2015-10-01

Acute and Chronic Effects of Botulinum Toxin Type-A on the structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits

Fortuna, Rafael


Downloaded from PRISM Repository, University of Calgary
Acute and Chronic Effects of Botulinum Toxin Type-A on the Structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits

by

Rafael Fortuna

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN KINESIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 2015

© Rafael Fortuna 2015
Abstract

Botulinum toxin type-A (BTX-A) injections have become a common treatment modality for a variety of neuromuscular disorders with the primary aim to relax spastic muscles, for example, in children with cerebral palsy, or following a stroke. Once injected, BTX-A prevents acetylcholine release at the motor nerve endings, thereby producing a dose-dependent muscle paralysis. Despite an exponential growth of patients receiving BTX-A treatment, there has been no systematic evaluation of the effects of the toxin on target and non-target muscles. Therefore, the general purpose of this PhD project was to evaluate muscle mass, strength, and contractile material in injected and contralateral non-injected quadriceps muscles of New Zealand White (NZW) rabbits following single and repeated BTX-A injections. Muscle mass was assessed as the wet weight of muscles following sacrifice, strength was assessed by stimulating the knee extensor muscles via femoral nerve stimulation and quantifying the knee extensor force, the amount of contractile material was quantified histologically.

We found that six-monthly BTX-A injections into the quadriceps caused substantial muscle weakness, atrophy, and contractile material loss in the injected and the contralateral non-injected muscles. Adding direct electrical muscle stimulation during the BTX-A injection help to alleviate muscle mass, strength and contractile material loss and the injected and contralateral-non injected muscles, and finally, BTX-A injections had long lasting effects that were not fully recovered at six months following the end of the injection protocol.

We concluded from the results of this series of studies that BTX-A treatment resulted in adverse effects on the injected and contralateral non-injected musculature up to six months following the
injection protocol. Future studies should be aimed at identifying strategies that minimize/prevent adverse effects of BTX-A injections on target and non-target muscles.
Preface

Each of the following four chapters is based on scientific manuscripts:


*Used with permission – Elsevier Ltd.*


*Used with permission – Elsevier Ltd.*


*Used with permission – Elsevier Ltd.*


*Used with permission – Elsevier Ltd.*
This dissertation is based on a collection of manuscripts, and therefore has some redundancy in the introduction and methods sections of chapter three through six.
Acknowledgements

I would like to express my deep gratitude and appreciation to the following individuals:

Walter Herzog for taking me under his wings over the last seven years, providing guidance, advice, and scientific knowledge throughout this endeavour.

Dr. Brian Macintosh and Dr. Steven Boyd for serving on my supervisory committee.

Dr. Cheryl Kozey and Dr. John Bertran for being on my examining committee, and Dr. Benno Nigg for acting as a Neutral Chair.

Tim Leonard, Hoa Nguyen, Andrzej Stano, Andrew Sawatsky, Azim Jinha, Ruth Seerattan, Monika Horisberger, Robert van der Marel, and Dawn Martin for their technical help and support for data collection.

Tak Fung for assistance with statistical analysis.

Holly Hanna and Amanda Lottermosser for their administrative help.

Dr. David Hart for his scientific contribution in chapter 6.

Dr. Marco Aurelio Vaz for introducing me into research during my undergraduate and for his mentorship since then.

The Human Performance Laboratory professors, colleagues, and staff for making the lab such a pleasant environment.

To all the visiting students I was fortunate enough to cross path with and share the office space at KNB207. I’m convinced that they made my journey much more entertained and productive.

The government of Brazil for making this work possible through their funding during the last four years.
To my Mexican friends: Francisco Alaniz Uribe, my very first and devoted friend that helped me to get adapted to life in Calgary and for introducing me to Avalanche FC; and Karelia Tecante, for her kindness, compassion, and for always been there for me.

Dr Eng Kuan Moo, one of the most loyal friends I have ran into my life, who joined me in every activity in and out of the UofC campus, including exploring the Rocky Mountains, our favorite weekend getaway.

Last, but not least, my family. Many thanks to my mother, Neusa Veirich Fortuna, and my father, Newton Fernando Fortuna, for being devoted and altruistic parents, and to my brother, Jose de Oliveira Fortuna Neto, and despite the fact that we don’t talk much and don’t hang out together all the time, I know from the bottom of my heart my brother will always be there for me when I holler.
Dedication

To my parents and my brother who have always supported and believed in me, especially when things went a bit off the track.

To the patients who suffer from muscle spasticity, I hope this work may help alleviate the social and economic burden related to spasticity and improve the quality of life for you.
Table of Contents

Abstract ............................................................................................................................... ii
Acknowledgements ............................................................................................................ vi
Table of Contents ............................................................................................................... ix
Epigraph ................................................................................................................................ xix

CHAPTER ONE: INTRODUCTION ..................................................................................1
  1.1 Motivation and context ..............................................................................................1
  1.2 Objective ....................................................................................................................5
    1.2.1 Specific objectives: ............................................................................................5
  1.3 Significance and clinical relevance ............................................................................6
  1.4 Thesis outline .............................................................................................................7

CHAPTER TWO: REVIEW OF SELECTED AND RELEVANT LITERATURE ...........9
  2.1 Cerebral palsy ............................................................................................................9
  2.2 Botulinum toxin type-A (BTX-A) ...........................................................................12
    2.2.1 Structure and mechanism of action .................................................................12
    2.2.2 Recovery following BTX-A injection .............................................................14
    2.2.3 Clinical application of BTX-A ........................................................................15
      2.2.3.1 History ...................................................................................................16
      2.2.3.2 Clinical studies in children with cerebral palsy .....................................17
    2.2.4 Adverse effects ................................................................................................25
      2.2.4.1 Local adverse effects .............................................................................26
      2.2.4.2 Systemic adverse effects ........................................................................28

CHAPTER THREE: CHANGES IN THE RABBIT QUADRICEPS CONTRACTILE
    PROPERTIES FOLLOWING REPEAT BTX-A INJECTIONS .............................32
  3.1 Introduction ..............................................................................................................32
  3.2 Methods ...................................................................................................................33
    3.2.1 Experimental Design .......................................................................................33
    3.2.2 Botulinum Toxin Type-A: injection protocol ..................................................34
    3.2.3 Knee extensor torque and muscle mass ...........................................................34
    3.2.4 Contractile material .........................................................................................35
    3.2.5 Data analysis ....................................................................................................36
  3.3 Results ......................................................................................................................37
    3.3.1 Muscle weakness .............................................................................................37
    3.3.2 Muscle atrophy ................................................................................................39
    3.3.3 Contractile material .........................................................................................42
  3.4 Discussion ................................................................................................................44
    3.4.1 Knee extensor torque .......................................................................................44
    3.4.2 Muscle mass ....................................................................................................45
    3.4.3 Contractile material .........................................................................................47
  3.5 Conclusions ..............................................................................................................50

CHAPTER FOUR: THE EFFECTS OF ELECTRICAL STIMULATION TRAINING
    EXERCISE ON MUSCLES INJECTED WITH BTX-A ........................................51
4.1 Introduction ..............................................................................................................51
4.2 Methods .....................................................................................................................52
  4.2.1 Experimental design .............................................................................................52
  4.2.2 Botulinum Toxin Type-A: injection protocol ......................................................53
  4.2.3 Electrical stimulation (ES) exercise in BTX-A+ES group ....................................54
  4.2.4 Knee extensor torque, glycogen depletion protocol, and muscle mass ...............54
  4.2.5 Contractile material ...........................................................................................57
  4.2.6 Data analysis .......................................................................................................57
4.3 Results .......................................................................................................................58
  4.3.1 Muscle weakness ...............................................................................................58
  4.3.2 Muscle atrophy ....................................................................................................60
  4.3.3 Contractile material ...........................................................................................62
  4.3.4 Femoral nerve vs. direct muscle stimulation in BTX-A+ES group rabbits ..........63
  4.3.5 Glycogen depletion protocol in BTX-A+ES group rabbits ....................................64
4.4 Discussion ..................................................................................................................66
  4.4.1 Muscle weakness ...............................................................................................66
  4.4.2 Muscle atrophy and contractile material .............................................................68
  4.4.3 Femoral nerve vs. direct muscle stimulation and glycogen depletion in BTX-A+ES group rabbits .................................................................70
4.5 Conclusion ................................................................................................................71

CHAPTER FIVE: DO SKELETAL MUSCLE PROPERTIES RECOVER FOLLOWING REPEAT BTX-A INJECTIONS? .............................................................................72
5.1 Introduction ..............................................................................................................72
5.2 Methods .....................................................................................................................72
  5.2.1 Experimental design .............................................................................................72
  5.2.2 Botulinum Toxin Type-A: injection protocol ......................................................73
  5.2.3 Knee extensor strength, glycogen depletion protocol, and muscle mass ..........74
  5.2.4 Contractile material ...........................................................................................76
  5.2.5 Data analysis .......................................................................................................76
5.3 Results .......................................................................................................................77
  5.3.1 Muscle weakness ...............................................................................................77
  5.3.2 Muscle atrophy ....................................................................................................79
  5.3.3 Contractile material ...........................................................................................80
  5.3.4 Femoral nerve vs direct muscle stimulation on BTX-A+6M group rabbits .........83
  5.3.5 Glycogen depletion protocol on BTX-A+6M group rabbits ....................................84
5.4 Discussion ................................................................................................................85
  5.4.1 Muscle weakness ...............................................................................................86
  5.4.2 Muscle atrophy ....................................................................................................88
  5.4.3 Contractile material ...........................................................................................89
  5.4.4 Femoral nerve vs. direct muscle stimulation and glycogen depletion protocol in BTX-A+6M group rabbits .................................................................90
  5.4.5 Clinical significance ..........................................................................................91
5.5 Conclusion ................................................................................................................93

CHAPTER SIX: A CLINICALLY RELEVANT BTX-A INJECTION PROTOCOL LEADS TO PERSISTENT WEAKNESS, CONTRACTILE MATERIAL LOSS, AND AN
List of Tables

Table 1 Validated rabbit primers used for qPCR ................................................................. 100
Figure 3-1 Mean muscle weakness (± 1 SE) following 1, 3, and 6 months BTX-A injections for injected (dark bars) and contralateral non-injected hind limbs (light bars) normalized to the values of Control group rabbits (100%). A significant difference ($p<0.0001$) was observed for the injected hind limbs compared to control for all experimental group animals. Muscle weakness was also observed in the contralateral non-injected hind limbs of all experimental group rabbits, but this difference was only statistically significant for group 4 animals (6 monthly injections of BTX-A) ($p<0.0001$). * compared to control group rabbits. 

Figure 3-2 Mean muscle atrophy (± 1 SE) following 1, 3, and 6 months of BTX-A injection for injected (dark bars) and contralateral non-injected (light bars) hind limbs. Significant atrophy ($p<0.0001$) was observed for the injected hind limbs for all experimental group rabbits compared to control group rabbits. Muscle atrophy was also observed in the contralateral non-injected hind limbs of all experimental group rabbits. However, this atrophy was only significant for the 6 months BTX-A group ($p<0.0001$). * compared to control group rabbits.

Figure 3-3 Mean muscle atrophy (± 1 SE) following 1, 3, and 6 months of BTX-A injection for the different portions of the quadriceps femoris (VL=vastus lateralis; RF=rectus femoris; VM=vastus medialis). Significant muscle atrophy ($p<0.05$) was observed for the different portions of the quadriceps femoris of the experimental group rabbits compared to the control group rabbits. * compared to control group rabbits.

Figure 3-4 Exemplar histological cross-sectional images showing the contractile material (H&E; red staining) and non-muscle contractile material (white color; assumed to be primarily fat and connective tissue) for muscles from injected (left column) and contralateral non-injected (right column) for control, 1 BTX-A, 3 BTX-A, 6 BTX-A group rabbits (first, second, third, and fourth row, respectively). There was a significant reduction in the percent contractile material. Contractile material seemed to be replaced primarily by fat at 3 months following BTX-A injection on the injected side and this fat infiltration was even more pronounced for 6 BTX-A group rabbits. A significant loss of contractile material in the contralateral hind limbs was only observed for 6 BTX-A group rabbits, and the contractile material seemed to be replaced by connective tissue, rather than fat, as observed in the experimental hind limbs.

Figure 3-5 Estimated loss in knee extensor torque in the BTX-A injected hind limbs of 1, 3, and 6 BTX-A group rabbits associated with the inhibition caused by BTX-A denervation (BTX-A weakness, white bars), loss of muscle mass (muscle atrophy, dark bars), and/or the loss of contractile material (contractile material loss, grey bars). While the loss in knee extensor torque associated with BTX-A injections remained about constant throughout the experimental period, the loss was associated primarily with BTX-A induced chemical ablation initially (1 BTX-A group rabbits) and the loss of muscle mass and contractile material at the end (6 BTX-A group rabbits).
Figure 4-1 Quadriceps musculature stained with PAS. Left: control muscle not exposed to the glycogen depletion protocol shows an even distribution of glycogen throughout the entire cross-sectional area. Right: muscle exposed to the glycogen depletion protocol is completely devoid of glycogen in the contractile material. .......................................................... 56

Figure 4-2 Mean muscle weakness (± 1 SE) normalized to the values of the control group rabbits (dark bars-100%). Knee extensor torque in the BTX-A group (light bars), and the BTX-A+ES (shaded bars) group rabbits was significantly reduced for the BTX-A injected and contralateral non-injected hind limbs compared to control group rabbits. BTX-A+ES group rabbits had significantly increased knee extensor torque in the BTX-A injected and the contralateral non-injected hind limbs compared to the corresponding hind limbs of the BTX-A group rabbits. However, muscle strength in the BTX-A+ES group rabbits did not reach control values, neither for the ES exercised nor the contralateral non-injected hind limbs. * compared to control group rabbits; † compared to BTX-A group rabbits. ....................................................................................................... 59

Figure 4-3 Mean muscle atrophy (± 1 SE) normalized to the values of control group rabbits (dark bars – 100%). Muscle mass in the BTX-A (light bars), and the BTX-A+ES (shaded bars) group was significantly reduced for the BTX-A injected and contralateral non-injected hind limbs. BTX-A+ES group rabbits had significantly increased muscle mass in the BTX-A exercised and the contralateral non-injected and non-exercised hind limbs compared to the corresponding hind limbs of the BTX-A group rabbits. Muscle mass for the contralateral non-injected hind limbs of BTX-A+ES group rabbits was fully preserved and similar to the values of the control group rabbits. * compared to control group rabbits; † compared to BTX-A group rabbits. ........................................................................................................ 61

Figure 4-4 Exemplar histological cross-sectional images showing the muscle contractile material (H&E, red staining) and non-muscle contractile material (white color; assumed to be primarily fat and connective tissue) for muscles from injected (first row) and contralateral non-injected hind limbs (second row) for control group muscles (left), BTX-A group muscles (middle), and BTX-A+ES group muscles (right). There was a significant loss of contractile material for the injected and contralateral non-injected muscles for the BTX-A group rabbits (middle column) compared to control group muscles. The percentage of contractile material was significantly greater for the injected and contralateral non-injected muscles of the BTX-A+ES group compared to BTX-A group rabbits. The percentage of contractile material for the contralateral muscles of BTX-A+ES group rabbits was fully preserved and similar to control values. .......................... 62

Figure 4-5 Knee extensor torque obtained by femoral nerve stimulation (dark bars) and by direct muscle stimulation (light bars) for the injected and contralateral non-injected hind limbs of BTX-A+ES group rabbits. Torque values were normalized relative to control group values (100% - not shown). Direct muscle stimulation produced significantly greater muscle force than femoral nerve stimulation in BTX-A injected and contralateral non-injected hind limbs, suggesting that BTX-A was partially blocking acetylcholine release at the motor axons of the target injected and non-target contralateral musculature. * compared to femoral nerve stimulation. ....................................................... 64
Figure 4-6 Histological sections of the quadriceps musculature using PAS staining for glycogen content. The left section is from BTX-A injected muscle of a BTX-A+ES group rabbits and shows a substantial amount of glycogen following the glycogen depletion protocol, suggesting an almost complete blockage of acetylcholine release through BTX-A, and thus little glycogen depletion. The right section is from a contralateral non-injected muscle of a BTX-A+ES group rabbits and shows some remnant glycogen depletion following the glycogen depletion protocol, suggesting that there is also some blockage of acetylcholine release through BTX-A in the non-target contralateral non-injected musculature.

Figure 5-1 Mean muscle weakness (±1 SE) normalized to the values of the control group rabbits (dark bars – 100%). Knee extensor strength in the injected quadriceps of BTX-A+0M group rabbits (light bars) was significantly reduced compared to control, while strength in BTX-A+1M/3M/6M (shaded bars) group rabbits recovered significantly compared to BTX-A+0M group rabbits during the recovery period, but remained lower than control group values. Knee extensor strength in the contralateral non-injected quadriceps of BTX-A+0M group rabbits was significantly reduced compared to control values, but recovered completely for BTX-A+1M/3M/6M group rabbits. * compared to control group rabbits; † compared to BTX-A+0M group rabbits.

Figure 5-2 Mean muscle atrophy (±1 SE) normalized to the values of control group rabbits (dark bars – 100%). Quadriceps atrophy was significant in the injected muscles of BTX-A+0M group rabbits (light bars) compared to control values, and was significantly reduced for BTX-A+3M/6M group rabbits (shaded bars), but did not reach control group values in the six months recovery period. Quadriceps atrophy was significant in the contralateral non-injected BTX-A+0M group rabbits compared to control group rabbits, but recovered to control values for BTX-A+1M/3M/6M group rabbits. * compared to control group rabbits; † compared to BTX-A+0M group rabbits.

Figure 5-3 Exemplar histological cross-sectional images showing the muscle contractile material (H&E; red staining) and non-muscle contractile material (white color; assumed to be primarily fat and connective tissue) for muscles from injected (left column) and contralateral non-injected muscles (right column) for control (first row), BTX-A+0M/1M/3M/6M group rabbits (second, third, fourth, and fifth row), respectively. There was a significant loss of contractile material for the injected and contralateral non-injected quadriceps for BTX-A+0M group rabbits (second row) compared to control group rabbits. The contractile material for the injected quadriceps muscles only showed significantly recovery for BTX-A+6M (first column; fifth row) compared to BTX-A+0M group rabbits (first column; second row), but did not reach control group values during the recovery period. There was no significant recovery in contractile material for the contralateral non-injected quadriceps muscles of BTX-A+1M/3M/6M (second column; third, fourth, and fifth row) compared to BTX-A+0M group rabbits, and the percentage of contractile material remained smaller compared to control values throughout the recovery period.

