Above and Below: Changes in Conduit Artery after Spinal Cord Injury, 

Autonomic Dysreflexia, and Passive Exercise

by

Mei Mu Zi (Annie) Zheng

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Abstract

Spinal cord injury (SCI) is a devastating condition that not only results in motor and sensory loss, but also autonomic dysfunctions. Individuals with SCI experience a 3-4 fold increased risk of cardiovascular disease (CVD), the leading cause of mortality in this population. Endothelial dysfunction is among the earliest markers of CVD progression. This thesis aims to: 1) clarify previous reports showing a counterintuitive improvement in endothelial function after SCI, 2) examine the effect of autonomic dysreflexia (AD) on conduit vasculature, and 3) assess the efficacy of passive exercise (PE) to reverse vascular dysfunction. In uninjured controls (CON), T3-complete spinal cord transected Wistar rats (SCI), T3-transected with induced AD by colorectal distension (SCI+CRD), and T3-transected with PE (SCI+PE), we assessed endothelium-dependent vasodilation and specific mechanisms for relaxation in brachial (BA) and femoral artery (FA) using wire-myography. Sympathetic innervation, mechanotransducer expression [transient receptor potential channel V4 (TRPV4)], arterial morphology, and profibrotic markers were assessed using immunohistochemistry. Impaired reactivity to acetylcholine was seen in FA after SCI via decreased contribution of endothelium dependent hyperpolarizing factor (EDHF) mediated pathways, while BA showed preserved endothelial function. Moreover, FA in SCI exhibited inward remodelling, 37.7% less sympathetic nerve fiber density, and increased collagen I expression (53.0%). Chronic repetitive AD resulted in a shift in vasodilatory mechanisms away from nitric oxide and towards EDHF, hypersensitivity to phenylephrine, and reduced elastin expression (13.9%). Passive hind-limb exercise after SCI led to improved sensitivity of FA to acetylcholine, through an increase in TRPV4 and prostacyclin-mediated pathways for vasodilation. Outward remodelling, as well as decreased expression of transforming growth factor beta (47.7%) and collagen I (39.0%) was seen in FA after PE. We
have shown, for the first time, the expected endothelial dysfunction in the inactive/supraspinally disrupted FA after SCI and that chronic repetitive AD resulted in exacerbation of vascular dysfunction caudal to injury. Furthermore, PE was effective in reversing endothelial dysfunction and provided atheroprotective benefit, indicating PE may be a viable therapeutic intervention for preventing CVD after SCI. The observed changes provide insight into the mechanisms of endothelial dysfunction and possible directions on improvement of vascular health after SCI.
Preface

This thesis is based on work conducted in the Autonomic Laboratory at the International Collaboration on Repair Discoveries (ICORD), in collaboration with Dr. I. Laher from the Department of Anesthesiology, Pharmacology and Therapeutics, at the University of British Columbia. All experiments were approved by the University of British Columbia’s Animal Care Committee under the certificate A14-0152.

I assisted Drs. Aaron Phillips and Barbara Frias with spinal cord transection surgeries. I performed animal care and experimental treatments in chapters 1-3, with help from Mr. Rayshad Gopaul and Ms. Mengyao Jia. Wire myography was performed by Dr. Saeid Golbidi in Dr. Laher’s laboratory. I performed all tissue harvesting and processing for downstream applications. Immunohistochemistry was carried out with help from volunteers (Sophia Shen, Alyssa Thurston, Yuchen Bo, and Katherine Lam). I performed all data analyses and composed this thesis, with edits by Dr. Aaron Phillips.

A version of chapter 1 and 3 has been combined and is in process of being submitted for publication in the Journal of Clinical Investigations. A version of chapter 2 will also be prepared for submission. Authors of the manuscripts are: Mei Mu Zi Zheng, Aaron A. Phillips, Saeid Golbidi, Ismail Laher, and Andrei V. Krassioukov. I played a large role in the experimental design, data collection, data analyses, and composition of the manuscript. In addition to manuscripts directly related (two in preparation) to my thesis, I have completed and published five manuscripts in total.
Table of Contents

Abstract.......................................................................................................................... ii
Preface............................................................................................................................ iv
Table of Contents .......................................................................................................... v
List of Tables ................................................................................................................ ix
List of Figures ................................................................................................................ x
List of Abbreviations ..................................................................................................... xii
Acknowledgements ....................................................................................................... xiv
Dedication ...................................................................................................................... xvi

Chapter 1: Impaired endothelial function in rat femoral artery and preserved brachial artery after spinal cord injury ......................................................................................... 1

1.1 Introduction ............................................................................................................... 1

1.2 Methods ................................................................................................................... 4

1.2.1 Experimental design ........................................................................................... 4

1.2.2 Spinal cord transection surgery and animal care ................................................... 4

1.2.3 *In vitro* wire myography ..................................................................................... 6

1.2.4 Perfusion ............................................................................................................... 8

1.2.5 Tissue processing and immunofluorescence ......................................................... 8

1.2.6 Quantification ....................................................................................................... 9

1.2.7 Immunohistochemical morphometric assessment of the BA and FA ................. 10

1.2.8 Tyrosine hydroxylase (TH) analysis of whole mounts ....................................... 10

1.2.9 Analysis of TH-innervation in whole-mounts ......................................................... 11
1.2.10 Statistical analyses ........................................................................................................ 11

1.3 Results .................................................................................................................................. 11

1.3.1 Impaired endothelial function in femoral artery and preserved endothelial function
in brachial artery after SCI ........................................................................................................ 11

1.3.2 Decrease in contribution of EDHF-mediated vasodilation in the femoral artery . 15

1.3.3 Inward eutrophic remodelling of femoral artery after SCI ........................................... 17

1.3.4 Structural changes after SCI .......................................................................................... 18

1.3.5 Decreased sympathetic fiber density in femoral artery and increased sympathetic
fiber density in brachial artery .............................................................................................. 19

1.4 Discussion .......................................................................................................................... 20

1.4.1 Endothelial dysfunction and inward remodelling of the femoral artery after SCI 20

1.4.2 Sympathetic disruption and profibrosis of the femoral artery after SCI ................. 23

1.4.3 Preserved endothelial function of the brachial artery after SCI .............................. 25

1.4.4 Increased sympathetic innervation and unremarkable arterial morphological
changes in the brachial artery after SCI ................................................................................. 25

1.4.5 Clinical implications ...................................................................................................... 26

1.5 Conclusion .......................................................................................................................... 27

Chapter 2: Chronic autonomic dysreflexia results in maladaptive changes in femoral artery
after spinal cord injury .............................................................................................................. 28

2.1 Introduction ......................................................................................................................... 28

2.2 Methods .................................................................................................................................. 31

2.2.1 Experimental design ........................................................................................................ 31

2.2.2 Repetitive colorectal distension ..................................................................................... 31
2.3 Results................................................................................................................................. 32

2.3.1 No change in absolute relaxation but shift in contribution from NO- to EDHF-mediated vasodilation .................................................................................................................. 32

2.3.2 Femoral artery hyperreactivity and hypersensitivity to phenylephrine after SCI with chronic autonomic dysreflexia ............................................................................................................. 35

2.3.3 Structural changes with chronically induced AD .................................................................. 36

2.4 Discussion ............................................................................................................................. 38

2.5 Conclusion ............................................................................................................................ 40

Chapter 3: Passive exercise improves endothelial function and arterial structure in femoral artery after spinal cord injury .................................................................................................................. 42

3.1 Introduction .......................................................................................................................... 42

3.2 Methods .................................................................................................................................. 44

3.2.1 Experimental design ............................................................................................................. 44

3.2.2 Passive hind-limb cycling ..................................................................................................... 45

3.2.3 Endothelial TRPV4 staining and analysis ............................................................................ 45

3.3 Results .................................................................................................................................... 46

3.3.1 Endothelial dysfunction in femoral artery after SCI is reversed with passive exercise ................................................................................................................................. 46

3.3.2 Outward remodelling of femoral artery after passive exercise .............................................. 48

3.3.3 Changes in vascular protein expression after passive exercise ............................................ 49

3.3.4 Shift in relative contribution between prostacyclin and EDHF vasodilation ............... 51

3.4 Discussion ................................................................................................................................ 53

3.4.1 Passive exercise reverses endothelial dysfunction after SCI ................................................ 53
3.4.2 Passive exercise prevents inward remodelling and induces anti-fibrotic structural changes in femoral artery ................................................................. 56

3.4.3 Clinical implications ..................................................................................... 57

3.5 Conclusion ........................................................................................................ 57

Concluding remarks ................................................................................................ 58

Bibliography ........................................................................................................... 60
List of Tables

Table 1-1. Absolute contribution of each vasodilator to total relaxation in FA after SCI.......... 15
Table 2-1. Contribution of each vasodilator in BA and FA relaxation with chronic AD.......... 33
Table 3-1. Contribution of each vasodilator to total relaxation in FA with PE......................... 51
List of Figures

Figure 1-1. Impaired vasodilation of the FA after SCI .......................................................... 13
Figure 1-2. Preserved vascular reactivity of the BA after SCI. ........................................... 14
Figure 1-3. Less contribution by EDHF in FA dilation after SCI. ...................................... 15
Figure 1-4. Less contribution by EDHF-mediated relaxation in FA after SCI ................. 16
Figure 1-5. SCI resulted in inward eutrophic remodelling of the FA ................................ 17
Figure 1-6. Markers of profibrosis in conduit arteries after SCI ....................................... 18
Figure 1-7. Sympathetic fiber density after SCI ................................................................. 19
Figure 1-8. SCI resulted in a decrease in the TRPV4-EDHF pathway for vasodilation. ...... 22
Figure 2-1. Recording of blood pressure during CRD bouts ............................................. 30
Figure 2-2. Hyper vasoconstrictive response in FA after chronic autonomic dysreflexia (AD). . 32
Figure 2-3. Less contribution of NO in FA of with chronic AD. ....................................... 33
Figure 2-4. Shift in relative contribution from NO to EDHF in FA after chronic AD .......... 34
Figure 2-5. No change in vascular reactivity of the BA after chronic AD ....................... 34
Figure 2-6. No change in sympathetic fiber density after chronic autonomic dysreflexia .. 35
Figure 2-7. Profibrotic markers in BA and FA after chronic AD .................................... 36
Figure 2-8. Morphology of BA did not change with chronic autonomic dysreflexia ............ 37
Figure 2-9. No change in morphology of FA with chronic autonomic dysreflexia .......... 37
Figure 2-10. Shift in relative contribution to dilation towards the TRPV4-EDHF pathway, and away from eNOS with chronic AD .................................................................................. 39
Figure 3-1. Improved vascular reactivity of the FA after PE ................................................. 47
Figure 3-2. Passive exercise resulted in outward remodelling of the FA ......................... 48
Figure 3-3. Less expression of profibrotic markers in FA after PE ................................... 49
Figure 3-4. Greater endothelial TRPV4 expression in FA after PE. ................................. 50
Figure 3-5. Greater contribution by prostacyclin in FA with passive exercise. ......................... 51
Figure 3-6. Greater contribution of prostacyclin in FA after SCI with passive exercise............ 52
Figure 3-7. Greater contribution on TRPV4- prostacyclin pathway with passive exercise......... 55
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>autonomic dysreflexia</td>
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<tr>
<td>BA</td>
<td>brachial artery</td>
</tr>
<tr>
<td>Col I</td>
<td>collagen I</td>
</tr>
<tr>
<td>Col III</td>
<td>collagen III</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CRD</td>
<td>colorectal distension</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of agonist to give half-maximal response</td>
</tr>
<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal response</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FA</td>
<td>femoral artery</td>
</tr>
<tr>
<td>FMD</td>
<td>flow-mediated dilation</td>
</tr>
<tr>
<td>IEL</td>
<td>internal elastic lamina</td>
</tr>
<tr>
<td>Indo</td>
<td>indomethacin</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<tr>
<td>L-NAME</td>
<td>N-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS containing Triton X-100 (PBST)</td>
</tr>
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<td>passive exercise</td>
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<tr>
<td>PF</td>
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<tr>
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<td>physiological salt solution</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>SCI</td>
<td>spinal cord injury</td>
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<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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<td>transforming growth factor beta</td>
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<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPV4</td>
<td>transient receptor potential cation channel, subfamily V, member 4</td>
</tr>
</tbody>
</table>
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Special thanks are owed to my parents, who have always loved and supported me unconditionally. Thank you for believing in me when sometimes I even doubted myself.
Dedication

