contribute to autonomic dysreflexia. Further, intrathecal anti-NGF reducing autonomic dysreflexia in rats [8] demonstrated that drugs with anti-NGF properties such as BTX-A might block dysreflexia following SCI. In fact, our data shows that BTX-A when given intravesically has the ability to block dysreflexia pressor effects possibly through reducing NGF content in the bladder and dorsal root ganglia.
4.7 Role of BTX-A in the pathogenesis of detrusor hyperreflexia and autonomic dysreflexia

Recent reports have found that increased NGF in the spinal cord after spinal cord injury is responsible for inducing hyperexcitability of C-fibre bladder afferent pathways [83-85], and that intrathecal application of NGF antibodies, which neutralized NGF in the spinal cord, suppressed detrusor overactivity and detrusor-sphincter-dyssynergia in spinal cord injured rats [44]. In addition, intravesical BTX-A injection lowered NGF content in the bladder tissue of patients with neurogenic detrusor overactivity [11, 60]. Together, these reports support our findings that BTX-A suppressed detrusor overactivity, lowered voiding pressure and blocked autonomic dyreflexia response during bladder distension via CMG in SCI rats could be mediated by BTX-A action on NGF.

Following SCI, increased NGF content in the bladder, dorsal root ganglia, and spinal cord have been reported [42, 84]. During development, NGF is released by the target tissue, taken up in responsive neurons by receptor-mediated endocytosis and transported retrogradely to the cell body where it exerts its trophic/differentiative effects [41], whereas, intrathecal administration of NGF at the L6-S1 level of spinal cord for 1 or 2 weeks caused bladder hyperactivity and hyperexcitability of bladder afferent neurons [86].

It has been proposed that detrusor-sphincter-dyssynergia (DSD) is the initial insult following SCI [27]. DSD, then, leads to bladder outlet obstruction and subsequently bladder hypertrophy. Bladder smooth muscle and urothelium in turn produce NGF; hence the increased NGF levels in bladder [27]. We believe that the increased NGF in the bladder is transported retrogradely to the dorsal root ganglia and the spinal cord. In the spinal cord, NGF is transported to the injury site via spinal interneuronal pathways. At the site of injury, NGF level is the highest because in addition to the NGF retrograde transport from visceral end organs, NGF is produced from local cells including oligodendrocytes, and Schwann cells [7].

In detrusor overactivity, intravesical BTX-A injection is believed to inhibit vesicular release of excitatory neurotransmitters and on the axonal expression of other SNARE-complex-dependent proteins in the urothelium/suburothelium [87]. In particular, BTX-A
inhibits the vesicular release of ACh, ATP, and SP and of the axonal expression of TRPV1 from the urothelium [88] and suburothelial nerve endings [79, 89]. Together, these findings support bladder afferent neuromodulation by BTX-A. The urothelial release of ACh and ATP on bladder filling has been also found to increase following spinal cord injury; thus, by inhibiting urothelial ACh release, BTX-A may block its proposed excitatory effect on suburothelial afferent and detrusor parasympathetic nerve endings [90]. Similarly, inhibition of the increased urothelial ATP release would reduce its proposed excitatory effect on suburothelial and urothelial P2X3 receptors on the myofibroblast network [91].

The urothelium is currently perceived as a sensing structure with signaling properties including neurotropins (trkA and p75) and a number of TRP channels (TRPV1) [92-97]. As ATP potentiates TRPV1 activity, a decreased release of ATP after BTX-A injection would also minimize potentiation of the TRPV1 receptor [53]. Furthermore, NGF has been shown to activate TRPV1 on small afferent nerves, which can promote the vesicular release of excitatory neuropeptides [98]. Thus, a reduction of bladder NGF could also lead to afferent pathway desensitization. A recent study, of biopsies from a mixed population of neurogenic and idiopathic bladder overactivity patients treated with intradetrusor BTX-A, also showed no change in suburothelial neuronal population during clinical response but it demonstrated progressive decrease of P2X3 and TRPV1 suburothelial nerve immunoreactivity, suggesting that BTX-A affects sensory receptors’ expression in suburothelial fibres [57]. Very recently, another study using over expression transgenic mice demonstrated that overexpression of NGF leads to urinary bladder enlargement characterized by marked nerve fibre hyperplasia in the submucosa and detrusor smooth muscle. They also found marked increase in the density of CGRP- and substance P-positive C-fibre sensory afferents, neurofilament 200-positive myelinated sensory afferents, and tyrosine hydroxylase-positive sympathetic nerve fibres in the suburothelial nerve plexus [97] (Figure 4.1).
In our study, BTX-A (without protamine sulphate) was given via intravesical instillation with minimal possibility of penetration to suburothelial tissues. Our findings therefore support a previous report which found that BTX-A was able to impair the bladder contraction frequency without being delivered to suburothelial locations [62]. Our certainty of BTX-A minimal urothelial penetration could be challenged because of the possibility of increased bladder urothelial permeability after SCI. However, a recent report demonstrated that urothelial transmembrane resistance is restored within 14 days of SCI have led us to believe that most of the therapeutic effects of intravesically applied BTX-A are secondary to a direct inhibition of urothelial function [99].
4.8 Limitations

There are several limitations associated with the animal studies that need to be taken into consideration. For instance, rats have a higher heart rate (400-500 beats/min.) compared to approximately (70 beats/min) in humans. Also, normal rats exhibit different micturation process such as the bursting external urethral sphincter activity during voiding as opposed to the inhibition of EUS activity that occurs in humans during voiding [100]. This, however, does not diminish the usefulness of rat models for studying the pathologic and pharmacologic features of neurogenic bladder dysfunction, because the neurotransmitter mechanisms involved in abnormal reflex activity may be similar in both species [101]. Furthermore, patients with SCI above T5 had pressor response and bradycardia following bladder distension; similar results were observed in rats with SCI suggesting that rats are appropriate experimental model for the human condition [6, 23].
Chapter 5