Figure 5-4 Knee extensor strength obtained with femoral nerve stimulation (dark bars) and by direct muscle stimulation (light bars) for the injected and contralateral non-injected
quadriceps of BTX-A+6M group rabbits. Knee extensor strength was normalized relative to control group rabbits (100%, not shown). Direct muscle stimulation produced significantly greater muscle force than femoral nerve stimulation in BTX-A injected quadriceps muscles, but was the same in the contralateral non-injected quadriceps muscles. * compared to femoral nerve stimulation.

Figure 5-5 Exemplar histological sections of the quadriceps muscles using PAS staining for glycogen for a BTX-A+6M group rabbit. The left section is from a BTX-A injected muscles following the depletion protocol, showing glycogen retention (pink stained cells – arrow) and glycogen depleted muscle fibers (unstained cell – “*” symbol). Fibers that remain stained contain glycogen, and thus were likely not activated during the depletion protocol because of remnant blocking of acetylcholine release following the six months recovery period. The right section contains a sample from the contralateral non-injected quadriceps muscles, which shows virtually complete glycogen depletion (unstained cells – “*” symbol) with only few fibers staining for glycogen (pink stained cells – arrow), suggesting that only few fibers were blocked by BTX-A following the six months recovery period.

Figure 6-1 Mean knee extensor strength (±1 SE) normalized to control group rabbits (100%) six months after the last BTX-A injection in rabbits receiving 0 (control), 1 (1-BTX-A), 2 (2-BTX-A), and 3 (3-BTX-A) BTX-A injections with a three months interval between injections. Knee extensor strength in the quadriceps femoris of all experimental group rabbits was reduced compared to control group rabbits, but was the same among the three BTX-A experimental groups. * compared to control group rabbits.

Figure 6-2 Mean muscle mass (±1 SE) normalized to the values of control group rabbits (100%) six months following the last BTX-A injection in rabbits receiving 0 (control), 1 (1-BTX-A), 2 (2-BTX-A), and 3 (3-BTX-A) BTX-A injection with a three months interval between injections. Muscle mass in all experimental BTX-A group rabbits was unaltered compared to control group rabbits.

Figure 6-3 Exemplar histological cross-sectional images showing the percentage of muscle contractile material (H&E – red staining) and non-contractile material (white color – primarily fat and connective tissue). The amount of contractile material for control group rabbits was 96.9 ±2.0% (top left). Following a single BTX-A injection, there was a significant reduction of contractile material for 1-BTX-A group rabbits (top right – 59.2 ±6.0%). There was no additional loss of contractile material for rabbits that received two injections (2-BTX-A; bottom left – 62.5 ±6.1%) and three injections (3-BTX-A; bottom right – 59.9 ±11.8%) group rabbits, respectively.

Figure 6-4 Knee extensor strength obtained with femoral nerve (dark bar) and by direct muscle stimulation (light bar) for the injected quadriceps femoris musculature of 3-BTX-A group rabbits. Knee extensor strength was normalized relative to control group values (100%, not shown). Direct muscle stimulation produced significantly greater muscle forces than femoral nerve stimulation. This result suggests that despite a six months recovery period following the last BTX-A injection, there is a persistent
blockage of acetylcholine release at the neuromuscular junction. * compared to 3-BTX-A femoral nerve stimulation. ........................................................................................................................................ 105

Figure 6-5 mRNA expression normalized to the values of control group rabbits (dark bars – 100%). Gene expression of Collagen I, Collagen III, IGF-1, TGFβ, and MuRF1 were significantly increased compared to control group rabbits. Similar to the mechanical and histological data, there was no difference in mRNA expression between the three BTX-A experimental groups. * compared to control group rabbits......................................................... 107

Figure 6-6 Average muscle strength in BTX-A group rabbits averaged across all three experimental groups (light gray bar), and estimated loss in strength associated with reduction in contractile material (dark gray bar), and the remnant effects associated with BTX-A blockage (shaded bar). Six months following the last BTX-A injection, muscle strength was still significantly reduced to 55% of the strength in control group rabbits (white bar). The loss in strength associated with the loss in contractile material was obtained by assuming a linear relationship between the amount of contractile material and strength. This assumption likely underestimates the real loss in strength associated with the loss of contractile material................................................................. 110
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX-A</td>
<td>Botulinum toxin type-A</td>
</tr>
<tr>
<td>ES</td>
<td>Electrical stimulation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>VL</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>RF</td>
<td>Rectus femoris</td>
</tr>
<tr>
<td>VM</td>
<td>Vastus medialis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>MuRF1</td>
<td>Muscle RING finger protein-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messengerRNA</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosomal associated protein 25</td>
</tr>
<tr>
<td>PRS</td>
<td>Physician rating scale</td>
</tr>
<tr>
<td>GMFM</td>
<td>Gross motor function measure</td>
</tr>
<tr>
<td>MAS</td>
<td>Modified Ashworth scale</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance image</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription-quantitative polymerase chain reaction</td>
</tr>
</tbody>
</table>
Epigraph

“You miss 100% of the shots you don’t take”

-Wayne Gretzky
Chapter One: Introduction

1.1 Motivation and context

Cerebral palsy is the most common cause of motor disability in childhood, affecting 2-3 children per 1000 live births (Heinen et al.; Love et al., 2010). It is an umbrella term covering a group of neuromuscular disorders that permanently affect body movement and posture secondary to a non-progressive central nervous system lesion arising at any time during brain development. It is estimated that sensory, motor, and cognitive functions are affected to variable extents, depending on the magnitude and/or location of the lesion (Bax et al., 2005). Topographically, cerebral palsy may involve a single extremity (monoplegia) or a combination of upper and lower extremities (quadriplegia) (L Andrew Koman, Smith, & Shilt, 2004). Independent of the magnitude, cerebral palsy impairs muscle function, thus joint functionality, often compromising daily life activities (Barber, Hastings-Ison, Baker, Barrett, & Lichtwark, 2011; Molenaers, Van Campenhout, Fagard, De Cat, & Desloovere, 2010).

Spastic cerebral palsy is the most common subtype and is defined as a “motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes, with exaggerated tendon jerks resulting from hyperexcitability of the stretch reflex, as one component of the upper motoneuron syndrome” (Lance, 1980). Spasticity presents with increased muscle tone (“stiff muscles”) in a growing child. Spasticity leads to limited motor learning and locomotor abilities, which contributes to the development of joint contractures and bony deformities, thus causing further functional impairments (Barber et al., 2011; Eames et al., 1999; Sutherland, Kaufman, Wyatt, Chambers, & Mubarak, 1999). Furthermore, spasticity can also result in alterations of skeletal muscle structure and function (Foran, Steinman, Barash, Chambers, & Lieber, 2005;
Muscle contractures, which restrict joint range of motion, are commonly assumed to be caused by “fixed” and “shortened” muscles that are not properly growing in length (Lieber & Fridén, 2002; Malaiya et al., 2007). Contractures lead to muscle weakness and reduced functional capacity in cerebral palsy children (Damiano & Abel, 1998; Elder et al., 2003; Rose & McGill, 2005). As a result, children often develop compensatory mechanisms, or coping responses, to overcome spasticity during daily life activities (Mockford & Caulton, 2010).

Since cerebral palsy is incurable, treatment modalities have been focused on reducing or normalizing muscle tone in order to delay or prevent further progression of muscle contractures and bone deformities (Brin, 1997). Current treatment modalities can be divided into non-surgical and surgical interventions. Non-surgical interventions comprise treatments such as physical therapy, orthoses, and oral medication. Surgical interventions include neurological procedures, such as selective dorsal rhizotomy (Wright, Sheil, Drake, Wedge, & Naumann, 1998), and orthopedic surgeries, such as tendon transfer (de Roode, James, & Van Heest, 2010), and muscle and tendon lengthening procedures (Lynn, Turner, & Chambers, 2009). The goals of surgical interventions are not different from those of non-surgical interventions: pain relief, improvement of joint function, and improvement of the quality of life. Surgery is usually recommended when non-surgical options have failed. However, surgeries always present a risk, especially for fragile cerebral palsy children (Damiano & Abel, 1998). Additionally, surgical interventions are usually postponed until a certain “stability age” has been reached, as neurological conditions may be dynamic and unpredictable at a very young age, thus potentially requiring additional surgeries and producing post-surgical complications that can be avoided once the disease has stabilized.
In the past two decades, botulinum toxin type-A (BTX-A) has been introduced as a selective treatment option for spasticity in children with cerebral palsy (Koman, Mooney, Smith, Goodman, & Mulvaney, 1993). Once injected into the target muscles, BTX-A prevents acetylcholine release at the motor nerve endings, thereby inducing a dose-dependent muscle paralysis (Burgen, Dickens, & Zatman, 1949; Schiavo, Matteoli, & Montecucco, 2000). BTX-A injections have been proved to reduce muscle tone, allowing muscles to lengthen, thus increasing joint range of motion and functionality in patients (Eames et al., 1999; Sutherland et al., 1999; Ubhi, Bhakta, Ives, Allgar, & Roussounis, 2000). These outcomes will transiently increase a patient’s independence and help delay or obviate the need for assistive devices and may postpone invasive surgical interventions (Hägglund et al., 2005; Molenaers, Desloovere, Fabry, & De Cock, 2006). Additionally, BTX-A treatments have been combined with muscle strengthening (Damiano, Kelly, & Vaughn, 1995; Williams et al., 2012), casting (Ackman et al., 2005; Bottos, Benedetti, Salucci, Gasparroni, & Giannini, 2003), or electrical stimulation (Kang, Bang, & Jung, 2007), and such combined interventions have been associated with better functional outcomes in subjects with spastic muscles (Baricich, Carda, Bertoni, Maderna, & Cisari, 2008; Eleopra, Tugnoli, & De Grandis, 1997; Hesse, Reiter, Konrad, & Jahnke, 1998). It has become clear that the use of BTX-A for treating spastic muscles is a major advance and has become a widely accepted treatment modality in the management of muscle spasticity in cerebral palsy (Bjornson, Belza, Kartin, Logsdon, & McLaughlin, 2007; Boyd & Hays, 2001).

Despite its beneficial effects, being considered safe, and approved by the US Food and Drug Administration (FDA), BTX-A injections have been associated with undesired adverse
effects of target and non-target muscles (Ansved, Odergren, & Borg, 1997; Coté, Mohan, Polder, Walton, & Braun, 2005; Girlanda, Vita, Nicolosi, Milone, & Messina, 1992; Naidu et al., 2010). Specifically, Schroeder et al. reported a significant muscle atrophy of healthy human gastrocnemius lateralis one year following a single, clinically relevant BTX-A injection, suggesting that the toxin induced atrophy and potential muscle weakness may last well beyond the expected treatment period of BTX-A (Schroeder et al., 2009). Furthermore, Bakheit et al. described two patients suffering from muscle spasticity who developed generalised muscle weakness with widespread electromyography abnormalities, suspecting that the toxin was not confined to the injection site and affected distant non-target muscles (Bakheit, Ward, & McLellan, 1997).

However, the studies mentioned above were pilot experiments involving two healthy subjects (Schroeder et al., 2009) and a two study case report (Bakheit et al., 1997). Currently, clinical applications of BTX-A injections, and the timing between repeat injections, are based on clinical experience and anecdotal evidence (Barber et al., 2013). Furthermore, clinical evaluation for repeat BTX-A injections are focused on gain and/or maintenance of joint function and the ability to perform daily life activities, disregarding the effects the toxin may have on muscle strength and muscle structure (Bjornson et al., 2007; Love et al., 2001; Sutherland et al., 1999; Ubhi et al., 2000). To date, there is no systematic evaluation of muscle strength and muscle structure following repeat BTX-A injection protocols. The argument that recovery of muscle structure and function in BTX-A treated patients, such as children with cerebral palsy, is not important because BTX-A treatment is merely a precursor to more radical surgical intervention (Koman et al., 1993; Molenaers et al., 2006), should not be accepted, as the aim should be to keep
muscles functional during the treatment and post-treatment periods, so that non-surgical treatment options remain open and are encouraged.

1.2 Objective

The general objective of the research described in this thesis was to determine the structural and functional changes in the rabbit quadriceps femoris musculature subjected to BTX-A injections, and to identify possible strategies for avoiding and/or limiting undesired adverse effects and improve functional recovery of the target muscles following a period of BTX-A treatment. The New Zealand White (NZW) rabbit model was used for these studies, as this model has been established as an effective muscle weakness model (Longino, Frank, Leonard, Vaz, & Herzog, 2005).

1.2.1 Specific objectives:

The specific purposes of this thesis were to:

1. Determine the changes in muscle strength, mass, and contractile material following 1, 3, and 6 monthly BTX-A injections in the injected and contralateral non-injected quadriceps femoris muscles of NZW rabbits.

2. Determine the effects of a unilateral electrical stimulation training protocol exercise on strength, mass, and contractile material after a six-month BTX-A injection protocol in the injected quadriceps femoris of NZW rabbits, and to quantify the effects of BTX-A injections and electrical stimulation exercise on the contralateral non-injected and non-exercised musculature.
3. Investigate the rate of recovery of muscle strength, mass, and contractile material following a six-monthly BTX-A injection protocol in the injected and contralateral non-injected quadriceps femoris musculature.

4. Evaluate muscle strength, mass, contractile material, and selected mRNA expression profiles six months following a single or repeat BTX-A injections into the quadriceps femoris of NZW rabbits. An interval of three months between repeat injections was chosen to approximate clinically accepted practices.

1.3 Significance and clinical relevance

Currently, 70-80% of children with cerebral palsy suffer from muscle spasticity that impairs daily life function (Love et al., 2010). Even though cerebral palsy is a non-progressive disorder, spasticity can inhibit normal longitudinal muscle growth (Lieber & Fridén, 2002; Lieber, Runesson, Einarsson, & Fridén, 2003), thus children progressively lose joint range of motion and functionality as they grow. My research was aimed at characterizing the changes in muscle strength and structure following a period of BTX-A treatments and to evaluate the potential unwanted adverse effects induced to target muscles, and non-target muscles that are far away from the injection site. Additionally, the use of a strength training protocol, or changes in the frequency and timing of the BTX-A injection protocol might affect the adverse effects induced by the toxin on target and non-target muscles. Strength training, or variations in treatment protocols, can easily be implemented into clinical practice and into the care of children with cerebral palsy. Therefore, the results of this study could have clinical implications for the treatment of children with cerebral palsy.
1.4 Thesis outline

Including the current introductory chapter, this thesis is composed of 7 chapters. The content of these chapters is briefly described in the following:

Chapter 2 includes a select review of the relevant literature. It provides general information on the structure and mechanism of action of BTX-A as well as the usage of the toxin as a treatment modality for children with cerebral palsy and the potential adverse effects of BTX-A treatments.

Chapter 3 covers the first set of experiments in which the acute and chronic effects of BTX-A were quantified for the quadriceps femoris musculature of NZW rabbits.

In Chapter 4, I describe the potential benefits of electrical muscle stimulation during a BTX-A injection protocol for target and non-target muscles.

In Chapter 5, I describe the recovery of target and non-target muscles following a six month BTX-A injection protocol. Recovery of the muscles was assessed at 1, 3, and 6 months following the injection protocol.

In Chapter 6, I summarize the results of a study aimed at evaluating muscle recovery from a clinically relevant BTX-A injection protocol. BTX-A injections were given at intervals of three months and recovery was assessed at six months following the last injection.
Finally, in chapter 7, the significant findings of this thesis are summarized, general conclusions are discussed, and possible future work is proposed.
Chapter Two: **Review of Selected and Relevant Literature**

2.1 Cerebral palsy

Cerebral palsy is the most common cause of motor disability in childhood, affecting 2-3 children per 1000 live births (Heinen et al., 2010; Love et al., 2010). The term cerebral palsy refers to any one of a number of neurological disorders that appear in infancy or early childhood and permanently affect body movement, posture, and muscle coordination (Corry, Cosgrove, Duffy, Taylor, & Graham, 1999; Cosgrove, Corry, & Graham, 1994). Cerebral palsy is a stationary brain lesion, and as such does not worsen throughout the life span (Bax et al., 2005). However, the functional capacity of children with cerebral palsy typically deteriorates as children grow while the muscles do not grow adequately (Barber et al., 2011; Barrett & Barber, 2013; Barrett & Lichtwark, 2010). Children born with cerebral palsy typically show early impairments before they are three years old (Barber et al., 2011). The most common signs of cerebral palsy are stiff muscles, a lack of muscle coordination during voluntary movements, muscle weakness, and abnormal gait patterns, such as tip-toe, crouched, or scissored gait depending on the severity of spasticity (Bax et al., 2005; Corry et al., 1999). It has been suggested that sensory, motor, and cognitive functions are affected to variable extents, depending on the magnitude and/or location of the lesion (Bax et al., 2005). Independent of the magnitude, cerebral palsy impairs muscle function, thus joint functionality, often compromising daily life activities.

Cerebral palsy is divided into different subtypes to describe different movement impairments reflecting the areas of the brain that are damaged (Bax et al., 2005). About 70-80% of children suffering from cerebral palsy have spastic cerebral palsy, making it the most common
subtype (Love et al., 2010). Spasticity is defined by “a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes, with exaggerated tendon jerks resulting from hyperexcitability of the stretch reflex, as one component of the upper motoneuron syndrome” (Lance, 1980). Spasticity presents with increased muscle tone (“stiff muscles”) in a growing child, leading to limited motor learning and locomotor abilities, which contributes to the development of joint contractures and bony deformities, thus causing further functional impairments (Koman et al., 2003). Furthermore, spasticity can also result in alterations of skeletal muscle structure and function (Lieber & Fridén, 2002; Lieber et al., 2003). Muscle contractures, which restrict joint range of motion, are commonly assumed to be caused by “fixed” and “shortened” muscles that are not properly growing in length, which leads to muscle weakness and reduced functional capacity in cerebral palsy children (Damiano & Abel, 1998; Elder et al., 2003; Stackhouse, Binder-Macleod, & Lee, 2005). As a result, children often develop compensatory mechanisms, or coping responses, to overcome spasticity during daily life activities (Dietz & Sinkjaer, 2007; Stewart & Shortland, 2010).

Currently, cerebral palsy has no cure and although spasticity could be considered as a compensation for muscle weakness, the increased muscle tone may interfere with normal movement coordination patterns, imposing limitations on daily life activities. Despite being neural in origin, spasticity treatments are directed to the peripheral nerves and skeletal muscles in attempts to reduce muscle tone, thus preventing the progression of muscle contractures and bone deformities (Koman et al., 2004; Tilton, 2006). Conventional treatments are based on stretching of the affected muscles (Chung, Bai, Rymer, & Zhang, 2005), casting joints at angles that produce stretch to spastic muscle-tendon units (Bottos et al., 2003; Desloovere et al., 2001),
splinting joints to prevent excessive joint flexion and joint dislocation (Lai, Francisco, & Willis, 2009), using assistive walking devices (Davids, Rowan, & Davis, 2007), and physiotherapy aimed at stretching and strengthening the spastic and antagonist musculature (Dodd, Taylor, & Damiano, 2002; Williams et al., 2012). Despite such treatments, many children with cerebral palsy require corrective surgery for joint deformities.