To my grandparents.
Chapter 1: Impaired endothelial function in rat femoral artery and preserved brachial artery after spinal cord injury

1.1 Introduction

Spinal cord injury (SCI) is a devastating condition that not only results in motor and sensory loss, but also an array of autonomic impairments [1]. Individuals with chronic SCI suffer from a number of secondary complications including cardiovascular dysfunction. In fact, those with SCI experience a 3-4 fold increased risk of cardiovascular disease (CVD) compared to able-bodied individuals [2]. Moreover, CVD is the leading cause of death in the SCI population [3,4]. It is well established that endothelial dysfunction is one of the first identifiable steps in the pernicious decline of arterial health and CVD progression [5,6]. Endothelial function in the brachial artery can also be used to provide surrogate information of arterial health in primary end organs such as the cerebrovasculature and coronary arteries [7,8]. Because endothelial dysfunction is present well before overt clinical manifestations of vascular disease [8], examining endothelial function in the SCI population may provide early insight into the high propensity of CVD, as well as help identify a potential therapeutic target.

The vascular endothelium is a single layer of cells lining the vasculature that has a number of functions essential for vascular growth, vasoregulation, and vasoprotection. More specifically, the endothelial layer is responsible for inhibition of smooth muscle cell growth, suppression of inflammatory responses, modulation of vascular permeability, as well as vasodilation [9]. Endothelial cells can convey the vascular effects of various chemical (e.g. acetylcholine and bradykinin) and physical stimuli (e.g. shear stress) through the release of three vasoactive mediators: nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived...
hyperpolarizing factors (EDHF) [10]. Dysfunction in the endothelium can be associated with impairment of any of the three pathways [11,12], and potentially a shift in the relative contribution of three pathways [13,14].

Endothelial function can be assessed in a number of ways [6]. Flow-mediated dilation (FMD), a non-invasive measure of endothelial function that was developed in 1992, has been widely used in endothelial function measurement of various populations [15]. Large peripheral conduit arteries such as the brachial and femoral artery are commonly used to assess FMD in humans. FMD has been widely adopted to measure endothelial response in various pathological populations as it is easy to administer, non-invasive, and can be measured at various different time points [16].

However, findings on endothelial function in the SCI population have been contentious. Endothelial function of the superficial femoral artery after SCI can be preserved [17,18], or even improved [19]. Such findings are unexpected considering individuals with chronic SCI have immobile lower limbs, and are likely to experience significant alteration in the structure and function of the leg vasculature. Aside from the apparent loss of motor and sensory functions below the level of spinal cord lesion, there is also a disruption of the centralized control of vasculature by the sympathetic nervous system, which is responsible for blood pressure regulation [20]. A likely reason for why endothelial function is reported to be improved or preserved after SCI most probably lies in the mathematics underlying the calculation of FMD. Specifically, the percent dilation in response to increased shear is dependent on the baseline diameter as the denominator in the equation for calculation of FMD:

\[
\%\text{FMD} = \frac{(\text{Diameter}_{\text{Peak}} - \text{Diameter}_{\text{Base}})}{\text{Diameter}_{\text{Base}}} \times 100
\] (1)
As such, the rapid inward remodelling in the immobile lower limbs after SCI, where arterial diameter can reduce by as much as 50%, is likely undermining the accurate determination of endothelial function in this population [17,19,21,22]. Although commonly used in various healthy and pathological populations, FMD may not be appropriate for the SCI population, where arteries in the chronically inactive limbs undergo inward remodelling and have disrupted sympathetic control [21,23]. Clearly, an accurate evaluation of endothelial function after SCI that is independent of morphometrics would be of significant clinical importance, and would provide critical insight into the cardiovascular health and the elevated CVD risk in this population [24–26]. In addition to changes in endothelial function and arterial morphometrics, there are a number of maladaptations after SCI, including profibrotic remodelling of the heart and cerebrovasculature [26,27]. The changes in composition of the peripheral conduit artery are yet to be elucidated.

The objective of this study is to use a preclinical model to examine the effect of SCI on conduit artery endothelial function, both above and below the level of spinal cord lesion. We also aim to examine the changes in arterial composition and sympathetic innervation after SCI. We hypothesize that conduit arteries below of level of injury will have endothelial dysfunction, a shift in the relative contribution of vasodilators, profibrotic remodelling, and a decrease in sympathetic nerve fiber density.
1.2 Methods

1.2.1 Experimental design

In this study, we used an animal model of SCI and *in vitro* myography to assess the endothelial function of clinically relevant conduit arteries above and below the level of spinal cord lesion. Experiments were conducted on twelve male Wistar rats (250-350g; Harlan Laboratories, Indianapolis, IN, USA). Animals were assigned to either the uninjured control group (CON; n=6), or the T3 complete spinal cord transection group (SCI; n=6). Results from the SCI group in this chapter are used in chapters 2 and 3. Six weeks after SCI, the brachial (BA) and femoral arteries (FA) were assessed for endothelial function and vasoconstrictive responsiveness using wire myography. This time frame is analogous to what is considered “chronic” SCI in the clinical SCI population [1]. In addition, vascular composition, innervation, and protein expression were examined using immunohistochemistry. All experimental procedures were carried out with accordance to the Guide to the Care and Use of Experimental Animals established by the Canadian Council on Animal Care and approved by the University of British Columbia Animal Care Committee.

1.2.2 Spinal cord transection surgery and animal care

Surgery and animal care were conducted according to standard procedures in our laboratory [24,27]. Enrofloxacin (Baytril; 10 mg/kg, s.c., Associated Veterinary Purchasing [AVP], Langley, British Columbia, Canada) was administered for 3 days before surgery. Enhanced caloric provision (i.e., Ensure, fruit, spinach, cereal) was provided to animals 5 days prior to surgery.

On the day of the surgery, sedation of adult male Wistar rats (250–300 g; Charles River Laboratories, Inc., Wilmington, MA, USA) was carried out with inhalant isoflurane (AErrane;
5%, AVP), delivered in 100% oxygen at a flow rate of 1 L/min. General anesthesia was induced with ketamine hydrochloride (Vetalar; 70 mg/kg, i.p., University of McGill Animal Resources Centre, Montreal, Canada) and medetomidine hydrochloride (Domitor; 0.5 mg/kg, i.p., AVP). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (Temgesic; 0.02 mg/kg, s.c., University of McGill Animal Resources Centre), and ketoprofen (Anafen; 5 mg/kg, s.c., AVP) were administered preoperatively, and the skin at the surgical site was shaved, and treated with three cycles of chlorhexidine gluconate (Hibitane), iodine, and 70% ethanol washes.

To expose the spinal cord, a midline incision was made through the skin and superficial muscles as well as blunt dissection of the muscles covering the C8-T3 vertebrae. The dura at the T2–T3 intervertebral gap was carefully opened with microscissors and the spinal cord was completely transected with surgical scissors. The separation and retraction of both cut ends of the spinal cord was observed under the surgical microscope by two surgeons to verify for completeness of the transection. Absorption sponges and Gelfoam (Pharmacia & Upjohn Company, Pfizer, New York, USA) were used to achieve hemostasis. The muscle and skin of the surgical site were closed with 4–0 Vicryl and 4–0 Prolene sutures, respectively.

Animals received warmed lactated Ringer’s solution (5 mL, s.c.) and were positioned in a temperature-controlled environment (Animal Intensive Care Unit, HotSpot for Birds, Los Angeles, CA, USA) for recovery. Anesthesia was reversed with atipamezole hydrochloride (Antisedan; 1 mg/kg, s.c., Novartis, Mississauga, Ontario, Canada). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (0.02 mg/kg, s.c.), and ketoprofen (5 mg/kg, s.c.) were administered once per day for 3 days post-surgery.

Animals with SCI required specialized cages, with rubber matting to facilitate movement, low-reaching water bottles, and food scattered on the cage bottom to encourage foraging.
Animals were supported with an enriched diet, including meal replacement shake (Ensure, Abbott Laboratories, Abbott Park, Illinois, USA), fruit, spinach, cereal, commercially available rat treats and kibble (LabDiet, Rodent Diet 5001; PMI International, St. Louis, MO, USA). The bladder was manually emptied 3 times a day until spontaneous bladder function returned (about 10 days after injury). The health condition of the animals was monitored and recorded daily for the first 2 weeks after surgery and every 2 days thereafter. Objective criteria were used to assess body mass, activity level, social behavior, healing at the surgery site, and clinical signs of morbidity. All assessments and analyses were completed blinded of each condition/group.

1.2.3 In vitro wire myography

In vitro myography was used to examine vasoconstriction with phenylephrine (PHE), an α-adrenoceptor agonist and vasodilation with acetylcholine (ACh), an endothelium-dependent dilator [28,29]. Brachial and femoral arteries were isolated from deeply anesthetized control and SCI animals. The isolated arteries were cut into 2-mm segments, mounted isometrically onto a four-channel wire myograph (JP Trading, Aarhus, Denmark), and immersed in physiological salt solution (PSS) gassed with 95% O₂ and 5% CO₂. Two arterial segments were prepared for each artery, such that two replicates were studied in parallel for the vascular responses of each animal. However, an average value (n=1) was taken of the two segments was used for purposes of data analysis.