5 Conclusions

Spinal cord injury (SCI) remains a significant cause for morbidity and mortality in North America. As medical protocols have significantly improved, patients are expected to live longer, and demand a better quality of life. Bladder dysfunction following SCI is very common and significantly affects patients’ quality of life; also, it can also lead to severe complications such as renal failure and autonomic dysreflexia. Intravesical BTX-A treatment has recently shown remarkable results in controlling bladder dysfunction symptoms. These symptoms and urodynamics changes were quite remarkable and hence warranted further explanation. Recent evidence has suggested a sensory involvement via modulation of sensory neuropeptides, neurotransmitters, and receptors. It is still, however, unclear whether BTX-A can affect putative spinal neurons involved in detrusor overactivity and autonomic dysreflexia.

To test this hypothesis, we developed a clinically relevant, complete spinal cord transection at T4 segment rat model. We triggered the dysreflexia responses via bladder distension during cystometrogram which provides a consistent and clinically relevant assessment tool. We showed that intravesical BTX-A significantly improved cystometrogram parameters including maximum vesical pressure and number of uninhibited contractions following SCI. The CMG findings were also associated with significant reduction in the pressor response following bladder distension via cystometrogram.

To understand how intravesical BTX-A blocks autonomic dysreflexia, we measured CGRP and NGF content in the bladder and dorsal root ganglia. We showed that BTX-A has reduced CGRP content in T4, L5 spinal segment dorsal root ganglia. This support previous reports, that demonstrated a modulatory effect of BTX-A on the C-afferent fibres supplying the urinary bladder. Furthermore, we showed that intravesical BTX-A has lowered NGF content in the bladder tissue, and T4, L5 dorsal root ganglia. Again, this support previous reports, that linked BTX-A actions to lowered NGF levels in the neurogenic bladders.
We, therefore, propose a novel role for intravesical BTX-A treatment in the control of autonomic dysreflexia. As increased NGF proved to be a culprit for development of the dysreflexia response, agents that could lower NGF is thought to block this response. Our results showed an increased NGF content in the bladder, T4, and L5 dorsal root ganglia after SCI. Given that NGF is known to transport retrogradely to the dorsal root ganglia and the spinal cord up to the site of injury. We propose that following SCI, loss of coordination between the bladder and its sphincter (DSD) has lead to bladder hypertrophy because of outlet obstruction. This caused an increase in the NGF level in the bladder. NGF then transported retrogradely to the dorsal root ganglia and the spinal cord to the site of injury contributing to the development of autonomic dysreflexia. Furthermore, we showed that after BTX-A treatment, bladder, T4, and L5 dorsal root ganglia NGF content was reduced which provides evidence that BTX-A treatment at the bladder level has reduced NGF content at the site of injury.
Finally, the findings of this study shed a light on potential benefits of intravesical BTX-A treatment in patients with SCI, and also provide a novel mechanism for the control of autonomic dysreflexia via a minimally invasive treatment modality.
5.1 Future Directions

Based on our findings, BTX-A blocks autonomic dysreflexia following SCI. Further lines of investigation are required, however, to assess this novel role of BTX-A.

1. *Can intravesical BTX-A administration prevent primary afferent sprouting in the dorsal horn of the thoracic cord?*

Previous experiments have shown that concurrent with the development of autonomic dysreflexia in rats [45], afferent fibres that are immunoreactive for calcitonin gene-related peptide (CGRP-IR) increase their central terminal arbours in the dorsal horn of the thoracolumbar cord. Although very little NGF is present in the spinal cord under normal conditions, NGF protein levels near and within a cord injury site rise to a peak at 1 week after injury, and remain increased for up to 4 weeks [72]. Moreover, the presence of NGF in the spinal cord has caused sprouting of CGRP-immuno-reactive fibres. Based on our current findings, it is important to understand whether the reduced level of NGF that we showed at the dorsal root ganglia and was caused by BTX_A treatment is also associated with a concomitant prevention of sprouting of CGRP-immuno-reactive fibres.

2. *Determining the optimal time for BTX-A administration.*

In this study, we chose to assess autonomic dysreflexia and bladder dysfunction after 3 weeks of SCT because NGF levels in spinal cord within a few segments of a cord injury site have been reported to increase to a peak at 1 week post injury, remaining increased for 4 weeks [72]. Hence, blocking NGF at 2-3 weeks provides the highest possibility of blocking the dysreflexia response. It is important however to investigate the effects of BTX-A administration soon after the spinal cord injury and conduct the autonomic dysreflexia assessment 3 weeks after the SCT. This will enable us to compare these effects to the results of the current study, and understand whether BTX-A could have long-term modulatory effects.

3. *Understanding the mechanisms of BTX-A action on the urothelium and the bladder smooth muscle.*
In this study, we showed that BTX-A lowers NGF levels in the bladder. Previous reports demonstrated that bladder smooth muscle and urothelium produce NGF causing the increased NGF levels in bladder [27]. It has been also suggested that bladder hypertrophy following SCI plays a major role in the increased NGF production of the bladder [27]. Whether, BTX-A intravesical administration could affect bladder hypertrophy is still unknown. Also, investigating the molecular determinants that could play a role in the mechanism of BTX-A ability to inhibit bladder hypertrophy seems equally important.
References