Surgical interventions can be divided into neurological procedures, such as selective dorsal root rhizotomy (Engsberg, Ross, & Park, 1999; Wright et al., 1998), and orthopedic surgeries, such as tendon transfer, and muscle and tendon lengthening procedures with the goal to improve joint mobility and functionality (de Roode et al., 2010; Lynn et al., 2009), thereby enhancing independence and the quality of life of patients. However, surgical interventions always present a risk, especially for weak and fragile cerebral palsy sufferers. Therefore, surgical procedures are preferably postponed until a certain age, when neurological conditions have stabilized and growth is limited, thus hopefully avoiding multiple surgeries and post-surgical complications. The use of anti-spasticity drugs, such as baclofen, although effective, is limited by drowsiness and generalised muscle weakness (Koman et al., 2004). Local drug therapies, targeted at specific muscle groups, reduce the risk of drowsiness and generalised muscle weakness. Drug therapies, such as phenol nerve blocking, can create lesions in the nerve, which may be accompanied by sensory loss. On the other hand, BTX-A offers a transient local chemical denervation of the target musculature in cerebral palsy children without causing loss of sensation, and it can be used in combination with conventional treatments for children with cerebral palsy. BTX-A thus has become a widely used treatment approach with documented clinical successes that often delay the need for more aggressive surgical intervention (Molenaers et al., 2006).
2.2 Botulinum toxin type-A (BTX-A)

2.2.1 Structure and mechanism of action

Botulinum neurotoxin is produced by the anaerobic bacterium *Clostridium botulinum* and is currently one of the most potent neurotoxins known (Brin, 1997). The toxin is synthesized in seven different serotypes (A, B, C, D, E, F, G) and although all serotypes have a high affinity for motor nerve endings, they differ in neurotoxin complex size, activation level, intracellular site of action, muscle weakness efficacy, duration of action, and target affinity (Simpson, 1981). Independent of the serotype being used, they all share the same end result: they prevent acetylcholine release at the motor nerve endings (Burgen et al., 1949). Botulinum neurotoxins exert a dose-dependent muscle paralysis on the injected target musculature. Due to its chemical ablation of nerves from the target muscles, botulinum neurotoxin has become an attractive, non-invasive treatment modality for an increasing number of neuromuscular disorders with the primary aim to relax spastic muscles, for example, in children with cerebral palsy or in patients following a stroke.

Botulinum toxin type-A (BTX-A) has been the serotype of intense animal and clinical studies with the main objective to study its effectiveness on skeletal muscles (Aoki, 2001; Eleopra, Tugnoli, Rossetto, De Grandis, & Montecucco, 1998). Clinical studies have shown that BTX-A serotype is more potent and has a longer effect duration compared to serotypes B and F (Billante et al., 2002; Mezaki et al., 1995; Sloop, Cole, & Escutin, 1997). Therefore, BTX-A is the most frequently used serotype by clinicians, making it the best suitable option for the studies described here in the next chapters. Specifically, BTX-A serotype is a single polypeptide with a
molecular mass of about 150kDa (Tighe & Schiavo, 2013). It is composed of a heavy chain (100kDa) and a light chain (50kDa), linked by a disulfide bond. The heavy chain is responsible for binding and internalization of the toxin via specific synaptic vesicle (SV2) receptors at the motor nerve endings (Dong et al., 2006), while the light chain produces the paralysis effect. During physiological muscle contraction, vesicles storing acetylcholine fuse with the nerve membrane and release its content to acetylcholine receptors located on the skeletal muscle membrane. Specific proteins that are embedded in the nerve membrane are essential for the docking-fusion complex of the vesicles. However, once BTX-A is injected into the muscles, it prevents acetylcholine release by cleaving and removing nine C-terminal amino acid residues from SNAP-25 (synaptosome associated protein of 25kDa) (Schiavo et al., 1993), thereby impairing the docking, fusion and the release of acetylcholine at the terminal presynaptic membrane (Schiavo et al., 1993).

The purpose of BTX-A injections in the management of spasticity is to reduce muscle tone produced by an unwanted overactive contracting muscle. A reduction in muscle tone leads to an improvement in the joint range of motion, allowing for stretching of the tight, spastic musculature, thereby presumably promoting longitudinal muscle growth (Cosgrove & Graham, 1994). This in turn helps facilitate the application of braces, splints, and casts (Bottos et al., 2003; Desloovere et al., 2001). Furthermore, BTX-A injections have been linked to functional improvements in motor control and posture (Corry et al., 1999; Eames et al., 1999; Ubhi et al., 2000), providing the patient with the opportunity to develop compensatory behaviors during functional task activities (Bottos et al., 2003; Tilton, 2006; Ward, 2008). Also, a reduction in tone of spastic muscles may induce changes in muscle tone in other muscle groups of the limb,
causing a reduction in the overall effort required to perform movement tasks (Frasson et al., 2012; Misiaszek & Pearson, 2002).

2.2.2 Recovery following BTX-A injection

The chemical denervation induced by BTX-A is considered to be fully reversible (Tighe & Schiavo, 2013), suggesting that the toxin causes a time limited paralysis at the motor nerve endings. The clinical response of the toxin have been documented to last 3-6 months (Graham et al., 2000; Heinen et al., 2010). Therefore, if the goal of a treatment intervention is to keep muscles in a non-spastic state, repeat injections are required approximately every 3 months.

Following BTX-A injection, recovery of muscle function occurs initially by collateral nerve growth, which is known as nerve sprouting (Angaut-Petit, Molgó, Comella, Faille, & Tabti, 1990; de Paiva, Meunier, Molgó, Aoki, & Dolly, 1999). It has been demonstrated that fine nerve outgrowths occur from the nodes of Ranvier (nodal sprouting) and from the nerve terminal (terminal sprouting), running parallel along the muscle fibers (Angaut-Petit et al., 1990; de Paiva et al., 1999). It has been shown, via stained FM1-43 fluorescent labeling of sternomastoid nerve endings in living mice, that nerve sprouting appeared as early as 3 days following the initial BTX-A injection, and that these sprouts extended well beyond the original motor endplate and continued to grow up to 28 days following a first injection (de Paiva et al., 1999). After 28 days, the newly formed nerve sprouts exhibit a novel neuromuscular complex system with many key proteins present to allow for exocytosis, including SNAP-25, synaptobrevin, syntaxin, synaptotagmin, voltage-activated Na\(^+\), Ca\(^{2+}\), and acetylcholine receptors (Meunier, Schiavo, & Molgó, 2002). Furthermore, Paiva et al. showed that a new system to mediate neuromuscular
transmission at the sprouts had been formed at 28 days past the first BTX-A injection, and that exocytosis via nerve stimulation occurred exclusively in these newly formed sprouts and not in the original BTX-A poisoned parent terminal (de Paiva et al., 1999; Duchen & Strich, 1968). However, the parent terminal retained its structure. Therefore, one month following BTX-A injection, the newly formed synapses started to take over the role of the BTX-A poisoned parent terminals, allowing for muscle activation to commence approximately one month following BTX-A injection.

Following the initial nerve sprouting, a second distinct phase on muscle recovery following BTX-A injection is observed. This second stage is much slower but allows for complete recovery of the neuromuscular transmission. Approximately seven weeks following BTX-A injection, nerve sprouting is diminished and its exocytosis activity is vastly reduced. At the same time, the parent terminal recovers its function and takes over the connection of nerve to muscle via renewed acetylcholine release. The recovery of the parent terminal progresses over the next 4 weeks, and at 3 months following BTX-A injection, the parent nerve ending regains its pre-injection morphology and function. Simultaneously, the nerve sprouts are deactivated and they disappear (de Paiva et al., 1999; Holland & Brown, 1981; Meunier et al., 2002). It is not completely understood how BTX-A is metabolized and eliminated from the body, but liver involvement via hepatic biotransformation, has been suggested (Lance Simpson, 2013).

2.2.3 Clinical application of BTX-A
2.2.3.1 History

Botulinum neurotoxin was first suggested as a therapeutic treatment by a German medical doctor, Justinus Kerner, who described 155 case reports of patients who had contracted botulism from food poisoning in 1822. The patients had clinical symptoms that suggested that the toxin acted by interrupting the transmission between the nervous and musculoskeletal system. He wrote: “the capacity of nerve conduction is interrupted by the toxin in the same way as in an electrical conductor by rust”. In the final chapter of his monograph, Dr. Kerner proposed the possibility of using the toxin as a remedy for a variety of neuromuscular disorders and concluded that if the toxin is applied in minimal dosages it might help alleviate hyperactivity and hyperexcitability in patients (Erbguth, 2007; Meyler & Cooper, 2007).

Since its early discovery, BTX-A has been the object of intense study. In 1895, a Belgium microbiologist, Emile Pierre Marie Van Ermengem, isolated the bacteria from food poisoned ham and corpses of victims of botulism. He grew the bacteria and used them initially in animal experimentation. The pathogen was later named *Clostridium botulinum*. Next, Burgen *et al.* discovered that the toxin acted by preventing acetylcholine release at the nerve endings (Burgen et al., 1949). Later, Edward Schantz developed the first batch of toxin and, together with Alan Scott, developed the toxin for use in human disease in 1968 (Erbguth, 2007).

In 1973, Scott published promising preliminary results describing how minute doses of BTX-A injected into the ocular muscles of monkeys was effective at treating strabismus. The main objective was to prevent acetylcholine release at the motor nerve endings and balance neural input to the extraocular rectus muscles, thereby balancing the muscle forces that would
straighten the eye. Scott said: “an injection of a few picograms would induce paralysis confined to the target muscles, long in duration, and with no side effects whatsoever” (Scott, 2004). Next, human experiments were performed that showed promising effects in patients suffering from strabismus, blepharospasm, and hemifacial spasms (Scott, 2004). These experiments led to a first approval of the toxin for human use by the US Food and Drug Administration (FDA) in 1989 (Erbguth, 2007). The toxin was later also FDA approved for muscle dystonia (2000) (Jankovic, 2004) and in aesthetic medicine for temporary improvement of glabellar lines associated with corrugator and/or procerus muscle activity in young adults (2002) (Ascher et al., 2010). These applications then turned into the now popular use of BTX-A for cosmetic applications.

Currently, BTX-A is FDA approved (2010) for the treatment of spasticity in the flexor muscles of the elbow, wrist, and finger in adults following stroke, traumatic brain injury, or in the progression of multiple sclerosis (Brashear et al., 2002; Childers et al., 2004). However, the toxin has not been shown to be safe and effective for spasticity in the lower limbs and for children with cerebral palsy, suggesting that children might be at greater risk of developing adverse side effects (Frasson et al., 2012; Naidu et al., 2010).

2.2.3.2 Clinical studies in children with cerebral palsy

In the late 1980s, Andrew Koman pioneered the use of BTX-A in pediatric treatment of lower extremity spasticity in children with cerebral palsy. A preliminary investigation published in 1993 showed that BTX-A at a dose of 1-2U/kg of body weight in 27 pediatric, 7 years old on average, reduced muscle spasticity, was considered safe, had lasting effects for 3-6 months and was repeatable. They concluded that BTX-A injections were a valuable adjunct therapy to manage spasticity in cerebral palsy (Koman et al., 1993). The patients in that initial study had
different extents of spasticity, the spasticity affected different muscle groups, and some were ambulatory while others were not. They were evaluated prior to BTX-A injections, 2 weeks, 2-3 months, and up to on average 15.5 months following injection by means of subject comfort, change in spasticity, range of motion, gait analysis, and subjectively by parent/guardian feedback. The authors suggested that although BTX-A may not permanently remove spasticity, and thus the need for surgical intervention, it may help in delaying aggressive surgical procedures until spasticity is stable and patients are older and at a lesser risk of potential complications from repeat surgical interventions (Eames et al., 1999; Koman et al., 1993; Molenaers et al., 2006).

Since the early studies by Koman et al. (1993), there has been an abundance of randomized controlled trials to investigate the efficacy and safety of BTX-A treatments in children with cerebral palsy. These studies were focussed primarily on the effects of BTX-A injection as a therapeutic tool aimed at decreasing muscle tone, thereby improving joint range of motion and joint function. Some studies were also aimed at evaluating the potential for combining BTX-A treatment with therapies such as stretching, casting, and electrical stimulation. Next, I will describe some of the research done in cerebral palsy children highlighting the beneficial effects following BTX-A injection on gait function, the optimal dosage, the effects of repeat BTX-A injection, the use of the toxin associated with other conservative treatments, and the adverse effects induced by BTX-A application.

2.2.3.2.1 Effects of BTX-A injections on gait function
During normal gait, the “first rocker” refers to the period immediately after heel strike, when the tibialis anterior eccentrically lowers the foot to the ground. This control part of the gait is frequently compromised in children with cerebral palsy, landing with a flat foot or directly with the toe (toe-tip gait) so that the “first rocker” is absent. The “second rocker” happens throughout the stance phase when the ankle progressively dorsiflexes, thereby lengthening the gastrocnemius muscle. Cerebral palsy is often associated with shortened and stiff musculature resulting in a lack of gastrocnemius lengthening during the “second rocker”. Last, the “third rocker” in the stance phase of walking consist of the phase associated with plantar flexion of the ankle due to activation of the gastrocnemius for propulsion. However this phase is also often absent in children with spastic cerebral palsy (Boyd, Pliatsios, Starr, Wolfe, & Graham, 2000; Engström et al., 2010). Studies on the gastrocnemius of hereditary spastic mice showed that BTX-A injections allow for normal muscle-tendon longitudinal growth when compared to untreated spastic animals (Cosgrove & Graham, 1994). Since a spastic gastrocnemius is the most common deficiency in children with cerebral palsy, and since this deficiency compromises standing balance and gait function (Boyd et al., 2000; Chan & Miller, 2014), clinical trials have been done to evaluate the efficacy of gastrocnemius BTX-A injections in children with spastic cerebral palsy.

Koman et al. (1994) confirmed his previous pilot findings (Koman et al., 1993) that a small dosage of BTX-A into the lower leg of twelve ambulatory spastic cerebral palsy children improved gait function, as assessed by a physician rating scale of gait (PRS) and video analysis when compared to placebo injections. Sutherland et al. (1999) and others (Koman, Mooney, Smith, Walker, & Leon, 2000; Ubhi et al., 2000) showed that BTX-A injections into the
gastrocnemius of children with cerebral palsy increased ankle range of motion and dorsiflexion during the stance phase of gait one month following treatment. Additionally, some children occasionally developed the “first rocker” by proper heel strike (Boyd et al., 2000). These studies showed benefits at 1-3 months following BTX-A injection. Others showed a persistent effect of BTX-A treatments lasting six months following injection. They suggested that this might be a “window of opportunity” to increase the strength of dorsiflexors during a period of artificially-induced weakness of the antagonist musculature (Love et al., 2001).

Improved gait function was associated with a reduction in muscle tone following BTX-A injection. Muscle tone, assessed by the Modified Ashworth Scale (MAS), has been shown to decrease significantly following injection (Bjornson et al., 2007; Love et al., 2001). However, studies using the Gross Motor Function Measure (GMFM), which is a clinical tool to evaluate changes in gross motor function in children with cerebral palsy, showed inconsistent results following BTX-A treatments (Baker et al., 2002; Bjornson et al., 2007).

2.2.3.2.2 Optimal BTX-A dosage

The literature offers a large range of dosages to obtain optimal effects on muscle function following BTX-A injection. In the first investigations on BTX-A interventions (Cosgrove et al., 1994; Koman et al., 1993) a dosage of 1-2U/kg of body weight was used in children with spastic cerebral palsy, with no reports of relevant adverse effects. In subsequent studies, higher dosages were used ranging from 4U/kg (Koman et al., 2001; Sutherland et al., 1999), 5-8U/kg (Corry et al., 1999), 10U/kg (Koman et al., 2000), 12U/kg (Bjornson et al., 2007), 15-25U/kg (Ubhi et al., 2000; Willis, Crowner, Brunstrom, Kissel, & Racette, 2007), up to a maximum of 30U/kg of
body weight (Baker et al., 2002; Naidu et al., 2010). The European consensus for BTX-A injections for children with cerebral palsy ranges from 4 to 30U/kg of body weight for single muscle and multi-muscle injections. Injections are prepared in a 25-100U/ml dilution, with a maximum total dosage of 400-600U (Heinen et al., 2010; Naidu et al., 2010). Currently, clinical applications of BTX-A are based on clinician experience, preference, and anecdotal evidence. There is no evidence of an optimal dosage per muscle or per child that maximizes the treatment response and minimizes adverse effects.

There is a limited number of studies aimed at investigating the dose-response curves of BTX-A treatments in children with spastic cerebral palsy. In a randomized placebo controlled trial, Baker et al. (2002) compared the magnitude and duration effects of low, medium, and high dose (10, 20, 30U/kg, respectively) BTX-A injections into the gastrocnemius musculature of children with cerebral palsy. They reported that the best dosage to increase gastrocnemius muscle length and improve gait was the medium dosage (20U/kg), which lasted up to four months following injection. Moreover, they showed that there was a smaller gain in muscle function with the highest dosage (30U/kg). The authors speculated that the highest dosage of 30U/kg might have caused spread of the toxin to neighbouring non-target muscles, thereby offsetting some of the beneficial effects that otherwise might have been achieved. They used the same injection volume for the three dosages, thus perfusion of the toxin should have been approximately equivalent (Baker et al., 2002). Similarly, Mancini et al. (2005) found that a medium dosage of BTX-A (320U) produced significant decreases in muscle tone and gait improvement up to four months following BTX-A injection into the spastic foot of post-stroke patients. The high dosage BTX-A group (540U) in their study had similar beneficial results.
However, the benefits in this group were associated with excessive muscle weakness of target and neighbouring non-target muscles, suggesting that an “optimal” dosage may indeed exist (Mancini, Sandrini, Moglia, Nappi, & Pacchetti, 2005).

2.2.3.2.3 Repeat BTX-A treatments

BTX-A injections produce a decrease in muscle tone lasting 3-6 months. Repeat injections may prolong the beneficial effects, preserving joint function and postpone the need for surgical interventions. Only a few studies have been performed in which multiple BTX-A treatments were administered. These studies were aimed at observing the persistence of clinical efficacy, the possibility of postponing surgical interventions, and the presence of adverse effects in children with cerebral palsy.