Arterial segments were equilibrated for one hour at the beginning of each test. During this time, the resting tension was gradually increased to an optimal value [24]. Tissues were maintained at this level of preload for 20 minutes. After equilibration, 80 mM of potassium chloride (KCl) was used to constrict arterial segments to ensure viability of the samples and this constriction was used as a standard to facilitate normalization of developed force later on in the
procedure. PSS washes were performed to restore the basal tension. Subsequently, PHE was added in a cumulative manner (10⁻⁹ M–10⁻⁵ M) to generate a concentration-response curve. Raw force was normalized to contractile force induced by KCl, and maximum contraction (E_max) and half-maximal effective concentration of PHE (logEC₅₀) were determined for tissues from each animal and each experimental group.

To examine endothelium-dependent vasodilation, arterial segments were treated with 3 x 10⁻⁶ M of PHE to first establish a stable contraction and then exposed to cumulative additions of ACh (10⁻⁹ M–10⁻⁵ M). Vasodilator responses were expressed as percent relaxation of PHE-induced contraction, and E_max and logEC₅₀ values of ACh-concentration curves were determined for each animal from both experimental groups. Data were obtained for PHE- and ACh-induced responses of the BA and FA from the uninjured CON (n=6) and SCI group (n=6). Endothelium-independent vasodilation was analyzed with cumulative additions of sodium nitroprusside (SNP, 10⁻⁹ M–10⁻⁵ M) after being preconstricted with 3 x 10⁻⁶ M of PHE.

To determine the relative contribution of NO and PGI₂, nitric oxide synthase blocker [N-nitro-L-arginine methyl ester (L-NAME), 200 uM], and non-selective cyclooxygenase blocker [indomethacin (Indo), 10 uM] were utilized, respectively. One segment from each artery was pretreated with L-NAME and another one with Indo for 30 minutes. The ACh dose-response curve protocol was then repeated to examine the contribution of each blockade to total vasodilation. The contribution (％) of NO was shown by the difference in area under the curve between the ACh-response curves in the absence and presence of L-NAME. The contribution (％) of PGI₂ was shown by the difference in area between the ACh-response curves in the absence and presence of Indo. The remaining area under the curve represented the contribution of EDHF in ACh-mediated vasodilation.
1.2.4  Perfusion

After removal of the required conduit arteries for wire myography, the animal was perfused transcardially with phosphate-buffered saline buffer (PBS) followed by 4% paraformaldehyde (PF). After the rat has reached the surgical plane of anesthesia (i.e., toe-pin punch response was absent), an incision was made in the midline immediately inferior to the rib cage using large scissors. The thoracic cavity was opened using sharp scissors. The rib cage and surrounding connective tissue was cut open to expose the heart. A needle was inserted into the left ventricle and upwards into the aorta and clamped. PBS was pumped at 80-90 mmHg to clear the blood in the vascular system. A small incision was immediately made in the right atrium to ensure free flow of the solution. Once the PBS solution flowing out of the animal becomes clear, around 300 mL of 4% PF was pumped into the system at 120-140 mmHg, or until the rat has become rigid. The BA and FA were excised and placed in Eppendorf tubes of 4% PF for 24 hours at 4°C. In the next three consecutive days, the tissue was transferred into 12%, 18%, and 24% sucrose solution, respectively. The tissue was stored in 24% sucrose before further processing.

1.2.5  Tissue processing and immunofluorescence

A segment of the BA and FA (5-10mm) from each animal was embedded in optimal cutting temperature (OCT) freezing medium. Using a cryostat microtome at -23°C, transverse 10 µm sections of the BAs and FAs were cut and collected on Superfrost Plus slides and stored at -80°C until further processing. At least five alternate (1 in 3) sections from each artery were collected on each slide. The morphological assessment and protein expression of the BAs and FAs were determined by immunofluorescence using the primary antibodies: rabbit α transforming growth factor beta receptor 1 (TGFβR1; 1:100, Santa Cruz, sc399), mouse α collagen I (Col I; 1:500,
Abcam, ab90395), mouse α collagen III (Col III; 1:300, Abcam, ab6310), rabbit α elastin (1:400, Millipore USA, AB2039), and rabbit α smooth muscle actin (SMA; 1:400, Abcam, ab5694).

Briefly, the slides with BA and FA sections were thoroughly washed in phosphate buffer saline (PBS) and 0.1 M PBS containing 0.3% Triton X-100 (PBST), and then incubated for 1 hour-incubation in 10% normal donkey serum in PBST. Slides were then incubated with the primary antibodies for 24h at room temperature in humidity chambers. Subsequently, sections were washed in PBST and incubated accordingly with Alexa™-fluor 488 donkey anti-mouse (1:1000; Molecular Probes©, USA) or donkey anti-rabbit conjugated Cy3 (1:1000; Jackson ImmunoResearch, USA) for 2 hours. Finally, all the sections were washed, mounted with Prolong Gold© mounting medium (Molecular Probes©, USA) and observed in an AxioImager.M2 Zeiss© microscope using Zen 2 Pro.

1.2.6 Quantification

The intensity of immunoreactivity and morphological assessment of arteries were assessed using the Fiji Software (based on ImageJ, http://fiji.sc/Downloads#Fiji). Intensity was averaged from at least 4 sections per artery for each animal. We generated a region of interest (ROI) using the adventitial border of the artery and the lumenal interface as the outer and inner boundaries, respectively [27]. Within this ROI, relative intensity was quantified. We were careful to obviate the surrounding connective tissue from within the region of interest. The area of the ROI was divided from the relative intensity to obtain the average intensity for each section and to obviate the bias introduced by arterial size. A negative control (i.e., only secondary antibody added in absence of primary antibody) was required for each protocol to ensure specificity of secondary to primary antibody [30]. For each antibody, microscope settings were kept consistent and specific.
threshold values were set in Zen 2 Pro for all of the images (using the CON group as a standard) so that comparisons could be made between animals. The specific threshold values may differ among different antibodies.

1.2.7 Immunohistochemical morphometric assessment of the BA and FA
Using the SMA staining of the FA and BA, outer diameter, intraluminal diameter, and arterial wall thickness were measured using Image J as published by our group previously [31]. For each section of an artery, four sites were selected representing each quadrant of the artery for the measure of wall thickness. A line was drawn across the span of staining perpendicular to the tangent line, and the length of this line was measured. An average of the four measurements was used to denote wall thickness for each section. Outer diameter was measured by taking the average of the widest and the narrowest portion of the artery, including the lumen and arterial wall. Similarly, intraluminal diameter was measured by taking the average of the widest and the narrowest portion of the lumen. Four sections were measured for each animal.

1.2.8 Tyrosine hydroxylase (TH) analysis of whole mounts
A small segment (~2mm) of each artery was used to determine tyrosine hydroxylase expression (TH; 1:1000; anti-rabbit; Millipore AB152, USA) by immunofluorescence using the donkey anti-rabbit conjugated Cy3 secondary antibody (1:1000; Jackson ImmunoResearch, USA). The segment was placed in a small Eppendorf tube and the immunoreaction proceeded as described above. The segment was cut on one side longitudinally and placed in a glass microscope slide with the lumen side facing upwards, mounted with Prolong Gold© mounting medium (Molecular Probes©, USA) and observed in the microscope.
1.2.9 Analysis of TH-innervation in whole-mounts

Quantification was carried out manually on Z-stack images from the BAs and FAs of each animal. A grid was superimposed over the entire imaging frame (44 um²/grid sector). From the grid, a 5x5 sector region of interest was selected (220 um²) randomly, given that the entire ROI is within the boundary of the artery. The quantification of the sympathetic nerve (TH+) fiber density was conducted within this region. A measurement was obtained for longitudinal and circumferential density by counting the number of times the sympathetic fibers crossed the horizontal and vertical lines of the ROI, respectively. A value for total innervation density (longitudinal + circumferential) then was calculated for the artery and then compared between groups [32].

1.2.10 Statistical analyses

Wire myography and immunohistochemistry data were analyzed by Student's t-test or a nonparametric alternative when the data did not fit a normal distribution model. Concentration–response curves for PHE, ACh, and SNP were analyzed by nonlinear regression (Hill equation) to calculate logEC₅₀ and Eₘₐₓ. All statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Difference between means was considered statistically significant when $p<0.05$. Data are shown as mean ± standard error.

1.3 Results

1.3.1 Impaired endothelial function in femoral artery and preserved endothelial function in brachial artery after SCI

Using in vitro myography, dose response curves to ACh and SNP were constructed for the FA. When looking at the vascular reactivity of the FA, the SCI group and the CON group did not
differ in their reactivity to KCl, which means the two groups had similar smooth muscle contractility (Figure 1-1D). SCI resulted in a greater logEC$_{50}$ value to ACh than that of the control group (p<0.0001), indicating an impairment in ACh sensitivity (Figure 1-1A). Specifically, the FA in the SCI group required 5x more ACh to reach 50% of the maximal dilation. Furthermore, using L-NAME and indomethacin we showed a decrease in contribution from EDHF-mediated pathways in FA vasodilation after SCI (Figure 1-4). The dilation curves to SNP were not different between the two groups, which indicate that the impaired sensitivity to ACh is endothelium-mediated (Figure 1-1B). Furthermore, the two groups were similar in their vasoconstrictive responses to cumulative additions of PHE, a selective $\alpha_1$-adrenergic receptor agonist and also an analogue for norepinephrine (Figure 1-1C).
Figure 1-1. Impaired vasodilation of the FA after SCI.
Dose-response curve of the FA in the control (CON; n=6) and T3-spinal cord transected rats (SCI; n=6) to cumulative additions of: A) acetylcholine (ACh); B) sodium nitroprusside (SNP); and C) phenylephrine (PHE). Endothelium-dependent vasodilation was impaired after SCI, as indicated by a greater logEC₅₀ value in the ACh-concentration curve. Vasoconstriction in response to PHE was not different between SCI and CON groups. D) Smooth muscle contractility in response to potassium chloride (KCl) was not significantly impacted by SCI. Values represent mean±standard error, unpaired t-test. *p<0.001.
Unlike the FA, no difference was observed in the logEC$_{50}$ or E$_{max}$ of the ACh dose-response curve for the BA (Figure 1-2A). Therefore, endothelium-dependent vasodilation of the conduit artery above the level of lesion (i.e., BA) is preserved. The BA of the SCI group showed hyporeactivity to PHE, a selective $\alpha_1$-adrenergic receptor agonist, as demonstrated by a 22.4% decrease in E$_{max}$ (p<0.001) compared to the CON group (Figure 1-2B). No difference was found in responses to KCl and SNP.

**Figure 1-2. Preserved vascular reactivity of the BA after SCI.**
Dose-response curve of the BA in the control (CON; n=6) and T3-spinal cord transected rats (SCI; n=6) to cumulative additions of: A) acetylcholine (ACh); and B) phenylephrine (PHE). Endothelium-dependent vasodilation was not significantly impacted by SCI. The BA from the SCI group exhibited smaller responses to PHE, indicated by smaller maximum vasoconstriction (in response to 10$^{-5}$ M PHE; E$_{max}$). Values represent mean±standard error, unpaired t-test. *p<0.001.
1.3.2 Decrease in contribution of EDHF-mediated vasodilation in the femoral artery

Blockade analysis using the dose-response curve to ACh before and after incubation of the FA with L-NAME and indomethacin (Figure 1-3) revealed a decrease in EDHF-mediated vasodilation (CON: 194.3±10.7 A.U. vs SCI: 112.4±4.6 A.U.; p<0.01) (Figure 1-4; Table 1-1). Contribution by NO and PGI2 was not significantly different between groups (Figure 1-4).

**Figure 1-3. Less contribution by EDHF in FA dilation after SCI.**
Dose-response curves to acetylcholine (ACh) with incubation of either L-NAME or Indo. The responses to increasing doses of ACh were examined in rings from femoral artery (FA) of the A) control (CON), and B) T3-transected (SCI) rats. The relaxations are expressed as a percentage of the preconstriction levels with phenylephrine. Indo, indomethacin; L-NAME, N-nitro-L-arginine methyl ester.

**Table 1-1. Absolute contribution of each vasodilator to total relaxation in FA after SCI.**

<table>
<thead>
<tr>
<th></th>
<th>NO (A.U.)</th>
<th>PGI2 (A.U.)</th>
<th>EDHF (A.U.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>119.4±4.9</td>
<td>22.7±8.4</td>
<td>194.3±10.7</td>
</tr>
<tr>
<td>SCI</td>
<td>137.7±3.8</td>
<td>13.0±3.3</td>
<td>112.4±4.6</td>
</tr>
<tr>
<td>p-value</td>
<td>0.229</td>
<td>0.59</td>
<td><strong>0.009</strong></td>
</tr>
</tbody>
</table>

Data presented as mean±standard error; FA, femoral artery; NO, nitric oxide; PGI2, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor.
Figure 1-4. Less contribution by EDHF-mediated relaxation in FA after SCI.
The absolute contributions of nitric oxide (NO), prostacyclin (PGI$_2$), and endothelium-derived hyperpolarizing factor (EDHF) in the ACh-induced relaxation for control (CON; n=4) and T3-transected animals (SCI; n=6). SCI resulted in smaller overall dilation and less contribution by EDHF, compared to CON. Values represent mean±standard error, unpaired t-test. *p<0.05.
1.3.3 Inward eutrophic remodelling of femoral artery after SCI

Using immunohistochemistry, we quantified morphometric measures of the FA (Figure 1-5) and BA. We observed a 16.8% decrease in outer diameter and 21.2% decrease in intraluminal diameter of the FA in the SCI compared to the control group (p<0.05), which is evidence for inward eutrophic remodelling. No difference existed in the wall thickness between the two groups. Moreover, outer diameter, intraluminal diameter, and wall thickness measures of the BA were not different between the SCI and CON groups.

![Morphometric analysis of smooth muscle actin immunohistochemical staining demonstrated evidence of eutrophic inward remodelling of the FA after SCI, as shown by reduced outer diameter (A), reduced intraluminal diameter (B), and unchanged wall thickness (C) in the SCI group. D) Representative images used to quantify FA morphology in both groups. *Significantly different from CON; mean±standard error, unpaired t-test, p<0.05.](image)

Figure 1-5. SCI resulted in inward eutrophic remodelling of the FA.

Morphometric analysis of smooth muscle actin immunohistochemical staining demonstrated evidence of eutrophic inward remodelling of the FA after SCI, as shown by reduced outer diameter (A), reduced intraluminal diameter (B), and unchanged wall thickness (C) in the SCI group. D) Representative images used to quantify FA morphology in both groups. *Significantly different from CON; mean±standard error, unpaired t-test, p<0.05.
1.3.4 Structural changes after SCI

The femoral artery underwent a number of structural changes after SCI (Figure 1-6B, 1-6C). Specifically, there is an increase in collagen I (53.0%), elastin (45.7%), and a significant decrease in SMA expression (31.1%). The brachial artery after SCI, on the other hand, is similar in expression of all markers of profibrosis (Figure 1-6A).

**Figure 1-6. Markers of profibrosis in conduit arteries after SCI.**

Immunofluorescence intensity of proteins in A) BA and B) FA associated with profibrosis between control (CON; n=5) and T3-transected (SCI; n=5) groups, standardized against the cross-sectional area for each artery. C) Representative images of profibrotic markers in the FA. TGFβ, transforming growth factor beta; Col I, collagen 1; Col III, collagen 3; SMA, smooth muscle actin. Values are mean±standard error. *Significance between CON and SCI, unpaired t-test, p<0.05.
1.3.5 Decreased sympathetic fiber density in femoral artery and increased sympathetic fiber density in brachial artery

SCI resulted in a 37.7% decrease in number and density of sympathetic nerve fibers below the level of injury (i.e., FA) and a 42.7% increase in sympathetic fibers above the level of injury (i.e., BA) (Figure 1-7).

**Figure 1-7. Sympathetic fiber density after SCI.**
Quantification of TH+ fiber density of the CON (n=6) and SCI (n=6) groups with representative images on the bottom panel. SCI resulted in A) more tyrosine hydroxylase (TH) positive fibers in the BA and B) less TH+ fibers in the FA. Values represent mean±standard error, unpaired t-test. *p<0.01.
1.4 Discussion

We have shown for the first time, using a well-controlled experimental model, that endothelial function of conduit artery below the level of spinal cord lesion is impaired, and that the femoral artery loses EDHF-mediated dilation. Our data further demonstrates that endothelial dysfunction is associated with profibrotic remodelling and potentially with the loss of supraspinal sympathetic support. This study provides strong and compelling evidence to refute previous clinical studies that showed counterintuitively improved endothelial function in the paralyzed and autonomically dysfunctional lower limbs after SCI. Considering the ubiquitous utility of endothelial health as a primary and highly sensitive marker of CVD progression, this study is of value both preclinically and clinically, and provides a foundation for future research aimed at understanding the causes of elevated CVD risk after SCI and the exploration of therapeutic strategies.

1.4.1 Endothelial dysfunction and inward remodelling of the femoral artery after SCI

According to our data, there is substantial decline in endothelial health after SCI in the vasculature caudal to injury (i.e., impaired sensitivity to ACh in FA). This result is in contrast to previous findings published in clinical studies over the past 15 years. These studies have surprisingly reported preserved [17,18], or even improved [19,33] endothelial function after SCI, which could be the result of two factors. First, the combination of physical inactivity, inflammation, oxidative stress, glucose intolerance, and autonomic dysfunction are leading to an unlikely improvement in endothelial function, through unknown mechanisms. Alternatively, and more likely, is the scenario where methodological considerations related to measuring endothelial function in humans are confounding clear conclusions. Specifically, endothelial function in humans is most commonly evaluated by the non-invasive FMD. Changes in %FMD
is critically dependent on the baseline diameter of the artery itself, which is drastically reduced after SCI [17–19,33]. In the present study we utilized a method of analyzing endothelial function that is independent of arterial morphometrics, and were thus able to obviate the bias introduced by reduced arterial size after SCI. Following this, our results agree with our hypothesis, and refute the majority of the literature on this topic. In fact, only one other study has reported impaired endothelial function below the level of spinal cord lesion. However, this study is not readily translatable to the clinical body of literature, as the analysis was completed on the posterior tibial artery, which is not a widely studied artery and did not undergo a reduction in cross-sectional area as is seen in the majority of sub-lesional conduit vasculature [34].

Our data showed that SCI resulted in less EDHF mediated dilation in the FA (Figure 1-8). Altered contributions of NO, PGI₂ and EDHF have been reported in a number of clinical models [35–38], and this is the first time it has been demonstrated after SCI. As hypertensive models (greater pressure) have been shown to result in greater EDHF-mediated pathways to compensate for the reduced dependency on NO [35–37], it may be that SCI (low pressure in combination with hypotrophic remodelling) results in a reduction of EDHF-mediated pathway.
Figure 1-8. SCI resulted in a decrease in the TRPV4-EDHF pathway for vasodilation.
The present study showed spinal cord injury to have resulted in less contribution of EDHF in FA vasodilation. Red dashed circle indicates significant decrease in relative contribution of SCI group (n=6), compared to CON (n=6), p<0.05. EC, endothelial cell; IEL, internal elastic lamina; SMC, smooth muscle cell; EDHF, endothelium-derived hyperpolarizing factor; PLA$_2$, phospholipase A2; AA, arachidonic acid; COX, cyclooxygenase; PGI$_2$, prostacyclin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TRPV4, transient receptor potential cation channel, subfamily V, member 4.
The vasoconstrictive response to phenylephrine in the FA was not different between the SCI and control groups, illustrating normal vascular reactivity to adrenergic stimulation in large conduit arteries caudal to the injury, where significant disruptions in supraspinal sympathetic regulation are known to occur [20,23,39]. This supports previous studies that reported similar changes in vascular resistance in response to phentolamine (a competitive antagonist of the α-adrenoceptor) infusion between the SCI and control subjects, indicative of preserved α-adrenergic tone of the leg skeletal muscle vascular bed in individuals without supraspinal sympathetic control [40]. Combined with studies on downstream vasculature, these findings suggest that the well-reported vascular hyper-sensitivity to adrenergic stimulation caudal to injury is specific to small conduit or resistance vessels [41,42]. This may be the result of differing sympathetic control, innervation, as well as the density and sensitivity of adrenergic receptors [43,44].

From a morphological perspective, high-thoracic SCI resulted in inward remodelling of the FA (i.e., reduced diameter) after chronic SCI. Similar results have been published previously, where inward remodelling of large conduit arteries in caudal to injury observed [23]. For example, diameter of the femoral artery can reduce by as much as 50% within six weeks in individuals with SCI [21,45]. The primary stimuli for inward remodelling after SCI is thought to be reduced metabolic demand in down-stream musculature, combined with profibrotic changes due to disrupted supraspinal sympathoexcitatory pathways [45,46]. Thus, profibrotic and sympathetic pathways were investigated further in the present study.