Koman et al. (2001) suggested that repeat BTX-A injections were safe and effective in the management of chronic equinus foot deformity in children with cerebral palsy. Repeat BTX-A injections were performed, on average, every three months. The effectiveness of the treatments was evaluated using a physician rating scale of gait and passive/active ankle range of motion for up to four years following BTX-A treatments. Eventual adverse effects were recorded at each visit, with 1-11% of patients reporting adverse effects such as stumbling, cramps, weakness, and calf atrophy (Koman et al., 2001). Eames et al. (1999) showed an increase in gastrocnemius muscle length with repeat BTX-A injections over a 24 week treatment period. However, only few patients showed continued benefits at the 12 month follow up evaluation, suggesting that the effects of the toxin are time-limited. Nevertheless, it was argued that if the toxin can prevent muscle shortening and prevent fixed contractures, it might be a suitable treatment modality for
delaying surgical treatments (Eames et al., 1999; Hägglund et al., 2005; Molenaers et al., 2006). Fattal et al. (2008) reported long-term effects on muscle tone, range of motion, and gross motor function classification scale, for the first two injections, but not for subsequent BTX-A injections in the lower limbs of children with spastic cerebral palsy (Fattal-Valevski, Domenievitz, Giladi, Wientroub, & Hayek, 2008) (Fattal-Valevski et al., 2008). Similarly, significant improvements in gait parameters were observed after the first and second BTX-A injections into equinus feet of children with cerebral palsy, but these improvements decreased with the third and fourth injections (Metaxiotis, Siebel, & Doederlein, 2002). The authors speculated that the decreased effect with multiple injections might have been caused by fixed contractures that occurred because of age and not because of the blunted effect of the BTX-A injections. Likely however, the beneficial effects of BTX-A injections decrease with repeat injections and duration of the treatment protocols. This suggestion is further supported by Barber et al. (2013), who found no differences in muscle volume, fascicle length, and physiological cross-sectional area in muscles exposed to single or repeat BTX-A injections (Barber et al., 2013).

In all the work done on repeat BTX-A injection and its benefits on gait function and safety in cerebral palsy children, there is no study in which the effects of BTX-A injections on muscle structure were studied systematically. If a single BTX-A injection can cause muscle atrophy (Koman et al., 2001), repeat injections might induce further atrophy and could potentially cause long-term functional deteriorations. Therefore, it seems paramount to evaluate muscle structure and function following repeat BTX-A injection treatments, and the recovery of muscle function and strength after long-term chemical denervation through BTX-A.
2.2.3.2.4 Combined treatments

BTX-A treatment has not been prescribed as a replacement therapy for physiotherapy or orthoses. Instead, it should be viewed as an adjunct therapy to current conservative treatment strategies. Indeed, it has been recommended that BTX-A treatments are accompanied by physiotherapy and/or splint stretching of spastic muscles to take advantage of the muscle relaxation effect produced by BTX-A, possibly causing prolonged reductions in muscle spasticity (Ryll, Bastiaenen, De Bie, & Staal, 2011).

It has been suggested that electrical stimulation applied immediately after BTX-A injection treatments enhances toxin uptake into target muscles (Hughes & Whaler, 1962). Kang et al. (2007) showed that BTX-A injections combined with electrical stimulation of the gastrocnemius in patients with cerebral palsy resulted in greater increases in the range of motion and improved gait function compared to BTX-A alone (Kang et al., 2007). Furthermore, in a preliminary study in post-stroke patients, it was found that there was no difference in muscle tone and gait variables when comparing a low BTX-A dosage associated with electrical stimulation with a high BTX-A dosage, suggesting that electrical stimulation helped reduce the amount of toxin required to produce the desired effects. Therefore, electrical stimulation could be used to reduce not only the cost of treatments, but also the risk of adverse effects (Bayram, Sivrioglu, Karli, & Ozcan, 2006). Finally, Minamoto et al. (2007) showed that electrical stimulation enhanced BTX-A uptake into target muscles and prevented the spread of the toxin into non-target musculature (Minamoto et al., 2007).
Similarly, it has been demonstrated that taping associated with BTX-A treatment reduces muscle tone significantly compared to BTX-A injections alone. The beneficial effects of taping have been demonstrated for wrist and fingers, as well for the ankle joint (Carda & Molteni, 2005; Reiter, Danni, Lagalla, Ceravolo, & Provinciali, 1998). The use of joint casting following BTX-A injection has also been shown to be more effective in correcting foot deformities in patients with cerebral palsy compared to BTX-A injections alone (Ackman et al., 2005).

It is well accepted that children with cerebral palsy are weaker, have greater co-contraction, and cannot activate their muscles as completely as typically developing children (D L Damiano & Abel, 1998; Stackhouse et al., 2005). Skeletal muscle training is now recognized as an effective intervention modality to increase muscle strength in cerebral palsy (Dodd et al., 2002) and may be combined with BTX-A treatment. While strength training targets muscle weakness, BTX-A aims to overcome muscle spasticity. Williams et al. (2013) reported a significant reduction in muscle spasticity and an increase in muscle volume and strength lasting up to six months when BTX-A treatments were combined with strength training in patients with cerebral palsy (Williams, Reid, Elliott, Shipman, & Valentine, 2013).

2.2.4 Adverse effects

Despite its beneficial effects, its safety record and its approval by the US Food and Drug Administration (FDA) for a variety of neuromuscular disorders, BTX-A treatments have been associated with undesired adverse effects on target and non-target muscles (Antonucci, Rossi, Gianfranceschi, Rosetto, & Caleo, 2008; Coté et al., 2005; Frasson et al., 2012; Koerte et al.,
2013; Lange, Brin, Warner, Fahn, & Lovelace, 1987; Schroeder et al., 2009; Yaraskavitch, Leonard, & Herzog, 2008). BTX-A treatments have been approved for spasticity in the flexor musculature of the elbow, wrist, and fingers in adults (Koman et al., 2013). However, due to a higher incidence of adverse effects in children with cerebral palsy compared to patients with other conditions, children with cerebral palsy appear at greater risk and further studies are necessary to guarantee the efficacy and safety of BTX-A treatment in this particular patient cohort (Albavera-Hernández, Rodríguez, & Idrovo, 2009; Naidu et al., 2010). The adverse effects induced by BTX-A injections can be divided into local and systemic adverse effects.

2.2.4.1 Local adverse effects

The most common local adverse effects induced by BTX-A injections reported in animal and clinical studies include injection site pain, edema, erythema, muscle atrophy, and excessive muscle weakness in target and non-target muscles adjacent to the injection site.

Injection pain, edema, and erythema are usually caused by the BTX-A administration and typically resolve without complications. Patients that suffer from neck dystonia have reported dose-dependent swallowing problems (dysphagia) following BTX-A injections (Dutton, 1996). Furthermore, patients with blepharospasm (excessive blinking), hemifacial spasm, or receiving BTX-A injections for the removal of wrinkles around the eye have suffered from ptosis, a weakness of the upper eyelid musculature which results in difficulties controlling eye opening (Eleopra, Tugnoli, Caniatti, & De Grandis, 1996; Naumann, Albanese, Heinen, Molenaers, & Relja, 2006). Also, in animal studies it has been shown that a single BTX-A injection into the cat soleus muscle produced significant weakness in the non-target neighboring plantaris muscle one
months following injection (Yaraskavitch et al., 2008). This weakness in non-target neighboring musculature has also been detected by single fiber electromyography (EMG) alterations and twitch responses in patients with writer’s cramp (Ross, Charness, Sudarsky, & Logician, 1997).

In studies on the rat tibialis anterior muscle, it has been found that BTX-A injections can cross inter-muscular fascial tissues even when administered at low, subclinical doses (Shaari, George, Wu, Biller, & Sanders, 1991; Shaari & Sanders, 1993). Furthermore, in a histologic study examining acetylcholinesterase staining and muscle fiber diameter, Borodic et al. (1990, 1994) demonstrated a dose-dependent gradient diffusion around the site of injection into the rabbit longissimus dorsi musculature (Borodic, Ferrante, Pearce, & Smith, 1994; Borodic, Joseph, Fay, Cozzolino, & Ferrante, 1990). Therefore, it has been recommended (Shaari et al., 1991; Shaari & Sanders, 1993) that BTX-A injections should be performed as close as possible to the motor nerve endings and at a low dilution in order to avoid BTX-A spread into neighboring muscles.

BTX-A injections have also caused prolonged muscle denervation and muscle atrophy. For example, patients who suffered from chronic anterior knee pain and received BTX-A injection into the distal third of the vastus lateralis musculature had persistent focal atrophy and muscle denervation signs revealed by single fiber EMG 12 months following the treatment (Dunne, Singer, Silbert, & Singer, 2010; Silbert, Singer, Silbert, Gibbons, & Singer, 2012). Similarly, Schroeder et al. reported a significant muscle and neurogenic atrophy of healthy human gastrocnemius lateralis one year following a single, clinically relevant BTX-A injection. Atrophy was indicated as a reduction in cross-sectional area assessed by means of magnetic
resonance imaging at 3, 6, and 12 months following injection. Neurogenic atrophy and degenerative changes at the neuromuscular junction were also confirmed in that study by muscle biopsy at 12 months (Schroeder et al., 2009). Also, the same group injected BTX-A into the forehead musculature to reduce glabellar lines. Again, they found significant muscle atrophy 12 months following injection. However the glabellar lines had returned to pre-injection severity at 6 months following the injection (Koerte et al., 2013). These two pilot investigations performed on healthy subjects demonstrated that muscle atrophy can persist well beyond the 3-6 months period which represents the consensus for the timing of repeat injections. The authors suggested that the neurogenic muscle atrophy should be monitored when repeat injections into the same musculature are indicated. Furthermore, they suggested that magnetic resonance imaging should be used to monitor the long-term effects of BTX-A injections into skeletal muscles.

2.2.4.2 Systemic adverse effects

Systemic adverse effects have been reported more recently than local adverse effects. They consist of nausea, general muscle weakness leading to frequent falls, difficulties in breathing and swallowing, seizures (Albavera-Hernández et al., 2009), and death (Coté et al., 2005; Naumann et al., 2006). More specifically, clinical studies have shown that patients receiving BTX-A injections into the neck region had abnormal EMG responses in remote forearm muscles (Garner, Straube, Witt, Gasser, & Oertel, 1993; Girlanda et al., 1992; Lange et al., 1987; Olney, Aminoff, Gelb, & Lowenstein, 1988). Moreover, children with cerebral palsy were found to develop bladder and bowel incontinence one week following BTX-A injection to treat spasticity of the hip adductor or the calf musculature. However, incontinence seems to resolve typically within six weeks (Naidu et al., 2010; O’Flaherty, Janakan, Morrow, Scheinberg,
& Waugh, 2011). Additionally, in case studies, it has been reported that patients receiving local BTX-A treatments suffered from general muscle weakness, with some patients not fully recovering within four months. Selected case reports talk of patients who were found to have difficulties breathing and swallowing that required hospitalization (Bakheit et al., 1997; Bhatia et al., 1999). Histologically, it has been shown that patients with cervical dystonia presented vastus lateralis atrophy after BTX-A injections compared to age-matched control (Ansved et al., 1997).

It is not entirely understood how BTX-A spreads systemically, thereby affecting muscles far from the injection site. However, two mechanism have been put forward as possible avenues for the systemic spread of BTX-A: (i) via the blood stream, and (ii) by retrograde transport along nerves. Regarding the spread of BTX-A via the blood stream, this has been suggested because of the essentially immediate observation of abnormal single fiber EMG in lower limb and upper limb musculature in patients receiving BTX-A injections in the neck area for dystonia. These observations suggest that the toxin can travel quickly throughout the body and affect distant muscles virtually immediately following injection (Garner et al., 1993). Regarding the idea of retrograde transport of BTX-A in nerves, Wiegand et al. injected I125 radiolabeled BTX-A into the cat gastrocnemius muscle and detected toxin activity in the sciatic nerve and in the ipsilateral half segment spinal cord within 48 hours following injection. Interestingly, the toxin was also detected in the spinal cord half segment contralateral to the injection (Habermann, 1974; Wiegand, Erdmann, & Wellhöner, 1976). At the time of first observation, it was not clear whether the radioactive toxin corresponded to the same potency as a catalytically active BTX-A. However, later studies in mice confirmed that the labelled toxin detected in nerves was indeed active (Antonucci et al., 2008; Matak, Riederer, & Lacković, 2012).
Taken together, the local and systemic adverse effects induced by BTX-A treatments suggest that the toxin affects muscle strength and muscle structure, as evidenced by muscle atrophy and weakness of injected target muscles, non-target neighboring muscles, and non-target remote muscles. Due to its prolonged but reversible action, there is a need for prospective studies aimed at evaluating muscle structure and function in the short- and long-term during and following BTX-A treatment. Although muscle function might be back to normal within 3-6 months of BTX-A treatments, muscle structure might still be compromised, suggesting that the toxin may have long-lasting adverse effects that have not been identified. It has been suggested that, because of the lack of information on chronic BTX-A exposure, particularly in the developing child, that children with cerebral palsy should receive repeat BTX-A injections only when alternative therapies have failed, or when the severity of the symptoms justifies the risks (L A Koman et al., 1993; Russman, Tilton, & Gormley, 1997).

At present, BTX-A treatments have not been approved by the US FDA for children with spastic cerebral palsy. This is probably due to a lack of information and the uncertainties regarding the effects of the toxin on the skeletal muscles of developing children. BTX-A injections are given with large variations in dosages and number of repeat injections. They are largely based on clinical experience rather than systematic evidence-based studies, thus we do not know the optimal dosage, the most efficient intervals between injections, or the maximum dosage that can be safely applied for children of different ages and with different severities of spasticity. BTX-A spreads in a manner whose details are not fully understood, and which require further investigation to avoid serious adverse effects. Also, rehabilitation protocols should be
designed specifically to overcome the adverse effects of BTX-A treatments so muscle structure and function is not compromised once BTX-A treatments have been finished.
Chapter Three: Changes in the Rabbit Quadriceps Contractile Properties Following Repeat BTX-A Injections

3.1 Introduction

BTX-A injections have become a common treatment modality for an increasing number of neuromuscular disorders with the primary aim to relax spastic muscles, for example, in patients with cerebral palsy or following stroke (Bakheit et al., 2001; Koman et al., 1993; Sutherland et al., 1999). Once injected into the target muscles, BTX-A prevents acetylcholine release at the motor nerve endings, thereby producing a dose-dependent muscle paralysis (Brin, 1997).

Although considered to be safe and approved by the US Food and drug administration (FDA) for neuromuscular disorders such as strabismus, dystonia, hemifacial spasm, and spasticity in adults following stroke, BTX-A treatment have been linked to regional and systemic adverse effects following the treatment period. Regional adverse effects include excessive weakness of the target (O’Flaherty et al., 2011) and neighboring non-target muscles (Yaraskavitch et al., 2008), while systemic adverse effects include generalized muscle weakness (Coté et al., 2005; Howell, Selber, Graham, & Reddihough, 2007), muscle atrophy (Ansved et al., 1997), and dysfunction of non-target neuromuscular junctions (Girlanda et al., 1992; Lange et al., 1987).

With an increase in the use of BTX-A treatment for an ever growing number of neuromuscular disorders, including not FDA approved repeat injections in young children with cerebral palsy, the effects of repeat injections on muscle strength, mass, and structure must be known. Although decreases in muscle strength and mass have been reported for specific time
points following BTX-A treatment (Longino et al., 2005; Longino, Butterfield, & Herzog, 2005), the associated changes in muscle structure for single and repeat injections remain unknown.

Therefore, the primary purpose of this study was to investigate the changes in muscle strength and mass in BTX-A injected muscles while simultaneously characterizing muscle structure and histology in order to determine possible changes in the amount of contractile material. A secondary purpose was to identify whether the changes in outcome measures are muscle specific, and if BTX-A injections affect non-target muscles. Therefore, mass and histological outcomes were determined for three target and three non-target muscles following 1, 3, and 6 injections of BTX-A separated by a month, and administered to the quadriceps femoris of New Zealand white (NZW) rabbits.

3.2 Methods

3.2.1 Experimental Design

Twenty skeletally mature, one year old, female NZW rabbits weighing an average of 5.5kg were used for this study. Approval of all procedures was obtained by the Animal Care Committee from the University of Calgary. Animals were allowed normal cage activity (65 x 45 x 30cm³), and received a standard diet.

Animals were divided into four groups as follow:

1. Control group – saline injection unilaterally (n=5; Control)
2. 1-months post-single BTX-A injection unilaterally (n=5; 1 BTX-A)
3. 3-months post-repeated monthly BTX-A injections unilaterally (n=5; 3 BTX-A)
4. 6-months post-repeated monthly BTX-A injections unilaterally (n=5; 6 BTX-A)
3.2.2 *Botulinum Toxin Type-A: injection protocol*

Rabbits were injected with *Clostridium botulinum* type-A neurotoxin complex (BOTOX, Allergan, Inc., Toronto, Ontario, Canada), which was reconstituted with 0.9% sodium chloride to a concentration of 20 units/ml. Rabbits received intramuscular BTX-A injections at a total dosage of 3.5U/kg of body weight. Injections were randomized to either the right or left quadriceps musculature. The anterior compartment of the thigh was isolated by manual palpation and the quadriceps was visually divided into superior and inferior halves. Each half was subdivided into medial, central, and lateral sections. One sixth of the total toxin was injected into each section to increase diffusion and to inject BTX-A directly into the vastus lateralis, rectus femoris and vastus medialis muscles.

Group 1 rabbits served as a control and received intramuscular saline injections randomized to either the right or left quadriceps musculature. The total volume of the injected saline was the same as the total volume of BTX-A. Group 2 rabbits received a one-time intramuscular BTX-A injection, while group 3 and 4 rabbits received monthly BTX-A injections into the same hind limb (randomized for the first injection) for 3 and 6 months, respectively. The primary outcome measures included knee extensor torque, quadriceps muscle mass, and the percentage of contractile material for the different quadriceps muscles (for details see appendix A-1).

3.2.3 *Knee extensor torque and muscle mass*

Isometric knee extensor torque was measured at the end of the 1 month (group 2 rabbits), 3 months (group 3 rabbits), and 6 months (group 4 rabbits) experimental periods. Knee extensor
torque in injected and contralateral non-injected hind limbs was obtained by stimulating the knee extensor musculature via a femoral nerve cuff electrode that was implanted prior to testing (D Longino et al., 2005). Following nerve cuff implantation, rabbits were secured in a stereotactic frame using bone pins at the pelvis and femoral condyles. Isometric knee extensor torques at 100° of knee flexion (full knee extension was defined as 180°) were measured using a strain-gauged bar placed over the anterior and distal portion of the rabbit’s tibia.

Stimulation of the knee extensor muscles was given through a Grass S8800 stimulator (Astro-Med Inc., Longueil, Quebec, Canada) at a voltage three times higher than the alpha motoneuron threshold, to ensure activation of all motor units (Herzog & Leonard, 1997). Stimulation duration was 500ms, pulse duration was 0.1ms, and the frequency of stimulation was 100Hz. A two minutes rest period was given between trials.

Following determination of knee extensor torque, animals were sacrificed by an overdose injection of Euthanyl (MTC Pharmaceutical; Cambridge, Ontario). Wet muscle mass for each quadriceps portion was determined using a commercial scale with a resolution of 0.001g.