1.4.2 Sympathetic disruption and profibrosis of the femoral artery after SCI

As expected, after complete high-thoracic SCI where descending sympathoexcititory fibres were disrupted, sympathetic innervation density was drastically reduced in large conduit arteries
caudal to injury (i.e., reduced TH+ innervation density). Based on the literature, we suspect that this change in neurogenic control of vascular tone is contributing to changes in arterial structure and function. The specific effect of chronic loss of supraspinal sympathetic excitatory signals on endothelial function of conduit vasculature has yet to be elucidated, however it has been shown that chronic pharmacological sympathectomy leads to a significant decrease in aortic endothelial NO synthase (eNOS) as detected by immunocytochemistry [47], and surgical sympathectomy blunts endothelial function [48]. Although these studies provide some insight, it should be noted that SCI is a unique model and condition, and not a pure sympathectomy, as the spinal reflexive sympathetic circuits are intact, while the supraspinal sympathetic regulation is disrupted [20,39]. Currently, we have no experimental insight into the effect of chronic loss of supraspinal sympathetic control on endothelial function. In this study, we observed impaired endothelium-dependent vasodilation in response to ACh, along with decreased sympathetic innervation density (which is directly related to sympathetic activity [49]) in the FA after SCI. While it is unclear whether decreased sympathetic activation is directly linked with endothelial dysfunction, the chronically impaired supraspinal sympathetic control in this case is likely contributing to the observed endothelial dysfunction.

The FA underwent significant changes in protein expression after high-thoracic SCI. Specifically, collagen I and elastin increased, while SMA decreased in the FA. Profibrotic changes of conduit arteries, characterized by an increase in collagen, are among the earliest processes taking place during arteriosclerotic progression [50]. Vascular stiffening develops from changes in the structural and cellular elements of the vessel wall, and are influenced by hemodynamic forces, as well as extrinsic factors such as hormones [51]. Increased collagen I expression seen in the FA indicates remodelling of the FA to become stiffer and less compliant.
Studies have shown structural stiffening of the arteries can lead to impaired endothelial function, which could further exacerbate stiffening [51,52]. Moreover, the reduced trophic effects of the sympathetic nervous system, along with reduced blood flow and arterial pressure, results in remodelling of the smooth muscle cell layer of the FA, as shown by a significant reduction in SMA [53]. Together, this data illustrates the molecular underpinning of vascular changes occurring caudal to SCI, and provide a link for vascular dysfunction and increased CVD risk.

1.4.3 Preserved endothelial function of the brachial artery after SCI

In contrast to the impaired endothelial function (i.e., decreased acetylcholine sensitivity) in the FA (below the level of injury), our data showed preserved vasoconstriction and endothelial-dependent vasodilation in the BA (above the level of injury). The SCI group retained their upper body function after the surgery because the upper limbs had to support and move their entire body, having higher functional demands than before surgery. In addition to this, normal endothelial function of the BA is likely attributed to preserved shear stress [54,55]. The present findings demonstrating preserved BA endothelial function confirms previous literature [18].

1.4.4 Increased sympathetic innervation and unremarkable arterial morphological changes in the brachial artery after SCI

We found preserved endothelial function and morphology in the BA, which is a conduit artery above the level of injury (in our animals model) and with intact sympathetic control. In fact, we identified for the first time that sympathetic innervation density (i.e., TH+ fibre density) was elevated in vasculature rostral to injury, which is corroborated by previous work evaluating cardiac innervation [56]. There is also further support for this contention in our human work, where in vivo surrogate marker of sympathetic activation rostral to injury were elevated in those with mid thoracic injury in comparison to uninjured controls [57]. Together, our present work
and previous findings indicate that mid-thoracic injury may produce a state of sympathetic hyperactivity rostral to injury; the etiology of this and consequences are currently unknown [58].

1.4.5 Clinical implications

Endothelial function has been suggested to reflect an overall integrative effect of all the atherogenic and atheroprotective factors present in an individual [59], and is an independent predictor of cardiovascular events [59,60]. Assessing brachial endothelial function was shown, in numerous large research studies in able-bodied individuals, to be independently predictive of long-term adverse cardiovascular events, and in addition to traditional risk factors [61–63]. These relations held true after adjusting for the Framingham Risk Score, and endothelial functional assessments in combination with the Framingham score helped to classify cardiovascular risk better than Framingham score alone [64], indicating that endothelial function represents distinct CVD risk that is not accounted for by current standard clinical assessments. According to large meta-analyses, endothelial dysfunction has predictive value for future cardiovascular events [65,66]. Although non-invasive measures for endothelial function (e.g., FMD) are already widely implemented in clinical studies and are being considered for clinical practice [67–69], methodological standardization is crucially needed. In light of the existing clinical work using FMD that is dependent on arterial diameter, and the present data using endothelial assessments that are independent of arterial morphometrics, it is most likely that the clinical studies in this field are not accurately identifying endothelial dysfunction after SCI, which is clearly occurring caudal to injury. At minimum future clinical research on endothelial function after SCI should apply allometric scaling for resting diameter [70,71].
1.5 Conclusion

In conclusion, the major conduit artery of the legs suffers from endothelial dysfunction after high-thoracic SCI. In addition to less capacity for muscle contraction, profibrotic remodelling and loss of supraspinal sympathoexcitatory signals underlie the impairment in FA health, and provide insight into the elevated CVD risk after SCI. Furthermore, our results demonstrate an elevation of sympathetic fiber density within the vasculature above the level of SCI, which suggest chronically elevated supraspinal sympathetic excitation. The next chapters will explore if the observed endothelial dysfunction is associated with autonomic dysfunctions after SCI (i.e., autonomic dysreflexia), as well as explore potential therapeutic interventions to combat endothelial dysfunction, such as passive hind-limb exercise.
Chapter 2: Chronic autonomic dysreflexia results in maladaptive changes in femoral artery after spinal cord injury

2.1 Introduction

Spinal cord injury (SCI) results in severe autonomic dysfunctions, including deleterious changes in cardiovascular function that predispose an individual with SCI to CVD, which are reported to be as much as 3-4 fold greater than the non-SCI population [20,72]. One primary suspect for the substantially greater CVD risk is autonomic dysreflexia (AD) [1], which is a life threatening episode of transient hypertension that occurs up to 30x/day (11x/day on average) in those with cervical or high thoracic SCI [73]. The most common triggers of AD are from afferent stimuli such as a full bowel and/or bladder, or sexual arousal [20]. Unfortunately, AD occurs on a daily basis in approximately 90% of those with high-level SCI, and persists for years throughout the patient’s lifetime [74–76]. Blood pressure elevations as high as 300 mmHg have been reported to occur during AD [20,77], and seizures, strokes, and even death have been reported [78–80]. Therefore, it is not surprising that people with SCI rank autonomic issues above regaining motor function when prioritizing clinical research objectives [81].

In addition to the acute effects of AD (i.e., cerebral hemorrhage) [79], recurrent and chronic exposure to transient hypertension is likely to have detrimental effects on the vasculature [82–85]. It is well established that chronic hypertension leads to marked vascular dysfunctions, such as exaggerated vascular reactivity [86,87], endothelial dysfunction [88,89], as well as vascular remodelling and profibrosis [90,91]. Moreover, previous studies have shown that hypertension leads to a shift with regard to the pathways involved in endothelial dilation, with a decreased contribution of NO- [35,36], and an elevation of the EDHF contribution [37]. We do not
presently understand how transient hypertension, in the form of AD, influences vascular health after SCI.

Our lab has developed a model of chronic AD in rodents using repetitive colorectal distension (CRD). Although our lab has evaluated the role of AD on vascular health after SCI [24], the aim of our previous study was to evaluate the effect of chronic AD on acute AD severity, and establish if resistance vascular changes were a linking mechanism. On the other hand, the present study is focused on understanding the effect of chronic AD on cardiovascular health, by evaluating the function and structure of large conduit arteries, which have well-established association with CVD risk [92–94]. The femoral (FA) and brachial arteries (BA) are conduit vessels that are used clinically to evaluate arteriosclerotic decline and endothelial function. No study to date has evaluated the role transient hypertension due to AD plays in FA or BA health after SCI.

To examine the effect of chronic AD, the present study employed a rigorous CRD protocol. We have shown the inflation of a balloon-tipped catheter in the descending colon/rectum reliably results in transient hypertensive episodes secondary to autonomic dysfunction after high thoracic SCI transection (Figure 2-1). Repetitive CRD, performed 6x/day, five days/week for four weeks, provides a clinically relevant model of AD. Four weeks of CRD simulates the burden of AD in those with chronic SCI, and is equivalent to two human years [95]. Furthermore, the BA and FA were chosen, because of their established associations with CVD risk and progression, and to parallel the clinic evaluation of endothelial function (i.e., flow-mediated dilation [FMD]) which is most commonly performed at these vascular sites [93,94].
The objective of this study is to examine changes in vasoreactivity, endothelial function, and structure of the rat conduit arteries both above and below the level of spinal cord lesion after chronically induced AD episodes. We hypothesize that experimentally-induced AD (i.e., 1 month of CRD) will worsen the effects of SCI on vascular health.

**Figure 2-1. Recording of blood pressure during CRD bouts.**
Blood pressure recordings from telemetry devices implanted into aorta during CRD. Each bout of CRD induced on average one AD episode. BP, blood pressure; CRD, colorectal distension. Reproduced with permission from Phillips et al. 2014 (unpublished data).
2.2 Methods

2.2.1 Experimental design

Experiments were conducted on ten male Wistar rats (250-350g; Harlan Laboratories, Indianapolis, IN, USA). Animals were assigned to either the T3 complete spinal cord transection group (SCI; n=6), or the group with chronic AD (SCI+CRD; n=4). Seven weeks after SCI, the brachial (BA) and femoral arteries (FA) were assessed for vasoconstrictive responsiveness and endothelial function using wire myography. This time frame is analogous to what is considered “chronic” SCI in the clinical SCI population [1]. In addition, vascular composition, innervation, and protein expression were examined using immunohistochemistry. Detailed methods have been described previously in Chapter 1, Section 1.2. Results in the SCI group in this chapter are the same as in Chapter 1. All experimental procedures were carried out with accordance to the Guide to the Care and Use of Experimental Animals established by the Canadian Council on Animal Care and approved by the University of British Columbia Animal Care Committee.

2.2.2 Repetitive colorectal distension

After SCI, one group of animals received six 5-minute bouts of CRD daily, 5 days/week for four weeks (SCI+CRD; n=4). A pediatric silicone Foley catheter (Fr10, Coloplast, Denmark) was inserted rectally, with a 10-mm latex balloon in the inserted end distending the colon of the animals. The catheter was secured to the tail with tape. A syringe was connected to the catheter via a one-way valve, and was used to inflate the balloon with 2 mL of air over a 10-second period. The inflated balloon was maintained in the conscious, free moving animal for five minutes per bout for three bouts, and then a 15-minute break ensued, followed by another three bouts of CRD. In between each 5-minute bout, the pressure in the syringe was released to ensure proper functioning of the catheter, and then the inflating procedure was repeated.
2.3 Results

2.3.1 No change in absolute relaxation but shift in contribution from NO- to EDHF-mediated vasodilation

Although the absolute endothelial dilation and sensitivity to ACh was not affected with chronically induced AD (Figure 2-2A), blockade analysis using the dose-response curve to ACh before and after incubation of the FA with L-NAME and indomethacin (Figure 2-3C-D) demonstrated a shift from NO-mediated vasodilation (SCI: 137.7±3.8 A.U. vs SCI+CRD: 95.7±6.7 A.U.; p<0.05) to greater contribution by EDHF pathways (SCI: 112.4±4.6 A.U. vs SCI+CRD: 162.8±11.0 A.U.; p<0.05) (Table 2-1; Figure 2-4B). No difference was found in endothelium-dependent vasodilation (Figure 2-5A) or relative contribution of vasodilators in the BA (Figure 2-4A) between the two experimental groups (Table 2-1; Figure 2-4A).

![Graph A: Vasodilation of FA](image1)

![Graph B: Vasoconstriction of FA](image2)

**Figure 2-2. Hyper vasoconstrictive response in FA after chronic autonomic dysreflexia (AD).**

Dose-response curve of the FA in the T3-transected (SCI; n=6) and SCI rats with chronic autonomic dysreflexia (SCI+AD; n=4) to cumulative additions of: A) acetylcholine (ACh); and B) phenylephrine (PHE). Maximum vasoconstriction (in response to 10^{-5} M PHE; E_{max}), and half-maximal effective concentration of ACh (logEC_{50}) was greater in arteries from SCI+CRD animals. Values represent mean±standard error, unpaired t-test. *p<0.05.
Figure 2-3. Less contribution of NO in FA of with chronic AD. Dose-response curves to acetylcholine (ACh) with incubation of either L-NAME or Indo. The responses to increasing doses of ACh were examined in rings from brachial artery (BA) of the A) SCI, and B) SCI+CRD group and femoral artery (FA) of the C) SCI, and D) SCI+CRD group. The relaxations are expressed as a percentage of the precontractions levels with phenylephrine. Indo, indomethacin; L-NAME, N-nitro-L-arginine methyl ester.

Table 2-1. Contribution of each vasodilator in BA and FA relaxation with chronic AD.

|       | BA | | | FA | | |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
|       | NO (A.U.)       | PGI₂ (A.U.)     | EDHF (A.U.)     | NO (A.U.)       | PGI₂ (A.U.)     | EDHF (A.U.)     |
| SCI   | 137.6±7.0       | 30.6±4.3        | 62.6±5.8        | 137.7±3.8       | 13.0±3.3        | 112.4±4.6       |
| SCI+CRD | 141.4±3.6      | 29.5±9.2        | 56.8±9.4        | 95.7±6.7        | 10.5±3.9        | 162.8±11.0      |
| p-value | 0.867           | 0.958           | 0.808           | **0.029**       | 0.837           | **0.027**       |

Data presented as mean±SEM; BA, brachial artery; FA, femoral artery; NO, nitric oxide; PGI₂, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor.
Figure 2-4. Shift in relative contribution from NO to EDHF in FA after chronic AD. The relative contributions of nitric oxide (NO), prostacyclin (PGI$_2$), and endothelium-derived hyperpolarizing factor (EDHF) in the ACh-induced relaxation for T3-transected (SCI; n=6) and SCI rats with chronic autonomic dysreflexia (SCI+AD; n=4). Autonomic dysreflexia resulted in a shift away from NO (p<0.05) to greater contribution by EDHF (p<0.01), compared to SCI. Values represent mean±standard deviation, *significant difference between SCI and SCI+CRD, unpaired t-test.

Figure 2-5. No change in vascular reactivity of the BA after chronic AD. Dose-response curve of the FA in the T3-transected (SCI; n=6) and SCI rats with chronic autonomic dysreflexia (SCI+AD; n=4) to cumulative additions of: A) acetylcholine (ACh); and B) phenylephrine (PHE). There was no difference in reactivity to ACh or PHE between SCI and SCI+CRD. Values represent mean±standard error, unpaired t-test.
2.3.2 Femoral artery hyperreactivity and hypersensitivity to phenylephrine after SCI with chronic autonomic dysreflexia

The FA of the SCI+CRD group demonstrated a 17% increase in $E_{\text{max}}$ ($p<0.01$) and a smaller logEC$_{50}$ value ($p<0.05$) in the dose-response curve to PHE, compared to the SCI group (Figure 2-2B). No difference was found in responses to KCl and SNP. There was no difference in TH expression in the SCI+CRD group (Figure 2-6B). In the BA, no difference was observed in PHE reactivity (Figure 2-5B) or sympathetic innervation density (Figure 2-6A).

![Bar charts showing sympathetic fiber density in BA and FA](image)

**Figure 2-6. No change in sympathetic fiber density after chronic autonomic dysreflexia.**
Quantification of TH+ fiber density was not different in A) BA and B) FA between the SCI (n=6) and SCI+CRD (n=5) groups. Values represent mean±standard deviation, unpaired t-test.
2.3.3 Structural changes with chronically induced AD

The FA of the SCI+CRD showed a 13.9% decrease in elastin expression (p<0.05), compared to SCI group (Figure 2-7B). Protein expression was not different in the BA of the two groups (Figure 2-7A). There was no difference between the SCI and SCI+CRD group in morphological measures (i.e., diameter, intraluminal diameter, and wall thickness) for both BA (Figure 2-8) and FA (Figure 2-9).

![Protein expression of BA and FA](image)

**Figure 2-7. Profibrotic markers in BA and FA after chronic AD.**
Immunofluorescence intensity of proteins in A) BA and B) FA associated with profibrosis between T3-transected (SCI; n=5) and T3-SCI rats with chronic autonomic dysreflexia (SCI+CRD; n=5), standardized against the cross-sectional area for each artery. TGFβ, transforming growth factor beta; Col I, collagen 1; Col III, collagen 3; SMA, smooth muscle actin. Values are mean±standard deviation. *Significance between SCI and SCI+CRD, unpaired t-test, p<0.05.
Figure 2-8. Morphology of BA did not change with chronic autonomic dysreflexia.
Morphometric analysis of smooth muscle actin immunohistochemical staining for outer arterial diameter (A), intraluminal diameter (B), and wall thickness (C) did not reveal a significant difference between the SCI and SCI+CRD groups. Unpaired t-test.

Figure 2-9. No change in morphology of FA with chronic autonomic dysreflexia.
Morphometric analysis of smooth muscle actin immunohistochemical staining for outer arterial diameter (A), intraluminal diameter (B), and wall thickness (C) did not reveal a significant difference between the SCI and SCI+CRD groups. Unpaired t-test.
2.4 Discussion

This study demonstrated for the first time that chronic repetitive AD resulted in impaired vascular function and atherogenic changes in composition of the conduit arteries. Specifically, these changes include a shift in the mechanisms of endothelial dilation away from NO and towards EDHF, hypersensitivity to vasoconstrictors, and reduced elastin expression. These findings indicate episodes of transient hypertension after SCI may be a primary causal factor in the drastically elevated CVD risk in this population. From a translational perspective, our data suggests that mitigating the frequency and severity of chronic episodic AD should be a primary target of future therapeutic interventions for reducing CVD risk after SCI.

The present data demonstrated a shift away from NO-mediated to EDHF-mediated relaxation in the femoral artery after chronic AD exposure (Figure 2-10). Shifts in the relative contribution of the three primary endothelial dilation pathways may occur before overt reductions in endothelium-mediated dilation. For example, while the relaxation response to ACh was similar in rats with a high-salt diet (i.e., high SBP) and in control rats, the high-salt group has a significantly enhanced dependence on EDHF-mediated relaxation to ACh [37]. It is possible that transient hypertensive episodes exert a similar effect on endothelium-dependent vasodilation as rats with a high-salt diet, where the contribution of the NO as a major dilating agent is shifted to EDHF [37].
Figure 2-10. Shift in relative contribution to dilation towards the TRPV4-EDHF pathway, and away from eNOS with chronic AD.

The present study showed chronic autonomic dysreflexia to have resulted in greater contribution of the EDHF-mediated pathway and less contribution of eNOS in FA vasodilation. Green circle indicates significant increase, and red dashed circle indicates significant decrease in relative contribution of SCI+CRD group (n=4), compared to SCI (n=6), p<0.05. EC, endothelial cell; IEL, internal elastic lamina; SMC, smooth muscle cell; EDHF, endothelium-derived hyperpolarizing factor; PLA₂, phospholipase A2; AA, arachidonic acid; COX, cyclooxygenase; PGI₂, prostacyclin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TRPV4, transient receptor potential cation channel, subfamily V, member 4.
Our data demonstrate that the conduit artery below the level of lesion (i.e., FA) is more sensitive to phenylephrine after repetitive episodes of AD, resulting from hyper-reactivity of smooth muscle α-adrenergic receptors. Our previous work has previously demonstrated that chronic exposure to AD further exacerbates α-adrenergic hyper-reactivity of resistance vasculature after SCI [24]. The present study supports this finding, and extends it to show that hyper-sensitivity to adrenergic agonists occurs even in the large conduit arteries. The mechanisms underlying this change have been speculated to be a compensatory response to elevated vasodilatory stimulation from calcitonin gene–related peptide and substance P (both potent vasodilators) that have collateral branches supplying blood vessels and are upregulated with chronic activation of colonic mechano-nociceptors [24,96].

Finally, the present study also demonstrated a significant reduction in femoral artery elastin expression after recurrent AD, which is fundamentally associated with increased vascular stiffness [97]. Previously, we found significantly increased collage I expression in the FA after chronic T3 SCI (Chapter 2). Combined with the results in the current study, profibrotic changes occur in the conduit artery below the level of spinal cord lesion, and is further exacerbated with chronic AD exposure. These changes undoubtedly contribute to increased arterial stiffness observed consistently in individuals with SCI [98,99], and considering the powerful predictive value of arterial stiffness for future CVD events, this data helps to explain the significant increase in CVD risk after SCI.