3.2.4 Contractile material

The contractile material was defined as the percentage of contractile material tissue quantified in histological samples of the quadriceps musculature. To determine the percentage of contractile material in rectus femoris (RF), vastus lateralis (VL), and vastus medialis (VM), the central third of each muscle was embedded in paraffin (automatic paraffin processor, Leica TP 1020) and cut cross-sectionally, with a microtome (Leica RM 2165). Every 100μm, an 8μm
section was collected for staining with hematoxylin-eosin (Leica ST5010). A total of 5 slides for each muscle were analyzed and averaged.

Photographs were taken for each stained muscle section using an Axionstar plus microscope (Carl Zeiss) using a 5x magnification. A customized Matlab program (Matlab 7.8, R2009a) was used to calculate the contractile material for at least 50% of the total muscle cross-sectional area relative to the total cross-sectional area of the muscles.

3.2.5 Data analysis

Knee extensor torque and muscle mass of the experimental hind limbs were expressed as a percentage relative to control group rabbits. The reduction in torque will hereafter be referred to as muscle weakness while the loss of muscle mass will be referred to as atrophy. The amount of contractile material for each muscle is given as the percentage area fraction calculated from the total cross-sectional areas of the analyzed sections.

A two-way ANOVA with repeated measured was used to assess muscle weakness with the main factors leg (injected and contralateral non-injected) and groups (control, 1, 3, and 6 months post BTX-A injections). A three-way ANOVA with repeated measured was used to assess muscle atrophy and contractile material with the main factor legs, groups, and muscle (VL, RF, and VM). A post-hoc Tukey test was performed if indicated. The level of significance was chosen as $\alpha=0.05$ a priori.
3.3 Results

The injection procedures were well tolerated by all rabbits. Data were analyzed from all animals, except for the percentage of contractile material from the rectus femoris of the control rabbits, as these samples were of bad quality.

3.3.1 Muscle weakness

There was no difference in muscle torque between hind limbs of the control group rabbits. The injected hind limb of group 2, 3 and 4 rabbits were significantly weaker (90-95%) than the non-injected controls ($p<0.05$; Figure 3-1).

The contralateral non-injected hind limbs of the experimental rabbits were consistently weaker than the hind limbs of the non-injected control rabbits. However, this difference (13%) was not significant for group 2 and 3 rabbits (1 and 3 months of BTX-A injections) but was significant (85%) for group 4 rabbits (6 months of BTX-A injections; Figure 3-1). Muscle weakness in the contralateral non-injected hind limb of group 4 rabbits was such that the difference between injected and contralateral hind limbs was not significant ($p>0.05$) in these animals.
Figure 3-1 Mean muscle strength (± 1 SE) following 1, 3, and 6 months BTX-A injections for injected (dark bars) and contralateral non-injected hind limbs (light bars) normalized to the values of Control group rabbits (100%). A significant difference ($p<0.0001$) was observed for the injected hind limbs compared to control for all experimental group animals. Muscle weakness was also observed in the contralateral non-injected hind limbs of all experimental group rabbits, but this difference was only statistically significant for group 4 animals (6 monthly injections of BTX-A) ($p<0.0001$). * compared to control group rabbits. For absolute values see appendix C-1.
3.3.2 Muscle atrophy

There was no difference in muscle mass between quadriceps muscles of control group rabbits. Muscle atrophy in the injected hind limbs of group 2, 3, and 4 rabbits (1, 3, and 6 months of BTX-A injections, respectively) was significantly greater compared to control rabbits. The mean muscle atrophy averaged across all muscles was 43%, 60% and 56% for groups 2, 3, and 4 rabbits, respectively (Figure 3-2). Muscle atrophy increased significantly from 1 to 3 months of BTX-A injections, but not from 3 to 6 months of BTX-A injections (Figure 3-2).

There was a loss in muscle mass of 15%, 15%, and 37% for the muscles of the contralateral non-injected hind limbs of 1, 3, and 6 months of BTX-A injection group rabbits, respectively, compared to control rabbits, although this difference only reached statistical significance for the 6 months BTX-A injection group rabbits (Figure 3-2).
Figure 3-2 Mean muscle mass (± 1 SE) following 1, 3, and 6 months of BTX-A injection for injected (dark bars) and contralateral non-injected (light bars) hind limbs. Significant atrophy ($p < 0.0001$) was observed for the injected hind limbs for all experimental group rabbits compared to control group rabbits. Muscle atrophy was also observed in the contralateral non-injected hind limbs of all experimental group rabbits. However, this atrophy was only significant for the 6 months BTX-A group ($p < 0.0001$). * compared to control group rabbits. For absolute values see appendix C-2.

The loss in muscle mass following BTX-A injections was muscle specific with VL showing the greatest losses (51%, 68% and 76% at 1, 3, and 6 months of BTX-A injections,
respectively), followed by RF (43%, 52%, and 44%, respectively) and VM (32%, 50%, and 14%, respectively) as shown in Figure 3-3.

Figure 3-3 Mean muscle atrophy (± 1 SE) following 1, 3, and 6 months of BTX-A injection for the different portions of the quadriceps femoris (VL=vastus lateralis; RF=rectus femoris; VM=vastus medialis). Significant muscle atrophy ($p<0.05$) was observed for the different portions of the quadriceps femoris of the experimental group rabbits compared to the control group rabbits. * compared to control group rabbits. For absolute values see appendix C-3.
3.3.3 Contractile material

The percentage of contractile material for the VL and VM for the control group rabbits was 96% (±0.8%), and 96% (±2.7%), respectively. Following a single BTX-A injection, these values remained the same for VL (94%; ±3.0) and VM (98%; ±0.5%), while the RF values (for which no reference values were obtained) had approximately the same amount of contractile material (94%; ±7.7%) as VL and VM. After 3 monthly BTX-A injections, the percent contractile material was 72% (±4.1%), 97% (±1.1%), and 73% (±6.7%) for VL, VM, and RF, respectively, which were statistically smaller for VL and RF ($p<0.05$) compared to control group rabbits. Furthermore, the percentage of contractile material for the 6 BTX-A group rabbits was 43% (±9.7%), 78% (±4.2%), and 70% (±8.0%) for VL, VM, and RF, respectively, all of which were significantly smaller ($p<0.05$) than all previous control and experimental group values (Figure 3-4; left column).

For the contralateral non-injected hind limbs of control group rabbits, the percentage of contractile material for VL and VM was 95% (±3.0%) and 94% (±1.7%), respectively. Following a single BTX-A injection, these values remained the same: 97% (±1.0%), 98% (±0.4%), and 97% (±0.8%) for VL, VM, and RF, respectively, as they did for the 3 months group rabbits (96% (±1.4%), 97% (±0.7%), and 96% (±1.2%) for VL, VM, and RF, respectively). However, the percent contractile material of the contralateral non-injected hind limbs of 6 BTX-A group rabbits decreased significantly to 74% (±6.5%), 82% (±5.2%), 81% (±6.5%) for VL, VM, and RF, respectively (Figure 3-4; right column).
Figure 3-4 Exemplar histological cross-sectional images showing the contractile material (H&E; red staining) and non-muscle contractile material (white color; assumed to be primarily fat and connective tissue) for muscles from injected (left column) and contralateral non-injected (right column) for control, 1 BTX-A, 3 BTX-A, 6 BTX-A group rabbits (first, second, third, and fourth row, respectively). There was a significant reduction in the percent contractile material. Contractile material seemed to be replaced
primarily by fat at 3 months following BTX-A injection on the injected side and this fat infiltration was even more pronounced for 6 BTX-A group rabbits. A significant loss of contractile material in the contralateral hind limbs was only observed for 6 BTX-A group rabbits, and the contractile material seemed to be replaced by connective tissue, rather than fat, as observed in the experimental hind limbs.

3.4 Discussion

With the increased use of BTX-A in medical applications, its mechanism of action has become well understood. However, its long-term effect on structure and function of target and non-target muscles has not been studied systematically. Repeated use of BTX-A, especially in children and adolescents suffering from neuromuscular disorders, such as cerebral palsy, makes understanding of BTX-A’s effects on muscles essential.

Here, we demonstrated that BTX-A not only creates a great loss of knee extensor torque due to its neuromuscular blockage, but also causes a substantial loss of muscle mass and a decrease in contractile material of both target and non-target muscles. Surprisingly, not all quadriceps femoris muscles were affected equally by BTX-A injections, with the vastus lateralis showing the largest loss in muscle mass and contractile material, followed by the rectus femoris and the vastus medialis.

3.4.1 Knee extensor torque

BTX-A injections caused large decreases in the knee extensor torque. These deficits were maintained through the entire 6-month experimental period. Dodd et al. (2005) found a similar
loss of force (88-90%) in rat gastrocnemius as observed here after a single BTX-A injection (Dodd, Selsby, Payne, Judge, & Dott, 2005). Our results are also consistent with those published previously on the rabbit knee extensor muscles (Longino et al., 2005; Longino, Frank, & Herzog, 2005).

Surprising, and new to the literature, is the result that knee extensor torque in the contralateral non-injected hind limb of 6 BTX-A group rabbits was also reduced. A small loss in torque that was not statistically significant was observed for 1 and 3 BTX-A groups rabbits, and a statistically significant loss was observed in the 6 BTX-A group rabbits. The experimental design of this study does not allow us to distinguish if this contralateral weakness is directly related to Botox or a result of atrophy caused by muscle disuse. However, this result suggests that repeated BTX-A injections can lead to general muscle weakness, as reported by Cote et al. (2005), that affects target and non-target muscles (Coté et al., 2005; Dodd et al., 2005).

3.4.2 Muscle mass

BTX-A injections caused a dramatic loss in muscle mass, reaching a peak value of approximately 60% after 3 months (Figure 3-2), which is comparable to results published in the literature (Dodd et al., 2005). Interestingly, muscle mass was the same for 3 and 6 BTX-A group rabbits. However, 3 months following BTX-A injection, muscle mass was primarily comprised of contractile tissue, while at 6 months, a great percentage of the muscle mass consisted of fat that had replaced the muscles contractile material. Thus, atrophy in terms of muscle mass stopped at 3 monthly injections, but further deteriorated from 3 to 6 months in terms of the percentage of contractile tissue.
For the knee extensor muscles of the contralateral non-injected hind limbs of the BTX-A experimental groups, no loss in muscle mass was observed in 1 and 3 BTX-A group rabbits. However, a statistically significant loss of muscle mass was observed in the 6 BTX-A group rabbits, reinforcing the idea that repeated BTX-A injections affect muscles far from the injection site (Dodd et al., 2005). Whether this loss in muscle mass of the contralateral non-injected knee extensors was a direct effect of Botox, or an indirect effect associated with disuse atrophy, cannot be resolved with the design of this study.

Muscle mass loss following BTX-A injections was greatest for the vastus lateralis and smallest for the vastus medialis (Figure 3-3). The injection protocol used in this study was carefully chosen to minimize the possibility that one of the knee extensor muscles would receive a disproportionate amount of the toxin. Therefore, we conclude that muscle atrophy is not homogeneous in a group of muscles exposed to BTX-A. Inagi et al. 2009 suggested that the effects of BTX-A injections are more evident in fast twitch than slow twitch fibers (Inagi et al., 1999). No differences were reported in the fiber type distribution of the quadriceps femoris of NZW rabbits, which is comprised of 100%, 90%, and 97% of fast twitch fibers for VL, RF, and VM, respectively (Hämäläinen & Pette, 1993; Rab et al., 2000). However, a difference is observed in the amount of type IIb/d fast twitch glycolytic fibers: VL has a higher proportion of type IIb/d than RF or VM, suggesting that BTX-A may have a greater effect on type IIb/d fibers than other fast or slow type muscle fibers.
3.4.3 Contractile material

Muscle strength does not only depend on the size of the muscle but also on the amount of contractile material. Although knee extensor torque and muscle mass decreased after a single BTX-A injections, the proportion of contractile material within a mid-cross section of the muscles remained constant. After 3 months of BTX-A injections, the proportion of contractile material relative to control rabbits was reduced in the vastus lateralis and rectus femoris, but not in the vastus medialis. This result suggests that BTX-A affects muscles differently, not only in terms of loss of muscle mass but also in terms of contractile material loss. The muscle with the greatest atrophy (VL) also showed the most pronounced loss of contractile material and vice versa (VM). For the 6 BTX-A group rabbits, all portions of the quadriceps showed a significant loss of contractile material (Figure 3-4; last row), emphasizing deterioration over time. Contractile material in the BTX-A injected hind limbs of 3 and 6 BTX-A group rabbits was replaced to a great degree with fat, thereby limiting the amount of loss in muscle mass of the tissue contained within the borders of the muscle. Therefore, using loss of muscle mass as an indicator of BTX-A induced atrophy may severely underestimate the amount of contractile material loss.

A loss in the proportion of contractile material was also found in the contralateral non-injected hind limbs of 6 BTX-A group rabbits. In contrast to the injected hind limb musculature, the loss of contractile material was not replaced by fat, but was a direct result of an increased amount of connective tissue (Figure 3-4; right column 6 BTX-A group rabbits). This result suggests that the loss of contractile material in the contralateral non-injected hind limbs is qualitatively different from that observed in the BTX-A injected hind limbs and could be due to
an indirect effect of BTX-A, which may induce general muscle weakness leading to a less active animal.

The injection doses used in the present study (3.5U/kg) are similar to those used in patients (3-6U/kg), including children with cerebral palsy (Graham et al., 2000; Heinen et al., 2010; Russman et al., 1997; Sutherland et al., 1999). However, therapeutic injections are typically given at intervals of 3-6 months, rather than monthly, as used in the present study (Graham et al., 2000; Russman et al., 1997; Wissel et al., 2009). In order to allow for optimal spreading of the toxin, we gave six injections for every treatment by carefully dividing the quadriceps into different portions as described in the methods section. This approach is consistent with clinical practice where multiple injection points are used for effective distribution of the toxin in the target musculature (Borodic et al., 1992; Brin, 1997; Shaari et al., 1991). Our treatment period was between 1 and 6 months which is consistent with some treatment protocols. However, multiple re-injections every 3-6 months and lasting up to three years have been used clinically with the average treatment period lasting for approximately one year (Borodic et al., 1990; Koman et al., 1993). Therefore, our injection protocols were aligned with common clinical practice with the exception of the frequency of injections which was greater in our study so as to not allow muscle recovery during the experimental period.

Throughout the experimental period, knee extensor torque in the BTX-A injected musculature was reduced to 90-95% of normal. Assuming that loss of muscle mass and contractile material relate linearly to loss of force, this loss in force can be explained in equal measures with atrophy and BTX-A inhibition for the 1 BTX-A group rabbits (Figure 3-5). For
the 3 BTX-A group rabbits, most of the force loss can be attributed to atrophy and the loss of contractile material, while the effect BTX-A becomes secondary. Finally, for the 6 BTX-A group rabbits, virtually all loss in knee extensor torque can be explained with atrophy and contractile material loss, while the actual chemo denervation induced by BTX-A becomes negligible.

**Figure 3-5** Estimated loss in knee extensor strength in the BTX-A injected hind limbs of 1, 3, and 6 BTX-A group rabbits associated with the inhibition caused by BTX-A denervation (BTX-A weakness, white bars), loss of muscle mass (muscle atrophy, dark bars), and/or the loss of contractile material (contractile material loss, grey bars). While the loss in knee extensor torque associated with BTX-A injections remained about constant throughout the experimental period, the loss was associated primarily with BTX-A induced chemical
ablation initially (1 BTX-A group rabbits) and the loss of muscle mass and contractile material at the end (6 BTX-A group rabbits).

3.5 Conclusions

The results of this study lead to the conclusion that BTX-A injections cause severe muscle atrophy and loss of contractile material, thereby affecting muscle strength and structure of the target muscles, while also causing atrophy and contractile material loss in non-target muscles distant from the injection site. It is not known if chronic BTX-A induced atrophy and contractile material loss is reversible and over what time frame a full muscle recovery might be possible. However, when using BTX-A clinically as a chemical denervation agent, it must be acknowledged that muscle atrophy and contractile material loss are side-effects with unknown consequences on structure and health of the target and non-target musculature.
Chapter Four: **The effects of electrical stimulation training exercise on muscles injected with BTX-A**

### 4.1 Introduction

Based on the results presented in the previous chapter (chapter 3), BTX-A injection into the quadriceps femoris musculature of NZW rabbits produces substantial muscle weakness, atrophy, and loss in the amount of contractile material in injected and non-injected musculature. These results suggest that the BTX-A causes undesired adverse effects, which compromise muscle and joint function following the BTX-A treatment period.

Since its approval by the US FDA for strabismus correction treatment in 1989, there is an ever growing increase in the number of applications of BTX-A for a variety of neuromuscular disorders. Consequently, there has also been a rise in the number of reported adverse effects following BTX-A treatments (Albavera-Hernández et al., 2009; Coté et al., 2005; Naidu et al., 2010).

Despite the beneficial effects of BTX-A treatments observed in clinical practice, such as reduced spasticity and co-contraction, thus improving joint range of motion, gait patterns, and overall, enhancing the quality of life for patients with spasticity, there are few studies focused on the adverse effects induced by BTX-A injections. Moreover, efforts to reduce or prevent the adverse effects induced by BTX-A treatments have been minimal. We hypothesize that the adverse effects induced by the chemical ablation of the toxin, as reported in chapter 3, can be minimized by strengthening the injected musculature through regular exercise. BTX-A injection precludes voluntary contractions. However muscles can be trained via direct electrical
stimulation (ES), which is widely used in rehabilitation for the recovery, preservation and/or increase in muscle mass and strength (Hortobágyi & Maffiuletti, 2011).

The purpose of this study was to determine the effects of ES training exercise on muscle strength, mass and contractile material in BTX-A injected quadriceps femoris muscles of NZW rabbits, and to quantify the effects of BTX-A injections and ES training exercise on contralateral non-injected and non-exercised muscles in an attempt to prevent or minimize adverse effects, thus maintaining muscle structure and functionality following BTX-A treatment.

4.2 Methods

4.2.1 Experimental design

Seventeen skeletally mature, one year old, female NZW rabbits were used for this study. Approval for all procedures was obtained from the Animal Care Committee of the University of Calgary. Rabbits were allowed normal activity in a cage (65 x 45 x 30 cm³), and received a standard diet.

Animals were divided into 3 groups as follows:

(1) Control group – saline injection unilaterally ($n=5$; Control) (same Control group rabbits as described in chapter 3)

(2) Six-months post-repeated monthly BTX-A injections unilaterally ($n=5$; BTX-A) (same 6 BTX-A group rabbits as described in chapter 3)

(3) Six-months post-repeated monthly BTX-A injection unilaterally + unilateral electrical stimulation (ES) exercise ($n=7$; BTX-A+ES)
4.2.2 *Botulinum Toxin Type-A: injection protocol*

The BTX-A injection protocol was similar to the one reported in the previous chapter. In summary, rabbits received intramuscular BTX-A injections at a total dosage of 3.5U/kg of body weight randomized to either the right or left quadriceps musculature. The anterior compartment of the thigh was isolated by manual palpation and the quadriceps was visually divided into superior and inferior halves. Each half was subdivided into medial, central, and lateral sections. One sixth of the total toxin was injected into each section to increase diffusion and to inject directly into the vastus lateralis, rectus femoris and vastus medialis muscles.