2.5 Conclusion
This is the first time that chronic repetitive AD has been demonstrated to result in impaired vascular function and structure of the conduit arteries below the level of lesion. Endothelial
dysfunction is further impaired after SCI by transient hypertension, where AD causes subtle but crucial changes in the vasculature, manifested by the decreased capacity to rely on NO for dilation, hypersensitivity to vasoconstrictors, and further profibrotic changes. Overall this data clearly indicate that repetitive AD exposure exerts its own deleterious effects on the conduit vasculature, in addition to SCI alone. Our data demonstrate that mitigating the frequency and severity of chronic episode of AD is a crucial aspect in preventing secondary cardiovascular consequences after SCI. The last chapter of this thesis will examine potential therapeutic interventions to ameliorate cardiovascular decline in the SCI population.
Chapter 3: Passive exercise improves endothelial function and arterial structure in femoral artery after spinal cord injury

3.1 Introduction

Individuals living with high-level SCI not only suffer from motor and sensory loss, but also severe autonomic dysfunctions, including deleterious changes in the cardiovascular system [20]. In fact, individuals with SCI are at a 3-4 fold elevated risk in cardiovascular disease risk [72]. Vascular endothelial impairment is one of the earliest markers of CVD progression, and precedes the development of morphological vascular changes [5,6]. Recently, we showed for the first time that chronic high-level SCI impairs endothelial function in the FA (i.e., below the level of lesion [FA]) using in vitro myography (i.e. a method independent of arterial morphometrics), which suggest increased CVD risk [100]. SCI also resulted in maladaptive remodelling of the FA to have reduced luminal diameter. Moreover, we observed a shift in relative contribution of vasodilators from endothelium derived hyperpolarizing factors (EDHF) to nitric oxide (NO) mediated pathways with SCI, where prostacyclin did not play a significant role in arterial relaxation.

Exercise is one of the most powerful therapeutic interventions capable of improving cardiovascular health and function, with diverse and robust positive effects on the vasculature [93,101]. In fact, the benefit of exercise outweights the combined effect of traditional cardiovascular risk factors [93]. The impact of exercise is exerted through a number of pathways, a primary one being the provision of increased blood flow and healthy shear stress on the endothelium [102], resulting in enhanced endothelial function and atheroprotective arterial remodelling [93,103]. Despite the well-known positive effects of exercise in able-bodied
individuals, the situation is complicated in the SCI population as lower limb paralysis prevents individuals with SCI from exercising the largest muscle groups in the body. Consequently, chronic arm exercise may not provide a sufficient stimulus to prevent the SCI-induced decline in the vasculature [104–106]. One feasible tool for engaging the lower limbs is passive exercise, where the lower limbs are mobilized manually or through machine. Studies in both SCI and non-SCI populations have demonstrated that passive lower limb exercise can increase blood flow through the legs, specifically through the femoral artery [107–110]. Critically, passive leg exercise has been reported to enhance femoral artery hemodynamic response in people with paraplegia [111]. Considering the positive endothelial effect of increased blood flow and anterograde shear, it is possible that passive exercise will improve FA endothelial function in the lower limbs after SCI.

The endothelial layer is a critical component of the vasculature that is responsible for mediating vessel reactivity and tone to changes in mechanical stimulation such as shear stress, and therefore is crucially involved in ensuring end-organs receive appropriate level of blood flow. Transient receptor potential (TRP) ion channel superfamily is involved in sensing a broad variety of stimuli, including mechanical shear [112]. TRPV4 is a well characterized TRP channel in endothelial cells that is not only thought to play a role in mediating cholinergic receptor-mediated dilation [112], but also to be a key mechanosensor through which the shear stimulus alters the permeability of the mechanosensitive receptor and allows mechano-deformation to be directly transmitted into intracellular Ca\(^{2+}\) signaling [113,114]. Because TRPV4 is necessary for shear-mediated vascular adaptations in the endothelium [115], it is likely crucial in endothelial functional changes in response to chronic alterations in shear stimuli, such as those resulting from passive hind limb exercise after SCI.
The current study aims to test whether passive exercise can reverse endothelial
dysfunction in the FA, a clinically relevant conduit artery, in an experimental animal model of
SCI. Moreover, we aim to examine if passive exercise can offset the inward remodelling and
profibrosis previously seen in the FA, in order to better understand the mechanisms underlying
cardiovascular changes after SCI. We hypothesize that passive exercise will lead to improved
endothelial function, mediated by an increase in TRPV4 expression, as well as prevention of
inward remodelling seen after SCI.

3.2 Methods

3.2.1 Experimental design

Experiments were conducted on twelve male Wistar rats (250-350g; Harlan Laboratories,
Indianapolis, IN, USA). Animals were assigned to either the T3 complete spinal cord transection
group (SCI; n=6), or the group with passive exercise intervention (SCI+PE; n=6). Six weeks
after SCI, the brachial (BA) and femoral arteries (FA) were assessed for vasoconstrictive
responsiveness and endothelial function using wire myography. This time frame is analogous to
what is considered “chronic” SCI in the clinical SCI population [1]. In addition, vascular
composition, innervation, and protein expression were examined using immunohistochemistry.
Detailed methods have been described previously in Chapter 1, Section 1.2. Results in the SCI
group in this chapter are the same as in Chapter 1. All experimental procedures were carried out
with accordance to the Guide to the Care and Use of Experimental Animals established by the
Canadian Council on Animal Care and approved by the University of British Columbia Animal
Care Committee.
3.2.2 Passive hind-limb cycling

The SCI+PE group (n=6) completed 5 weeks of passive hind-limb cycling using a customized cycle ergometer. Exercise began on day 7 post-SCI and was continued 5 days/week for 4 weeks. Each day consisted of 2 x 30 min sessions, with a 10 minute rest in between. Previous data showed that passive exercise in the early phase post-injury was effective in normalizing cardiac impairments after SCI [26]. At the start of the training session, rats were secured in a horizontal (prone) position on a sling, with the hind-limbs passing through large holes in sling to reach the pedals. The hind feet were secured to pedals with vet wrap bandaging tape. The ergometer was manually set at a frequency of 0.7 Hz (i.e., 42 rpm).

3.2.3 Endothelial TRPV4 staining and analysis

The FA was embedded, sectioned, and stained according to protocols in Section 1.2.5. Transient receptor potential cation channel, subfamily V, member 4 (TRPV4) expression was determined by immunofluorescence using the rabbit α TRPV4 (1:300, generous gift from Dr. Stefan Hellar at Stanford) as primary antibody, and donkey anti-rabbit conjugated Cy3 secondary antibody (1:1000; Jackson ImmunoResearch, USA). Slides were mounted with Vectashield® mounting medium for fluorescence with DAPI (Vector Laboratories, Inc, USA). Arterial sections were imaged with a laser confocal AxioImager.Z2 Zeiss© LSM 800 microscope (objective magnification 20X, ocular magnification 10X) using Zen 2 Pro.

Endothelial TRPV4 expression was assessed and quantified using the Fiji Software (based on ImageJ, http://fiji.sc/Downloads#Fiji). Staining intensity was averaged from at least 4 sections per animal. We generated a region of interest (ROI) using the internal elastic lamina of the artery and the lumenal interface (on the inside of the endothelial layer) as the outer and inner
boundaries. DAPI staining of nucleii helped to distinguish endothelial cells from the internal elastic lamina. Autofluorescence of the internal elastic lamina (IEL) was imaged to help distinguish between the endothelial cells and IEL. Within this ROI, intensity was quantified and was used to divide by the area of the ROI to obtain the average intensity for each section and to obviate the bias introduced by arterial size. The intensity settings were identical for each image. A negative control (i.e., only secondary antibody added in absence of primary antibody) was required to ensure specificity of secondary to primary antibody [30].

3.3 Results

3.3.1 Endothelial dysfunction in femoral artery after SCI is reversed with passive exercise

The FA of the SCI+PE group had a smaller logEC₅₀ value to ACh when compared to that of the SCI group (p<0.0001), indicating improved ACh sensitivity (Figure 3-1A). Our data showed that FA in the SCI+PE group required 5x less ACh to reach 50% of the maximal dilation. However, no difference in vasoconstrictive response to PHE was seen after the passive exercise intervention (Figure 3-1B). No significant difference was found in responses to KCl and SNP.
Figure 3-1. Improved vascular reactivity of the FA after PE.
Dose-response curve of the FA in the T3-transected (SCI; n=6) and SCI rats with passive exercise intervention (SCI+PE; n=6) to cumulative additions of: A) acetylcholine (ACh); and B) phenylephrine (PHE). Endothelium-dependent vasodilation was improved with passive exercise, as indicated by a smaller logEC$_{50}$ value in the ACh-concentration curve. Vasoconstriction in response to PHE was not different between SCI and SCI+PE groups. Values represent mean±standard error, unpaired t-test. *p<0.0001.
3.3.2 Outward remodelling of femoral artery after passive exercise

Passive hind-limb exercise led to outward remodelling in the FA, as shown by an increase in outer arterial diameter (29.3%; p<0.01) and intraluminal diameter (31.4%; p<0.05). No difference was seen in the wall thickness between the two groups (Figure 3-2).

Figure 3-2. Passive exercise resulted in outward remodelling of the FA. Morphometric analysis of smooth muscle actin immunohistochemical staining demonstrated evidence of outward remodelling of the FA after PE, as shown by increased arterial diameter (A), increased intraluminal diameter (B), and unchanged wall thickness (C) in the SCI+PE group. Values are mean±standard error. *Significantly different from SCI; unpaired t-test, p<0.05.
3.3.3 Changes in vascular protein expression after passive exercise

FA undergoes a number of structural adaptations after the passive hind-limb exercise intervention. Specifically, there is a significant decrease in TGFβ (47.7%; p<0.05), collagen I (39.0%; p<0.05), and a significant increase in SMA expression (29.4%; p<0.05) (Figure 3-3). Sympathetic innervation of the FA did not change with passive exercise. Furthermore, there was a significant increase in endothelial TRPV4 expression after five weeks of passive hind-limb exercise (Figure 3-4). In fact, TRPV4 expression in the SCI+PE in the FA was four times as much as the SCI group (p<0.01).

Figure 3-3. Less expression of profibrotic markers in FA after PE.
Immunofluorescence intensity of proteins in FA associated with profibrosis between T3-transected (SCI; n=5) and T3-SCI rats with passive exercise (SCI+PE; n=5), standardized against the cross-sectional area for each artery. TGFβ, transforming growth factor beta; Col I, collagen 1; Col III, collagen 3; SMA, smooth muscle actin. Values are mean±standard error. *Significance between SCI and SCI+PE, unpaired t-test, p<0.05.
Figure 3-4. Greater endothelial TRPV4 expression in FA after PE.

(A) Immunofluorescence intensity of endothelial transient receptor potential cation channel, subfamily V, member 4 (TRPV4) was greater in FA of T3-transected with passive exercise (SCI+PE; n=5) group, compared to SCI alone (SCI; n=5). Representative 60x images of TRPV4 staining of FA in SCI (B) and PE (C) groups. TRPV4=red, DAPI=blue, and IEL=green. Region of interest (ROI) for quantifying TRPV4 expression is shown (D). Values are mean±standard error. *Significant difference between SCI and SCI+PE, unpaired t-test, p<0.01.
3.3.4 Shift in relative contribution between prostacyclin and EDHF vasodilation

Blockade analysis using the dose-response curve to ACh before and after incubation of the FA with either L-NAME or indomethacin (Figure 3-5) revealed a higher contribution to vasodilation by prostacyclin (PGI\textsubscript{2}) in the SCI+PE group compared to the SCI group (SCI: 4.9\%±2.7\% vs SCI+PE: 12.6\%±2.0\%; p<0.05) (Table 3-1; Figure 3-6).

![Figure 3-5. Greater contribution by prostacyclin in FA with passive exercise.](image)

Dose-response curves to acetylcholine (ACh) with incubation of either L-NAME or Indo. The responses to increasing doses of ACh were examined in rings from femoral artery (FA) of the A) SCI, and B) SCI+PE group. The relaxations are expressed as a percentage of the precontractions levels with phenylephrine. Indo, indomethacin; L-NAME, N-nitro-L-arginine methyl ester.

<table>
<thead>
<tr>
<th></th>
<th>NO</th>
<th>PGI\textsubscript{2}</th>
<th>EDHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCI</td>
<td>137.7±3.8</td>
<td>13.0±3.3</td>
<td>112.4±4.6</td>
</tr>
<tr>
<td>SCI+PE</td>
<td>175.3±13.8</td>
<td>39.7±2.8</td>
<td>101.8±15.7</td>
</tr>
</tbody>
</table>

| p-value | 0.31    | **0.029**            | 0.796   |

Data presented as mean±standard error; FA, femoral artery; NO, nitric oxide; PGI\textsubscript{2}, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor.
Figure 3-6. Greater contribution of prostacyclin in FA after SCI with passive exercise.
The relative contributions of nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived
hyperpolarizing factor (EDHF) in the ACh-induced relaxation for T3-transected (SCI; n=6) and
SCI rats with passive exercise intervention (SCI+PE; n=6). Passive exercise resulted in a greater
contribution by PGI₂, compared to SCI. Values represent mean±standard error, unpaired t-test.
*p<0.05.
3.4 Discussion

In the present study, we demonstrate that passive hind-limb cycling is a viable therapeutic intervention to combat endothelial dysfunction, which is achieved by an increase in mechanosensitive channels (i.e., TRPV4 channels) in the endothelium, and an increase in prostacyclin mediated pathways for vasodilation. Passive exercise also resulted in atheroprotective vascular remodelling, specifically increased arterial diameter [116] and smooth muscle layer, as well as decreased expression in markers of profibrosis. Taken together, passive hind-limb cycling is able to improve both the function and structure of inactive and autonomically disrupted conduit artery (i.e., FA) after SCI.

3.4.1 Passive exercise reverses endothelial dysfunction after SCI

This study for the first time shows that passive exercise can restore endothelial function in conduit arteries that is associated with CVD risk. In a previous experiment, we saw impaired endothelium-mediated relaxation in the FA after complete spinal cord transection at the third thoracic level. After the 5-week passive exercise intervention in the current study, the FA had 5-fold increase in ACh sensitivity compared to that of the SCI group. Furthermore, we showed that the passive exercise intervention led to a specific increase in the PGI2-mediated pathway responsible for dilation.

Moreover, this study demonstrated significant increases in the expression of TRPV4 in the endothelium of the FA after passive exercise. The Ca^{2+}-permeable TRPV4 channel is critical for transmitting shear stimulus into intracellular Ca^{2+} signaling, leading to the release of endothelial relaxing factors [113], which mediates dilation in conduit arteries [113,115]. In fact, endothelial dependent vasodilation has been shown to be eliminated in TRPV4^{-/-} knockout mice [113,115]. The upregulated TRPV4 expression may be due to the elevated shear stress and blood
flow in the FA following 5 weeks of passive hind-limb cycling, and indicates that these stimuli may be leading to greater and more rapid calcium flux, resulting in the observed improvement in endothelial dilation.

In the present study, we saw an increase in PGI$_2$-mediated vasodilation in the SCI+PE group compared to the SCI group. Previous work has shown that there is an increase in endothelial production of prostacyclin in response to exercise and shear stress [117–119]. Overall, the improvement in endothelial function, mediated by increased PGI$_2$ contribution, as well as increased TRPV4 expression, suggests that passive exercise after SCI selectively upregulates the TRPV4- phospholipase A2 -arachidonic acid- cyclooxygenase pathway within the endothelium (Figure 3-7).
Passive exercise led to increases in blood flow and laminar shear stress in the FA, which activated TRPV4 channels. TRPV4-mediated rise in intracellular Ca\(^{2+}\) can mediate NO production by eNOS, production of PGI\(_2\) through COX action, and potentially also EDHF-mediated pathways. The present study showed passive hind-limb cycling in the FA to result in greater contribution of the PGI\(_2\)-mediated pathway responsible for vasodilation. Green circle indicates significant difference in relative contribution between SCI (n=6) and SCI+PE groups (n=6), p<0.05. EC, endothelial cell; IEL, internal elastic lamina; SMC, smooth muscle cell; EDHF, endothelium-derived hyperpolarizing factor; PLA\(_2\), phospholipase A2; AA, arachidonic acid; COX, cyclooxygenase; PGI\(_2\), prostacyclin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TRPV4, transient receptor potential cation channel, subfamily V, member 4.
3.4.2 Passive exercise prevents inward remodelling and induces anti-fibrotic structural changes in femoral artery

The FA underwent outward remodelling (i.e., increased outer diameter and intraluminal diameter) following the passive exercise intervention. In light of our previous study, passive hind-limb cycling appears to prevent inward remodelling in the FA after SCI (Chapter 2). Previous studies in non-SCI have shown similar outward remodelling in the femoral artery due to increased shear stress and blood flow with lower limb exercise [120–122]. Interestingly, both animal and clinical data indicate that exercise-induced functional changes in conduit arteries (i.e., endothelial function) precede structural adaptations (i.e., diameter), which may indicate that the positive vascular effects noted in the endothelium occur even earlier than the 5 weeks of passive exercise utilized in the present study [123]. Future studies should explore the time course of arterial functional and structural changes following passive hind-limb cycling in SCI models. The mechanisms by which passive exercise prevents inward remodelling are likely due to increased shear stress and blood flow, which are well established to increase arterial lumen diameter over time [21,123].

In addition to morphological changes, passive hind-limb cycling induced significant changes in protein expression in the FA. Specifically, there was an increase in SMA expression in the arterial wall, which can be attributed to the increase in shear stress and blood flow associated with exercising. Passive hind-limb cycling also resulted in reduced Col I and TGFβ expression. TGFβ is a potent stimulator of Col I synthesis [124]; both of these markers are indicative of a profibrotic environment. Following chronic SCI, arterial stiffening occurs, which is responsible for baroreflex dysfunction and contributes greatly to the elevated risk of cardiovascular events in this population [98,125]. The decrease in these profibrotic markers
further suggests that passive hind-limb cycling exerts powerful atheroprotective effects, which involves the molecular constituents of the vasculature.

3.4.3 Clinical implications

This study highlights that passive exercise is a viable tool to prevent or mitigate CVD risk. Previously, our lab has shown in a number of studies that passive exercise can reduce the aberrant neuroplastic changes occurring in dorsal afferent fibres that lead to autonomic dysfunction after SCI [126], and can offset cardiac decline [26]. Other groups have shown that these changes can even promote the restoration of motor recovery and reduction in spasticity [127,128]. Based on this, future studies should assess the efficacy of passive exercise in the sub-acute phase of SCI in a phase one clinical trial, to mitigating not only the deleterious cardiovascular and neuroplastic changes after SCI, but also a variety of important clinical issues afflicting this population.

3.5 Conclusion

Passive exercise after SCI results in functional and structural improvements in the conduit arteries that are associated with CVD risk, indicating that this may be a viable therapeutic intervention for reducing the drastically elevated cardiovascular morbidity and mortality in this population. Passive exercise restored FA endothelial dysfunction after high-thoracic SCI, largely through increases in prostacyclin pathways and increased mechanosensor expression. Passive hind-limb cycling also prevented inward remodelling of the inactive/supraspinally disrupted FA, as well as induced anti-fibrotic changes in arterial composition.
Concluding remarks

In accordance with our hypothesis, we have shown for the first time with a well-established experimental model, the expected endothelial dysfunction in conduit artery caudal to spinal cord lesion. There is a decrease in ACh sensitivity and less EDHF-mediated relaxation in the FA after SCI. Moreover, our data demonstrate that endothelial dysfunction after SCI is associated with profibrotic remodelling and potentially with the chronic loss of supraspinal sympathetic support. This study provides compelling evidence that contradicts previously published literature reporting counterintuitively improved [19] or preserved endothelial function [17,18] in the immobile and autonomically dysfunctional lower limbs after SCI, which indicates that studies utilizing FMD must provide appropriate allometric scaling in order to reflect the reality in endothelial health after SCI. Moreover, our study provides mechanistic insight to the causes of elevated CVD risk after SCI and potential therapeutic targets.

One of the major autonomic dysfunctions after SCI is autonomic dysreflexia, which we hypothesized would exacerbate the dysfunctions in conduit artery observed caudal to SCI. This is the first time that chronic repetitive AD has been demonstrated to result in impaired vascular function and structure of the conduit arteries below the level of lesion. With chronically induced AD, the femoral artery undergoes decreased capacity to rely on NO for dilation, hypersensitivity to vasoconstrictors, and further profibrotic changes, demonstrating deleterious effects on the conduit vasculature in addition to the effects of SCI. These findings indicate AD may be a primary causal factor that elevates CVD risk in individuals with SCI. From a translational perspective, mitigating the frequency and severity of chronic episodic AD is a crucial aspect in preventing secondary cardiovascular consequences after SCI, and should be a primary target of future therapeutic interventions.
Lastly, this study shows that passive hind-limb exercise after SCI results in functional and structural improvements in the conduit arteries that are associated with CVD risk, indicating that this may be a viable non-pharmacological intervention for reversing or preventing vascular dysfunctions in SCI. The endothelial relaxation in conduit artery below the level of spinal cord lesion is improved with the exercise regimen utilized in this study, through upregulation of the TRPV4-prostacyclin pathway. Moreover, passive exercise reverses the inward remodelling seen after SCI and provides atheroprotective changes in profibrotic markers. Taken together, passive hind-limb cycling is able to improve both the function and structure of inactive and autonomically disrupted conduit artery after SCI, and may provide a viable therapeutic intervention for preventing CVD in this population. Combined with other benefit of passive exercise reported in previous studies [126–128], future clinical trials should examine the effect of passive exercise in patients with the sub-acute phase of SCI on cardiovascular health. The effective time point to begin exercise and the frequency and intensity of exercise should be explored. We hypothesize that the benefit of passive exercise is exerted through increased healthy shear stress provided to the artery, future studies should examine anterograde vs. retrograde shear stress in conduit artery after passive hind-limb exercise, as well as change in blood flow and shear patterns with chronic AD.


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