Group 1 rabbits served as a control and received intramuscular saline injections randomized to either the right or left quadriceps musculature. The total volume of the injected saline was the same as the total volume of BTX-A injected into experimental group rabbits. Groups 2 and 3 received intramuscular monthly BTX-A injections into the same hind limb (randomized for the first injection) for a six months period. Additionally, group 3 rabbits received unilateral ES exercise for the BTX-A injected musculature during the six months experimental period (for details see appendix A-2).

The primary outcome measures were the knee extensor torque, muscle mass, and the percentage of contractile material in injected and contralateral non-injected hind limbs. Furthermore, a possible inhibition of activation of the contralateral non-injected hind limbs caused by BTX-A was assessed using a glycogen depletion protocol and measuring knee extensor torque by direct muscle stimulation rather than femoral nerve stimulation.
4.2.3 Electrical stimulation (ES) exercise in BTX-A+ES group

ES exercise sessions began 72 hours following the first BTX-A injection. BTX-A+ES rabbits received a total of 72 ES sessions, three sessions per week for the entire six months experimental period.

For each session, rabbits were anaesthetized using a 3.5% isoflurane/oxygen mixture. The area over the quadriceps musculature was prepared for electrode placement by shaving and cleaning with alcohol. The anaesthetized rabbits were placed on their back in a sling with the trained hind limb bluntly constrained in a stereotactic frame with the knee positioned at 90-100° of flexion.

The ES exercise was given only to the BTX-A injected hind limb musculature using a Grass S8800 stimulator (Astro-Med Inc., Longueil, Quebec, Canada). The quadriceps muscles were identified by palpation, and self-adhesive superficial electrodes were placed in the mid-belly region. A maximal contraction was elicited at the beginning of the session followed by 15 minutes of sub-maximal isometric contraction at 20% of the maximum contraction. Individual contractions were 0.5s long with a 1.5s rest interval. The sub-maximal force was maintained throughout the session by increasing the magnitude and/or frequency of stimulation.

4.2.4 Knee extensor torque, glycogen depletion protocol, and muscle mass

Isometric knee extensor torque was measured at the end of the six months experimental period. Knee extensor torque in the injected and the contralateral non-injected hind limbs was obtained by stimulating the quadriceps via a femoral nerve cuff electrode implanted prior to
terminal testing (Longino et al., 2005). Following nerve cuff implantations, rabbits were secured in a stereotactic frame using bone pins at the pelvis and femoral condyles. Isometric knee extensor torques at 100° of knee flexion were measured using a strain-gauged, calibrated bar placed over the anterior distal portion of the rabbit’s tibia.

Stimulation of the knee extensor musculature (Grass S8800 stimulator, Astro-Med Inc., Longuiel, Quebec, Canada) was given at a voltage three times higher than the alpha motoneuron threshold, to ensure activation of all motor units (Herzog & Leonard, 1997). Stimulation duration was 500ms, pulse duration 0.1ms, and the frequency of stimulation was 100Hz.

Additionally, knee extensor torque was also measured by means of direct muscle stimulation of the injected and contralateral non-injected hind limbs in BTX-A+ES group rabbits at the end of the six months experimental period. Rabbits were positioned as described previously. The quadriceps musculature was identified and self-adhesive superficial electrodes were placed in the mid-belly region. The maximum force value was obtained by increasing the voltage (frequency at 100Hz) of the stimulator until no further increases in force were observed.

Following knee extensor torque measurements, BTX-A+ES rabbits were subjected to a bilateral glycogen depletion protocol. Complete glycogen depletion was induced using fifteen minutes of continuous femoral nerve stimulation (20Hz) to all motor units of the quadriceps (Shaari et al., 1991; Shaari & Sanders, 1993). Immediately following the depletion protocol, rabbits were sacrificed by an overdose of Euthanyl (MTC Pharmaceuticals; Cambridge, Ontario) and the quadriceps musculature was excised bilaterally. The central third portion of the quadriceps
was embedded in paraffin (automatic paraffin processor, Leica TP1020) and cut cross-sectionally with a microtome (Leica RM 2165). For every 100µm, an 8µm section was collected for glycogen staining with a period acid-Schiff stain (PAS) (Sigma-Aldrich, USA). Photographs were taken using an Axionstar plus microscope (Carl Zeiss) with a 5x magnification lens.

A positive and negative control for PAS staining of the quadriceps was established using the muscles of two control rabbits (one exposed to the glycogen depletion protocol the other not) that were removed, cut, embedded, prepared, and stained for glycogen content with PAS (Figure 4-1).

![Figure 4-1 Quadriceps musculature stained with PAS. Left: control muscle not exposed to the glycogen depletion protocol shows an even distribution of glycogen throughout the entire cross-sectional area. Right: muscle exposed to the glycogen depletion protocol is completely devoid of glycogen in the contractile material.](image)

Next, muscle masses for the quadriceps muscles were determined using a commercial scale with a resolution of 0.001g.
### 4.2.5 Contractile material

The percentage of contractile material was determined histologically. The central third of each muscle was embedded in paraffin (automatic paraffin processor, Leica TP1020) and cut-cross-sectionally with a microtome (Leica RM 2165). For every 100µm, an 8µm section was collected for staining with haematoxylin-eosin (H&E) (Leica ST5010). A total of 5 slides for each muscle were analyzed and averaged.

Photographs were taken as described previously for the glycogen depletion protocol. A customized MatLab program (MatLab 7.8, R2010b) was used to calculate the percentage of contractile material for at least 50% of the total cross-sectional area of each muscle.

### 4.2.6 Data analysis

Knee extensor torque (femoral nerve stimulation) and muscle mass of injected and contralateral non-injected hind limbs of BTX-A injected groups were expressed as a percentage relative to values of the control group rabbits. Reduction in strength will hereafter be referred to as muscle weakness, while the loss of muscle mass will be referred to as atrophy. Knee extensor torque for direct muscle stimulation of BTX-A+ES group was expressed as a change in strength relative to the corresponding values obtained from femoral nerve stimulation. The amount of contractile material for each muscle is given as the percentage area of the contractile material relative to the total cross-sectional area of the analyzed sections. Glycogen staining was assessed qualitatively by visual inspection on the contralateral non-injected quadriceps to assess possible systemic effects on BTX-A+ES group rabbits.
A two-way ANOVA with repeated measures was used to assess knee extensor torque, muscle atrophy, and contractile material with the main factors leg (injected and contralateral non-injected) and groups (control, BTX-A, and BTX-A+ES). A post-hoc Tukey test was performed if indicated. Also, a paired $t$-test was used for comparisons between femoral nerve vs. direct muscle stimulation on BTX-A+ES group rabbits. The level of significance was chosen as $\alpha=0.05$ a priori.

### 4.3 Results

The first BTX-A injection induced loss of body weight and lack of appetite, however the subsequent injections were well tolerated by all rabbits (appendix B-1). Data were analyzed from all animals, except for the percentage of contractile material from the rectus femoris of the control rabbits, as these samples were of bad quality.

#### 4.3.1 Muscle weakness

There was no difference in knee extensor torque between saline injected and contralateral non-injected hind limbs of control group rabbits. Knee extensor torque was significantly reduced in the BTX-A injected (decreased of 95%) and the contralateral non-injected (85%) hind limbs of BTX-A group compared to control group rabbits. Following the ES exercise protocol on the BTX-A injected hind limb (BTX-A+ES), a small but significant increase in force was observed compared to the BTX-A group ($p<0.05$, Figure 4-2). Additionally, the contralateral non-injected hind limb of the BTX-A+ES group rabbits showed a significant force difference, even though it was not subjected to the ES exercise program ($p<0.001$, Figure 4-2). However, despite the ES
training protocol, muscle weakness persisted in the BTX-A injected and contralateral non-injected hind limbs of BTX-A+ES group compared to control group rabbits (p<0.001, Figure 4-2).

Figure 4-2 Mean muscle strength (± 1 SE) normalized to the values of the control group rabbits (dark bars-100%). Knee extensor torque in the BTX-A group (light bars), and the BTX-A+ES (shaded bars) group rabbits was significantly reduced for the BTX-A injected and contralateral non-injected hind limbs compared to control group rabbits. BTX-A+ES group rabbits had significantly increased knee extensor torque in the BTX-A injected and the contralateral non-injected hind limbs compared to the corresponding hind limbs of the BTX-A group rabbits. However, muscle strength in the BTX-A+ES group rabbits did not reach control values, neither for the ES exercised nor the contralateral non-injected hind
4.3.2 Muscle atrophy

There was no difference in muscle mass between saline injected and contralateral non-injected hind limbs of control group rabbits. Quadriceps atrophy was significantly increased in the BTX-A injected (increased by 52%) and contralateral non-injected (30%) hind limbs of the BTX-A group rabbits compared to control rabbits ($p<0.001$, Figure 4-3). Muscle mass was increased in the BTX-A injected hind limbs of BTX-A+ES group rabbits compared to BTX-A group rabbits ($p<0.05$), but remained smaller than the muscle mass values in the control group rabbits ($p<0.001$). Intriguingly, muscle mass on the contralateral non-injected and non-exercised hind limbs of the BTX-A+ES group rabbits was similar to control values (Figure 4-3)
Figure 4-3 Mean muscle mass (± 1 SE) normalized to the values of control group rabbits (dark bars – 100%). Muscle mass in the BTX-A (light bars), and the BTX-A+ES (shaded bars) group was significantly reduced for the BTX-A injected and contralateral non-injected hind limbs. BTX-A+ES group rabbits had significantly increased muscle mass in the BTX-A exercised and the contralateral non-injected and non-exercised hind limbs compared to the corresponding hind limbs of the BTX-A group rabbits. Muscle mass for the contralateral non-injected hind limbs of BTX-A+ES group rabbits was fully preserved and similar to the values of the control group rabbits. * compared to control group rabbits; † compared to BTX-A group rabbits. For absolute values see appendix C-5.
4.3.3 Contractile material

There was no difference in the area fraction of contractile material between the saline injected (96 ±1.6%) and the contralateral non-injected (94.5 ±2.6%) hind limbs of the control group rabbits. After repeated BTX-A injections for six months, the contractile material of the injected and contralateral non-injected hind limbs of BTX-A group rabbits was reduced to 63.9% (±16.9%) and 79.3 (±6.5%), respectively (p<0.001, Figure 4-4). Following ES exercise training of the BTX-A injected hind limbs, the percentage of contractile material for the injected and contralateral non-injected hind limbs of BTX-A+ES group rabbits was 77.1% (±14.3%) and 93.4% (±2.2%), respectively, which was significantly higher than the corresponding values for the BTX-A group rabbits (p<0.001, Figure 4-4). The percentage of contractile material of the contralateral non-injected hind limbs of BTX-A+ES was not different from control values (Figure 4-4).

Figure 4-4 Exemplar histological cross-sectional images showing the muscle contractile material (H&E, red staining) and non-muscle contractile material (white color; assumed to

62
be primarily fat and connective tissue) for muscles from injected (first row) and contralateral non-injected hind limbs (second row) for control group muscles (left), BTX-A group muscles (middle), and BTX-A+ES group muscles (right). There was a significant loss of contractile material for the injected and contralateral non-injected muscles for the BTX-A group rabbits (middle column) compared to control group muscles. The percentage of contractile material was significantly greater for the injected and contralateral non-injected muscles of the BTX-A+ES group compared to BTX-A group rabbits. The percentage of contractile material for the contralateral muscles of BTX-A+ES group rabbits was fully preserved and similar to control values.

4.3.4 Femoral nerve vs. direct muscle stimulation in BTX-A+ES group rabbits

Isometric knee extensor torque through direct muscle stimulation of the BTX-A+ES injected hind limbs produced greater quadriceps force than femoral nerve stimulation, as one would expect if BTX-A was still blocking acetylcholine release at the nerve endings following the experimental period ($p<0.05$, Figure 4-5). Direct muscle stimulation also produced a 48% higher quadriceps torque than femoral nerve stimulation in the contralateral non-injected hind limbs of BTX-A+ES group rabbits, suggesting that BTX-A also blocks the motor nerve endings of distant non-target muscles ($p<0.05$, Figure 4-5)
Figure 4-5 Knee extensor strength obtained by femoral nerve stimulation (dark bars) and by direct muscle stimulation (light bars) for the injected and contralateral non-injected hind limbs of BTX-A+ES group rabbits. Torque values were normalized relative to control group values (100% - not shown). Direct muscle stimulation produced significantly greater muscle force than femoral nerve stimulation in BTX-A injected and contralateral non-injected hind limbs, suggesting that BTX-A was partially blocking acetylcholine release at the motor axons of the target injected and non-target contralateral musculature. *

4.3.5 Glycogen depletion protocol in BTX-A+ES group rabbits

Histological muscle sections showed a significant amount of glycogen following the glycogen depletion protocol in the BTX-A injected muscles of BTX-A+ES group rabbits (Figure
Similarly, the histological sections of contralateral non-injected muscles from BTX-A+ES group rabbits also showed some glycogen staining following the depletion protocol, albeit to a lesser degree than the BTX-A injected musculature, suggesting that there was also blockage of acetylcholine release at the motor nerve endings of the contralateral non-injected musculature (Figure 4-6; right).

Figure 4-6 Histological sections of the quadriceps musculature using PAS staining for glycogen content. The left section is from BTX-A injected muscle of a BTX-A+ES group rabbits and shows a substantial amount of glycogen following the glycogen depletion protocol, suggesting an almost complete blockage of acetylcholine release through BTX-A, and thus little glycogen depletion. The right section is from a contralateral non-injected muscle of a BTX-A+ES group rabbits and shows some remnant glycogen staining following the glycogen depletion protocol, suggesting that there is also some blockage of acetylcholine release through BTX-A in the non-target contralateral non-injected musculature.
4.4 Discussion

The primary result of this second study was that low level ES training exercise partially prevented muscle weakness, atrophy, and contractile material loss in muscles injected with BTX-A over a six month treatment period. Furthermore, ES exercise partially prevented muscle weakness and fully prevented atrophy and contractile material loss in contralateral non-injected muscles in BTX-A+ES group rabbits. Finally, the contralateral non-injected hind limbs of BTX-A+ES group rabbits showed higher quadriceps forces when the muscles were directly stimulated compared to femoral nerve stimulation, and retained glycogen in some fibres following the depletion protocol, suggesting that BTX-A partially blocked muscle activation in non-target muscles far away from the injection site.

4.4.1 Muscle weakness

BTX-A injections caused substantial muscle weakness not only in injected but also in the contralateral non-injected hind limbs of BTX-A group rabbits, as described previously (Figure 4-2). This result suggests that BTX-A injections affected non-target muscles far away from the injection site. There are several possibilities of how weakness might have come about in the non-target musculature: (i) the animals might have become more sedentary because of the toxin injections and this might have produced a systematic disuse atrophy affecting all muscles (Dodd et al., 2005); (ii) BTX-A might have affected the animals’ metabolism and might have caused decreases in muscle mass and thus strength, or (iii) BTX-A might have spread from the injection site through the body and might have affected acetylcholine in motor axons of non-target muscles (Antonucci et al., 2008; Girlanda et al., 1992; Habermann, 1974; Lange et al., 1987). The finding that direct muscle stimulation produced more force than femoral nerve stimulation in
the contralateral non-injected quadriceps, and that glycogen depletion was incomplete in the contralateral non-injected muscles, suggests that the last of the above three options is at least partially responsible for the muscle weakness observed in the non-target musculature.

Direct ES exercise of BTX-A injected muscles helped reduce weakness in the quadriceps, but the effect of ES exercise was small (Figure 4-2). However, this result is not surprising as the muscle force values in this comparison are dominated by the BTX-A induced denervation of the muscles which persists throughout the six months period. Furthermore, previous studies suggested that ES exercise enhances BTX-A uptake, thus enhancing the BTX-A denervation of the muscles, thereby possibly underestimating the true preservation of strength in the ES trained muscles. This suggestion is supported by results from patients with spasticity which showed substantial increases in function when BTX-A injections were combined with ES exercise (Bayram et al., 2006; Kang et al., 2007; Seifart, Unger, & Burger, 2010).

Previous studies showed a substantial loss of strength, mass and contractile material in non-target muscles (Dodd et al., 2005; Fortuna, Vaz, Youssef, Longino, & Herzog, 2011). The results of this study, and those by others (Girlanda et al., 1992; Lange et al., 1987; Yaraskavitch et al., 2008) suggest that this might be caused by a spread of BTX-A to distant locations where the toxin inhibits acetylcholine release at the neuromuscular junction of non-target muscles. Probably the most exciting result of this study was that the severe muscle degeneration of the contralateral non-injected quadriceps observed here and in our previous study following BTX-A injections (Fortuna et al., 2011), was almost completely abolished. Mass and the area fraction of contractile material in the contralateral non-injected and non-exercised muscles of BTX-A
injected group rabbits was similar to control values. Furthermore, muscle weakness, when assessed by direct muscle stimulation, was less than 20% in the ES trained contralateral non-injected muscle while this value was on average more than 40% in animals just receiving BTX-A injections compared to control values (Figure 4-5). It appears therefore, that although the ES exercise protocol produced only small levels of force preservation in the BTX-A injected musculature, it helped preserve mass, contractile material and most of the strength in contralateral non-injected, non-target muscles.

We do not know why ES exercise had such a profound effect on the non-injected, non-exercised musculature. However, the following possibilities exist: (i) an increased uptake of BTX-A in the target muscles might have prevent spread of the toxin to non-target muscles. Minamoto et al. (2007) suggested that ES exercise of the rat tibialis anterior reduced spread of the toxin to the contralateral non-injected musculature (Minamoto et al., 2007); or (ii) ES exercise of the injected muscle might have produced a increase in strength on the contralateral non-exercised quadriceps, a phenomenon known as cross-over training. Although strength gains through cross-over training are typically found to be small (about 8%; Carroll, Herbert, Munn, Lee, & Gandevia, 2006), they might contribute to the force preservation observed in this study in the contralateral non-injected, non-exercised musculature.

4.4.2 Muscle atrophy and contractile material

BTX-A injections caused severe muscle atrophy and contractile material loss in injected and contralateral non-injected hind limbs of BTX-A group rabbits (Figure 4-3 and Figure 4-4). A detailed account of muscle atrophy and contractile material loss for the different portions of the
quadriceps has been presented previously (chapter 3; Fortuna et al., 2011). Muscle atrophy and loss of contractile material were partially prevented through the ES exercising program in the BTX-A injected musculature. However, the effects were smaller than we had hoped. Different ES exercise programs have been implemented successfully in a variety of neuromuscular disorders, such as cerebral palsy and after stroke. Possibly, the ES exercise program employed here was not optimally timed (relative to the BTX-A injections) or the frequency, magnitude, and duration were not optimal for achieving maximal muscle restoration. Future work should focus on optimizing the ES training program for patients undergoing long-term BTX-A treatments.

To our surprise, the muscle mass and the percentage of contractile material in contralateral non-injected hind limbs of BTX-A+ES group rabbits were fully preserved at the level observed for control group rabbits (Figure 4-3 and Figure 4-4). This result was found despite the fact that only the injected muscles were trained using ES exercise, while the contralateral non-injected muscles did not receive such training. This finding might have far reaching clinical implications, as it appears that adverse effects of BTX-A treatments in non-target areas can be limited using ES of the BTX-A injected target muscles. As described above, we speculate that the beneficial effects of ES on the contralateral non-injected hind limbs of BTX-A+ES group rabbits might be explained by a combination of (i) muscular cross-training and (ii) prevention of BTX-A spread. However, the detailed mechanism of muscle preservation in non-target muscles following ES are by no means clear and need to be addressed in future studies.
4.4.3 Femoral nerve vs. direct muscle stimulation and glycogen depletion in BTX-A+ES group rabbits

The injected hind limbs of BTX-A+ES group rabbits produced higher forces when stimulated directly through the muscle compared to stimulation through the femoral nerve (Figure 4-5). Furthermore, there was substantial lack of glycogen staining in the BTX-A injected musculature after the glycogen depletion protocol (Figure 4-6, left). These results were expected given that BTX-A blocks acetylcholine release at the motor nerve endings, and that this effect can be preserved with repeat injections over a six month period (Longino et al., 2005).

The contralateral non-injected hind limbs of BTX-A+ES group rabbits also showed higher forces through direct muscle stimulation compared to nerve stimulation (Figure 4-5), and also showed glycogen retention following the glycogen depletion protocol (Figure 4-6, right). These results provide strong, albeit indirect, evidence that BTX-A affects distant muscles either by diffusing through the blood stream (Garner et al., 1993) or by retrograde axonal transport (Restani et al., 2011). Measurement using SNAP-25 antibody (Marinelli et al., 2012) and $^{125}$I-labeled BTX-A (Habermann, 1974; Wiegand et al., 1976) demonstrated that the toxin can ascend motor neurons, enter the spinal cord, and then descend motor neurons leading to other muscles than those injected for treatment purposes.

We believe that our results have significant clinical implication. For example, children with cerebral palsy who receive multiple, high-dosage BTX-A injections were found to have more severe adverse effects than patients who only received a single injection, or multiple but low-dosage injections. There are no systematic studies on the long-term effects of repeat BTX-A
injections in patients with neuromuscular disorders, but in view of the current results, such studies are urgently needed. Naidu et al. (2010) concluded that high BTX-A doses were associated with an increased chance of systemic and respiratory complications in cerebral palsy children (Naidu et al., 2010). Furthermore, Bayram et al. (2006) suggested that low dose of BTX-A (100U) associated with ES had a similar clinical response as a high dose (400U) in post stroke patients (Bayram et al., 2006). Together, these results suggest that optimal treatments may combine small dosage BTX-A injections associated with ES exercise of the target muscles to enhance BTX-A uptake at the target site and limit BTX-A spread. The few studies combining BTX-A treatments with ES seem to produce better results in terms of gait and general muscle function in children with cerebral palsy (Kang et al., 2007; Seifart et al., 2010).

4.5 Conclusion

We conclude from the results of this study that ES exercise partially prevents degeneration of BTX-A injected muscles and fully prevents loss of mass and contractile material in contralateral non-injected muscles. Therefore, direct electrical muscle stimulation may provide opportunities to enhance treatment and recovery in patients receiving BTX-A injections for the purpose of “relaxing” hyperactive muscles and limbs.
Chapter Five: Do Skeletal Muscle Properties Recover Following Repeat BTX-A Injections?

5.1 Introduction

The results of our previous study (chapter 4) demonstrated that an electrical stimulation training protocol during a six-month BTX-A treatment period can help alleviate losses in muscle strength, mass and contractile material. Surprisingly, the training protocol produced significant improvements in muscle strength, mass, and contractile material on the contralateral non-injected non-exercised musculature.

Despite a variety of studies describing the short term effects of BTX-A on target and non-target muscles (Corry et al., 1999; Houltram et al., 2001; Sutherland et al., 1999; Ubhi et al., 2000), the detailed recovery of these muscles following BTX-A treatments remains unknown. At present, BTX-A injection protocols are based on clinical examination and functional assessment, thereby neglecting the natural recovery of the structural integrity of the injected and non-injected musculature.

The purpose of the present study was to investigate deterioration and recovery of muscle strength, mass, and contractile material of the quadriceps femoris musculature in NZW rabbits following a six months repeat BTX-A injection protocol.

5.2 Methods

5.2.1 Experimental design

Twenty seven skeletally mature, one year old, female NZW rabbits were used for this study. Approval of all procedures was obtained from the Animal Care Committee of the
University of Calgary. Rabbits were allowed normal cage activity (65 x 45 x 30cm$^3$) and received a standard diet.

Rabbits were divided into five groups as follows:

1. Control group – saline injection unilaterally ($n=5$; Control) (same Control group rabbits as described in chapter 3)

2. Six-months of repeated monthly BTX-A injections unilaterally + 0 month recovery ($n=5$; BTX-A+0M) (same 6 BTX-A group rabbits as described in chapter 3)

3. Six months of repeated monthly BTX-A injections unilaterally + 1 month recovery ($n=5$; BTX+1M)

4. Six-months of repeated monthly BTX-A injection unilaterally + 3 months recovery ($n=5$; BTX-A+3M)

5. Six-months of repeated monthly BTX-A injections unilaterally + 6 months recovery ($n=7$; BTX-A+6M)

5.2.2 Botulinum Toxin Type-A: injection protocol

The BTX-A injection protocol was similar to the one reported in the previous chapter. Rabbits received intramuscular BTX-A injections at a total dosage of 3.5U/kg of body weight randomized to either the right or left quadriceps musculature. The anterior compartment of the thigh was isolated by manual palpation and the quadriceps was visually divided into superior and inferior halves. Each half was subdivided into medial, central, and lateral section. One sixth of the total toxin was injected into each section to increase diffusion and to inject directly into the vastus lateralis, rectus femoris, and vastus medialis muscles.
Control group rabbits received intramuscular saline injections of equal volume as that given to the BTX-A injected experimental group rabbits. BTX-A experimental groups 2 to 5 (BTX-A+0M/1M/3M/6M) received intramuscular monthly BTX-A injections into the same hind limb (randomized for the first injection) for a six months period. Additionally, BTX-A experimental groups 3 to 5 (BTX-A+1M/3M/6M) were allowed to recover for periods of 1, 3, and 6 months, respectively, following the last BTX-A injection (for details see appendix A-3).

The primary outcome measurements were knee extensor strength, quadriceps muscle mass, and percentage of contractile material in the injected and contralateral non-injected quadriceps musculature. Additionally, the long-term effects of BTX-A injections on acetylcholine blockage were assessed using a glycogen depletion protocol and knee extensor strength measurements with direct muscle stimulation, rather than femoral nerve stimulation in BTX-A+6M group rabbits.

5.2.3 Knee extensor strength, glycogen depletion protocol, and muscle mass

Knee extensor toque was measured at 0, 1, 3, and 6 months following the BTX-A treatment period (BTX-A+0M/1M/3M/6M) in injected and contralateral non-injected quadriceps muscles using femoral nerve stimulation. For the strength measurements, rabbits were secured in a stereotactic frame using metal pins at the pelvis and femoral condyles. Isometric knee extensor strengths at 100° of knee flexion (full knee extension was defined as 180°) were measured using a strain-gauged bar placed over the anterior and distal portion of the rabbit’s tibia (Longino et al., 2005; Longino, Frank, et al., 2005).
Stimulation of the knee extensor musculature (Grass S8800 stimulator; Astro-Med Inc., Longueil, Quebec, Canada) was given at a voltage three times higher than the alpha motoneuron threshold, to ensure activation of all motor units (Herzog & Leonard, 1997). Stimulation duration was 500ms, pulse duration 0.1ms, and the frequency of stimulation was 100Hz.

Additionally, knee extensor strength was also measured by means of direct muscle stimulation of the injected and contralateral non-injected quadriceps muscles in BTX-A+6M group rabbits. For this procedure, rabbits were positioned as described above with self-adhesive electrodes placed in the mid-belly of the quadriceps group, and maximum force was obtained using a supra-maximal current and a stimulation frequency of 100Hz.

Following knee extensor strength assessment, BTX-A+6M group rabbits were subjected to a bilateral quadriceps glycogen depletion protocol using fifteen minutes of continuous femoral nerve stimulation at 20Hz. Next, rabbits were sacrificed and the quadriceps muscles were excised bilaterally. Wet quadriceps muscle mass was determined using a commercial scale (0.001g). After muscle mass determination, the central third portion of the quadriceps musculature was embedded in paraffin (automatic paraffin processor, Leica TP1020) and cut cross-sectionally with a microtome (Leic RM2165). For every 100µm, an 8µm was collected for glycogen staining using a periodic acid-Schiff (PAS) assay (Sigma-Aldrich, US). Photographs of the stained sections were taken using an Axionstar plus microscope (Carl Zeiss) with a 5x magnification objective. Positive and negative controls for PAS staining were established using quadriceps muscles from control group rabbits (Figure 4-1).
5.2.4 Contractile material

The percentage area of quadriceps contractile material was determined histologically using cross-sectional sections. In preparation for histology, the quadriceps musculature was harvested, fixed and photographed as described above for the glycogen depletion protocol, except that sections were stained with haematoxylin-eosin (H&E) (Leica ST5010). Five slides representing at least 50% of the total muscle cross-sectional area were analyzed. A customized Matlab program (MatLab 7.8, R2010b) was used to calculate the percentage cross-sectional area of contractile material relative to the total cross-sectional area of the muscles.

5.2.5 Data analysis

Knee extensor strength and muscle mass of the injected and contralateral non-injected muscles of BTX-A experimental group rabbits (groups 2-5) were expressed as a percentage of the values obtained in control group rabbits (group 1). Reduction in knee extensor strength will hereafter be referred to as muscle weakness, while the loss of muscle mass will be referred to as atrophy. Knee extensor strength values obtained from direct muscle stimulation was expressed as a change in strength relative to the corresponding values obtained from femoral nerve stimulation. The amount of contractile material is given as the percentage area of the contractile material relative to the total cross-sectional area. Glycogen staining was assessed qualitatively for its absence or presence.

A two-way ANOVA with repeated measured was used to assess muscle weakness, muscle atrophy, and contractile material with the main factors leg (injected and contralateral non-injected quadriceps) and groups (control, BTX-A+0M/1M/3M/6M). A Tukey post-hoc test was
performed, if indicated. A paired $t$-test was used for comparisons of strength between femoral nerve vs. direct muscle stimulation for the BTX-A+6M group rabbits. The level of significance was chosen as $\alpha=0.05$ a priori.

5.3 Results

The first BTX-A injection induced loss of body weight and lack of appetite, however the subsequent injections were well tolerated by all rabbits. Additionally, body weight recovered to before injection values for 1, 3, and 6 months recovery time period (appendix B-2). Data were analyzed from all animals, except for the percentage of contractile material from the rectus femoris of the control rabbits, as these samples were of bad quality.

5.3.1 Muscle weakness

There was no difference in knee extensor strength between the saline injected and contralateral non-injected quadriceps muscles of the control group rabbits. Knee extensor strength was significantly reduced in injected (decrease of 95%) and contralateral non-injected (85%) quadriceps of BTX-A+0M group rabbits compared to control group rabbits. One month following the BTX-A experimental period, a significant force recovery was observed in the BTX-A injected musculature of the BTX-A+1M compared to BTX-A+0M group rabbits ($p<0.001$). No additional recovery in knee extensor strength was observed for BTX-A+3M/6M group rabbits (Figure 5-1). Furthermore, the six months recovery period (BTX-A+1M/3M/6M groups) did not restore knee extensor strength to control values. The contralateral non-injected quadriceps of the BTX-A+1M/3M/6M group rabbits also showed a significant recovery in
strength compared to BTX-A+0M group rabbits ($p<0.001$), reaching similar values as the control group rabbits at 1, 3, and 6 months of recovery (Figure 5-1).  

Figure 5-1 Mean muscle strength (±1 SE) normalized to the values of the control group rabbits (dark bars – 100%). Knee extensor strength in the injected quadriceps of BTX-A+0M group rabbits (light bars) was significantly reduced compared to control, while strength in BTX-A+1M/3M/6M (shaded bars) group rabbits recovered significantly compared to BTX-A+0M group rabbits during the recovery period, but remained lower than control group values. Knee extensor strength in the contralateral non-injected quadriceps of BTX-A+0M group rabbits was significantly reduced compared to control values, but recovered completely for BTX-A+1M/3M/6M group rabbits. * compared to
control group rabbits; † compared to BTX-A+0M group rabbits. For absolute values see appendix C-6.

5.3.2 Muscle atrophy

There was no difference in muscle mass between saline injected and contralateral non-injected quadriceps muscles of control group rabbits. Quadriceps atrophy was 52% in BTX-A injected and 30% in contralateral non-injected quadriceps muscles of BTX-A+0M group rabbits compared to control group rabbits ($p<0.001$, Figure 5-2). Quadriceps muscle mass did not increase in the first month of recovery (BTX-A+1M group rabbits), but increased after 3 and 6 months of recovery (BTX-A+3M/6M group rabbits, $p<0.001$) However, muscle mass remained significantly smaller after six months of recovery compared to control group values (Figure 5-2). Quadriceps mass on the contralateral non-injected muscles of BTX-A+1M/3M/6M group rabbits increased significantly compared to BTX-A+0M group rabbits ($p<0.001$), and reached control values within the first month of recovery period (Figure 5-2)
Figure 5-2 Mean muscle mass (±1 SE) normalized to the values of control group rabbits (dark bars – 100%). Quadriceps atrophy was significant in the injected muscles of BTX-A+0M group rabbits (light bars) compared to control values, and was significantly reduced for BTX-A+3M/6M group rabbits (shaded bars), but did not reach control group values in the six months recovery period. Quadriceps atrophy was significant in the contralateral non-injected BTX-A+0M group rabbits compared to control group rabbits, but recovered to control values for BTX-A+1M/3M/6M group rabbits. * compared to control group rabbits; † compared to BTX-A+0M group rabbits. For absolute values see appendix C-7.

5.3.3 Contractile material

There was no difference in the area fraction of contractile material between saline injected (96% ±1.6%) and the contralateral non-injected (94.5% ±2.6%) muscles of control
group rabbits. The contractile material of injected and contralateral non-injected muscles of BTX-A+0M group rabbits was reduced to 63.9% (±16.9%) and 79.3% (±6.5), respectively ($p<0.001$). Following 1, 3, and 6 months of recovery, the percentage of contractile material in the BTX-A injected muscles was 70.8% (±11.0%), 67.1% (±9.5%), and 77.8 (±2.0%), with the six months value being the lone significant improvement compared to the BTX-A+0M group rabbits ($p<0.05$, Figure 5-3). The corresponding values for the contralateral non-injected muscles were 82.3% (±3.7%), 79.3% (±1.9%), 84.7% (±5.1%), at 1, 3, and 6 months of recovery, respectively, showing no recovery relative to the BTX-A+0M group rabbits. The percentage of contractile material did not recover to control values for any of the recovery group animals (Figure 5-3).
Figure 5-3 Exemplar histological cross-sectional images showing the muscle contractile material (H&E; red staining) and non-muscle contractile material (white color; assumed to be primarily fat and connective tissue) for muscles from injected (left column) and contralateral non-injected muscles (right column) for control (first row), BTX-A+0M/1M/3M/6M group rabbits (second, third, fourth, and fifth row), respectively. There was a significant loss of contractile material for the injected and contralateral non-injected quadriceps for BTX-A+0M group rabbits (second row) compared to control group rabbits. The contractile material for the injected quadriceps muscles only showed significantly recovery for BTX-A+6M (first column; fifth row) compared to BTX-A+0M group rabbits (first column; second row), but did not reach control group values during the recovery period. There was no significant recovery in contractile material for the contralateral non-injected quadriceps muscles of BTX-A+1M/3M/6M (second column; third, fourth, and fifth row) compared to BTX-A+0M group rabbits, and the percentage of contractile material remained smaller compared to control values throughout the recovery period.

5.3.4 Femoral nerve vs direct muscle stimulation on BTX-A+6M group rabbits

Direct muscle stimulation of BTX-A injected quadriceps muscles six months into recovery (BTX-A) produced 20% greater quadriceps forces than femoral nerve stimulation ($p<0.05$, Figure 5-4). Quadriceps forces were the same for direct muscle and femoral nerve stimulation in contralateral non-injected quadriceps muscles (Figure 5-4).
Figure 5-4 Knee extensor strength obtained with femoral nerve stimulation (dark bars) and by direct muscle stimulation (light bars) for the injected and contralateral non-injected quadriceps of BTX-A+6M group rabbits. Knee extensor strength was normalized relative to control group rabbits (100%, not shown). Direct muscle stimulation produced significantly greater muscle force than femoral nerve stimulation in BTX-A injected quadriceps muscles, but was the same in the contralateral non-injected quadriceps muscles. * compared to femoral nerve stimulation.

5.3.5 Glycogen depletion protocol on BTX-A+6M group rabbits

Glycogen depletion was incomplete in BTX-A injected quadriceps muscles six months following the BTX-A experimental period, while it was virtually complete in contralateral non-injected muscles of BTX-A+6M group rabbits (Figure 5-5).
Figure 5-5 Exemplar histological sections of the quadriceps muscles using PAS staining for glycogen for a BTX-A+6M group rabbit. The left section is from a BTX-A injected muscles following the depletion protocol, showing glycogen retention (pink stained cells – arrow) and glycogen depleted muscle fibers (unstained cell – “*” symbol). Fibers that remain stained contain glycogen, and thus were likely not activated during the depletion protocol because of remnant blocking of acetylcholine release following the six months recovery period. The right section contains a sample from the contralateral non-injected quadriceps muscles, which shows virtually complete glycogen depletion (unstained cells – “*” symbol) with only few fibers staining for glycogen (pink stained cells – arrow), suggesting that only few fibers were blocked by BTX-A following the six months recovery period.

5.4 Discussion

The results of this study demonstrate that target and non-target muscles did not fully recover within a six months recovery period following our BTX-A injection protocol. While isometric knee extensor strength and muscle mass recovered to a certain degree in the injected musculature, there was only a modest improvement in the percent contractile material for the
BTX-A+6M group rabbits, suggesting that quadriceps strength and muscle mass recover at a different rate than the contractile material. Furthermore, the BTX-A injected musculature showed greater quadriceps forces with direct muscle stimulation compared to femoral nerve stimulation and also showed islands of glycogen retention following the depletion protocol, providing evidence that BTX-A still blocked some acetylcholine release six months into the recovery period.

5.4.1 Muscle weakness

There was a substantial increase of knee extensor strength in the BTX-A injected quadriceps within the first month of recovery. This rapid strength increase in BTX-A+1M compared to BTX-A+0M group rabbits suggests that the effects of BTX-A on acetylcholine release are greatly diminished within a short time, which is consistent with previous findings. There could be at least two conceptual reasons for this finding. First, the effects of BTX-A on muscle contraction remain about the same from months 1-6 of the recovery period. Our results of increased force with direct muscle compared to femoral nerve stimulation at six months of recovery, and the retention of some glycogen following the depletion protocol suggest that BTX-A is still present at the end of the recovery period, and that this remnant BTX-A prevents increases in muscle force, as observed here for the latter part of the recovery period. However, this seems a rather unlikely scenario, as one would expect that BTX-A effects diminish over time and the toxin be cleared from the system, thereby allowing for continuous increases in strength over time.
A second possibility for our findings is based on the work by De Paiva et al. (1999) who found that nerves of muscles injected with BTX-A start to sprout new neuromuscular junctions about four weeks after BTX-A injection. These newly sprouted pathways are in parallel to the original blocked neurons, and they are thought to circumvent the blocked passages, thereby providing some innervation and activation of the BTX-A injected muscles. Once the original neurons recover their function, this new, parallel system is disassembled leaving the “old” innervation pathway to do the job. Possibly, the assembly and disassembly of parallel neural pathways, and the unblocking of the original pathways, are in some equilibrium that provides similar activation (and thus muscle force) in the BTX-A injected muscles, despite a continuous removal of BTX-A from muscles, and thus a continuous decrease in the inhibiting effects of BTX-A on muscle contraction and force production (de Paiva et al., 1999).

The contralateral non-injected quadriceps muscles also had a rapid recovery of muscle strength, reaching similar values to control rabbits within the first month of recovery. It has been shown that BTX-A can affect distant, non-target muscles in a manner that is still not fully understood (Dodd et al., 2005; Fortuna et al., 2011; Girlanda et al., 1992). For example, Naidu et al. reported that some cerebral palsy children receiving BTX-A injections into the calf muscles experienced loss of control in the sphincter muscle of the bladder. However, such adverse effects typically disappeared within one to six weeks after injection, which is similar to the results observed in our study (Naidu et al., 2010).
5.4.2 Muscle atrophy

Muscle mass did not significantly recover within the first month of the recovery period, but improved substantially after three and six months of recovery (Figure 5-2). However, even after six months of recovery, BTX-A injected muscles did not reach the muscle mass of control group animals. These results are consistent with others who found that a single injection into the rat gastrocnemius produced 38% muscle atrophy at 22 weeks after injection (Billante et al., 2002). Furthermore, a single injection into a healthy human gastrocnemius muscle resulted in a reduction of the cross-sectional area that was noticeable one year after the injection (Schroeder et al., 2009). These persistent atrophies following BTX-A injection are of concern, as they will affect muscle performance and function in everyday life.

Quadriceps mass was significantly decreased following the BTX-A injections in contralateral non-injected muscles, but recovered to control values within the first month of the recovery period and remained there for the three and six months recovery group rabbits (Figure 5-2). Although muscle mass recovered quickly, the corresponding contractile material did not, and a substantial part of the muscle mass was comprised of connective tissue that had replaced contractile material (Figure 5-3). This finding may potentially explain the reduced (albeit not statistically significant) force of the contralateral non-injected muscles throughout the six month recovery period (Figure 5-1).

Interestingly, there is a qualitative difference in the contractile material loss between the injected and the contralateral non-injected quadriceps musculature. The BTX-A injected muscles appear to have lost contractile material and was primarily replaced by fat, while the contralateral
non-injected muscles lost contractile material primarily at the expense of fibrous connective tissue (Figure 5-3; left vs right column). Although we did not stain the histological sections for fat and connective tissue specifically, this finding is fairly obvious nevertheless (Figure 5-3). The injected muscles regained a normal percentage of contractile material after the six months recovery period, while interestingly, the contralateral non-injected muscles did not. These results suggest that contractile material recovery following fat invasion and fibrosis have different time courses: fat invasion can be partially reversed within a six month period, while fibrosis cannot. It would be important to determine the rate of recovery for the contralateral non-target muscles, and ascertain that indeed they do recovery in due time.

5.4.3 Contractile material

While strength and muscle mass increased in the first month of recovery, the percentage of contractile material showed a significant increase from months 3-6 in BTX-A injected muscles, suggesting that strength and muscle mass recover at a different rate compared to contractile material. While muscle strength and muscle volume can easily be assessed in patients using dynamometry and ultrasound imaging, respectively (Fukunaga, Kawakami, Kuno, Funato, & Fukashiro, 1997), structural integrity is hard to quantify. Therefore, the recovery of muscles following BTX-A treatment in patients might be overestimated with strength and volume measurements alone. For example, the recovery of muscle mass to over 80% in BTX-A injected muscles of BTX-A+3M/6M group rabbits must be viewed by keeping in mind that less of the mass is contractile material in the recovery group compared to the control group rabbits. Of particular significance is also that neither BTX-A injected nor contralateral non-injected contractile material recovered to control values within the six months recovery period. Previous
studies have also reported an increase in connective tissue and fat infiltration in BTX-A injected muscles (Fortuna et al., 2011; Schroeder et al., 2009). Ideally, muscle composition should not be compromised during BTX-A treatment, so that muscles can function properly when treatments are terminated. In an attempt to minimize contractile material loss in BTX-A injected muscles, we used direct muscle stimulation during the treatment period with some success (Fortuna, Horisberger, Vaz, Van der Marel, & Herzog, 2013).

5.4.4 Femoral nerve vs. direct muscle stimulation and glycogen depletion protocol in BTX-A+6M group rabbits

The injected quadriceps of BTX-A+6M group rabbits produced higher forces with direct muscle compared to femoral nerve stimulation, suggesting that there are remnant effects of BTX-A at the neuromuscular junction (Figure 5-4). It has been suggested that new neuromuscular junctions are formed within 3-6 months of BTX-A injection (de Paiva et al., 1999), although abnormalities in neural innervation are observed up to 9 months (Duchen, 1970), thus supporting our results of incomplete activation of injected muscles through nerve stimulation six months after BTX-A injection. In agreement with our force results, a glycogen depletion protocol did not eliminate glycogen from all fibers, suggesting that these fibers were not activated by nerve stimulation because of remnant BTX-A blockage at the end of the six months recovery period (Figure 5-5).

The contralateral non-injected quadriceps muscles of BTX-A+6M group rabbits produced similar force with direct muscle and indirect femoral nerve stimulation (Figure 5-4), and glycogen depletion in the non-injected musculature was virtually complete (Figure 5-5). These
findings suggest that after six months of recovery, BTX-A paralysis effects on the non-injected musculature of the contralateral hind limb were minimal or completely absent. Therefore, BTX-A alone might not explain the persistent decrease in contractile material in the contralateral non-injected musculature of BTX-A+6M group rabbits. Possibly, the animals might have been less active following BTX-A injections (not monitored) and this might have led to structural changes associated with disuse atrophy (Dodd et al., 2005). However, this seems unlikely, as even normal rabbits move very little (200 hops in a 24h period), and ground reaction forces (and thus muscular forces) were increased in the contralateral hind limbs of unilaterally injected BTX-A animals, suggesting a compensation by the contralateral leg for the weakened BTX-A injected hind limb (Longino et al., 2005; Longino, Frank, et al., 2005). Another possibility is that the BTX-A effects that are present at recovery stages less than six months, might have initiated the observed structural changes of the non-target muscles, and these changes are not reversed between months 3 to 6 of the recovery period. However, in order to elucidate the detailed and time-dependent changes in structure in non-injected muscles following BTX-A injections, we need to study this phenomenon systematically in the future.

5.4.5 Clinical significance

There is little systematic research on the long-term recovery of BTX-A injected muscles. However, results of pilot studies suggest that muscle properties do not fully recover within a year of a single BTX-A injection (Schroeder et al., 2009), and adverse effects are dose-dependent (Albavera-Hernández et al., 2009; Naidu et al., 2010). Our results suggest that recovery of muscle properties is incomplete after six months of recovery in an aggressive BTX-A injection protocol. Unfortunately, we cannot compare our results directly to equivalent studies in human
patients receiving BTX-A treatments, as muscle recovery has not been a focus in human studies, while the effects of BTX-A in controlling muscle activation and increasing muscle and joint excursion have been well documented (Hesse et al., 1994; Hesse, Brandl-Hesse, Seidel, Doll, & Gregoric, 2000).

This study has several limitations and careful interpretation is required when trying to extrapolate our findings to clinical practice. First, our injection protocol, although not more severe in its total injection magnitude of botulinum toxin, was more frequent (once per month) than would be used in clinical practice. Second, the distances between target and non-target muscle, a higher dilution protocol (20U/ml), and the multiple injection sites might favour the systemic spread of the toxin into non-target muscles beyond that seen in patients. Third, the metabolic rate of small animals is greater than that in humans; hence the spread of toxin might occur faster in rabbits than in humans (Hulbert, Pamplona, Buffenstein, & Buttemer, 2007). Lastly, BTX-A injections have been applied in conjunction with modalities such as taping, stretching, and electrical stimulation in clinical practice, which were not applied in the current model, but might reduce toxin spread. Taken together, the spread of BTX-A, and its associated effects on non-target muscles, are likely exaggerated in our animal model compared to the clinical situation in humans. Therefore, extrapolation to clinical practice should be made with utmost caution.

Future research, including clinical trials, should not focus exclusively on functional outcomes for patients, but also on muscle structure and integrity. Further research may include systematic quantification of muscle structure and integrity during the BTX-A treatment and
recovery periods. Muscle imaging modalities could be used to assess changes in muscle volume (MRI) or changes in fascicle lengths, angles of pennation, tendon elongation, and fascicle excursions for prescribed joint movements (ultrasound imaging). Muscle strength could also be determined using dynamometry for voluntary contractions, or better yet, for controlled electrical stimulation which would eliminate problems with muscle activation and joint coordination and motivational factors of strength measurements. Finally muscle biopsies could be used to evaluate changes in contractile properties and structural protein isoforms, collagen content, stem cells, and changes in the molecular biology profiles associated with muscle atrophy and recovery of the target and non-target musculature.

Despite its limitations, we hope that the present study might motivate clinical studies in human subjects evaluating systematically the long term recovery of BTX-A injected muscles and non-target muscles. The argument that recovery of muscle function in BTX-A treated patients, for example children with cerebral palsy, is not important because BTX-A treatment is merely a precursor to more radical surgical intervention, is not acceptable, as the aim should be to keep muscles functional, so that non-surgical treatment options remain open and are encouraged.

5.5 Conclusion

We conclude from the results of this study that neither target nor remote non-target muscles in our animal model completely recover within six months from a repeat injection BTX-A treatment protocol. In the pursuit of non-surgical treatment options for skeletal muscle spasticity, systematic clinical studies of muscle recovery following BTX-A treatments should be pursued.
Chapter Six: A Clinically Relevant BTX-A Injection Protocol Leads to Persistent Weakness, Contractile Material Loss, and an Altered mRNA Expression Phenotype in Rabbit Quadriceps Muscles

6.1 Introduction

It has been demonstrated in the previous chapter (chapter 5) that six-monthly repeat BTX-A injections into the quadriceps followed by a six months passive recovery period was not sufficient for muscle strength, mass, and contractile material to recover to control values.

In a previous study, Schroeder et al. (2009) found a significant muscle atrophy in healthy human gastrocnemius one year following a single, clinically relevant BTX-A injection, suggesting that BTX-A may last well beyond the expected treatment period and that muscle structure takes a very long time to fully recover (Schroeder et al., 2009). Additionally, studies presented in chapters 2 and 5 demonstrated that an aggressive BTX-A injection protocol for six months led to substantial muscle weakness, atrophy, and contractile material loss, and that muscles did not fully recover within a six months period following the last BTX-A injection. However, the Schroeder et al. (2009) study was a pilot experiment involving two subjects only, while our studies used an aggressive BTX-A treatment protocol that does not reflect clinical practice (Fortuna et al., 2011). Currently, clinical application of BTX-A, and the timing between repeat injections, are based on clinical experience and anecdotal evidence, and studies quantifying muscle strength and structural damage following clinically relevant BTX-A treatment protocols have not been performed systematically.

Therefore, the purpose of the present study was to evaluate muscle strength, mass, and contractile material loss six months following a single or repeat (2 and 3) BTX-A injections into
the quadriceps femoris muscles of NZW rabbits. An interval of 3 months between repeat injections was chosen to approximate current clinical practice. Additionally, selected mRNA expression profiles were measured to assess protein expression turnover in connective tissue and muscle atrophy markers in order to better understand the recovery process following BTX-A injections.

6.2 Methods

6.2.1 Experimental design

Twenty three skeletally mature, one year old, female NZW rabbits were used for this study. All procedures were approved by the Animal Care Committee of the University of Calgary. Rabbits were allowed normal cage activity (65 x 45 x 30cm³) and received a standard diet.

Rabbits were divided into four groups as follows:

(1) Control group – saline injection unilaterally (n=5; control) (same Control group rabbits as described in chapter 3)

(2) Single BTX-A injection unilaterally (n=6); 1-BTX-A)

(3) Two BTX-A injection with a 3 month interval between injections (n=6; 2-BTX-A)

(4) Three BTX-A injections with a 3 month interval between injections (n=6; 3-BTX-A).

6.2.2 Botulinum toxin Type-A: injection protocol

The BTX-A injection protocol was similar to the ones reported in the previous chapters. Rabbits received intramuscular BTX-A injections at a total dosage of 3.5U/kg of body weight randomized to either the right or left quadriceps musculature. The anterior compartment of the thigh was isolated by manual palpation and the quadriceps was visually divided into superior and
inferior halves. Each half was subdivided into a medial, central, and lateral section. One sixth of the total BTX-A dose was injected into each section to increase diffusion and to equally distribute the toxin throughout the different portions of the quadriceps musculature.

Control group 1 received intramuscular saline injections randomized to left or right leg. The total volume of saline injection was the same as the total volume of BTX-A injected into the experimental group rabbits. Group 2 rabbits received a single intramuscular BTX-A injection and was evaluated six months post-injection. Group 3 and 4 rabbits received two and three BTX-A injections, respectively, with a three month interval period between injections, and rabbits were evaluated six months following the last injection (for details see appendix A-4).

The primary outcome measures were the isometric knee extensor strength (measured via femoral nerve stimulation and via direct muscle stimulation, the mass of the individual quadriceps muscles, the percentage of contractile material. Additionally, select mRNA expression profiles of connective tissue and muscle atrophy markers of the target muscles were measured to assess the recovery process six months following the BTX-A injection protocol.

### 6.2.3 Knee extensor strength and muscle mass

Isometric knee extensor strength was measured in the BTX-A injected musculature six months following the last BTX-A injection. Knee extensor strength was assessed by stimulating the quadriceps via a femoral nerve cuff electrode implanted prior to testing (Longino et al., 2005). Following nerve cuff implantation, rabbits were secured in a stereotactic frame using bone pins at the pelvis and femoral condyles. Isometric knee extensor strength at 100° of knee
flexion were measured using a strain-gauged, calibrated bar placed over the distal portion of the rabbit’s tibia.

Stimulation of the knee extensor musculature (Grass S8800 stimulation; Astro-Med Inc., Longueil, Quebec, Canada) was performed at a voltage three times higher than the alpha motoneuron threshold to ensure activation of all motor units (Herzog & Leonard, 1997). Stimulation duration was 500ms, pulse duration 0.1ms, and the frequency of stimulation was 100Hz.

Knee extensor strength was also measured by means of direct muscle stimulation of the injected musculature in the BTX-A experimental group rabbits. Rabbits were positioned as described above. The quadriceps musculature was identified and self-adhesive superficial electrodes were placed over the mid-belly region. Maximum forces were obtained by increasing the voltage (frequency at 100Hz) of stimulation until no further increases in force were detected (Fortuna et al., 2013).

Following knee extensor strength assessment, animals were sacrificed using an overdose of Euthanyl (MTC Pharmaceutical; Cambridge, Ontario) into the lateral ear vein. Wet mass for the individual quadriceps femoris muscles was determined using a commercial scale with a resolution of 0.001g.
6.2.4 Contractile material

The percentage of contractile material was determined histologically. The central third of the quadriceps muscles was embedded in paraffin (automatic paraffin processor, Leica TP 1020) and cut cross-sectionally with a microtome (Leica RM 2165). For every 100µm, an 8µm section was collected for staining with haematoxylin-eosin (H&E) (Leica ST5010). Five slides were analyzed and averaged for each muscle.

Photographs were taken from each section using an Axionstar plus microscope (Carl Zeiss) with a 5x magnification objective. A customized Matlab program (Matlab 7.8, R2010b) was used to calculate the percentage of contractile material for at least 50% of the total cross-sectional area of each muscle.

6.2.5 RNA isolation and RT-qPCR analysis of muscle tissue

Samples of frozen quadriceps femoris tissue were powdered at liquid nitrogen temperatures with a Braum Mikro-dismembrator (B. Braum Biotech International, Allentoen, PA, USA) and total RNA isolated using the TriSpin method (Reno, Marchuk, Sciore, Frank, & Hart, 1997). Briefly, following powdering, the samples were treated with Trizol Reagent (Life Technologies, Gaithersburg, MD, USA) and the RNA-rich layer was further fractionated and then treated with DNAase, as described previously (Hart & Achari, 2010; Leumann et al., 2012). Total RNA was quantified using a NanoVie Spectrophotometer (GE Healthcare, Baie d’Urfe, Quebec).
Aliquots (1µg) of each sample were then reverse transcribed using an Omniscript RT Kit (Qiagen Inc., Chatsworth, Calif., USA). All samples in a given set were reverse transcribed at the same time to avoid potential variation. qPCR was performed as described previously (Hart & Achari, 2010) with an iCycler (BioRad Laboratories Inc, Mississauga, ON) and validated using rabbit-specific primer sets for the molecules listed in table 1. All assessments were performed in duplicate or triplicate under optimal conditions that conformed to qPCR criteria.

### Table 1 Validated rabbit primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing T°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S (Housekeeping gene)</td>
<td>TGG TCG CTC GCT CCT CTC C</td>
<td>CGC CTG CTG CCT TCC TTG G</td>
<td>63</td>
</tr>
<tr>
<td>Collagen I</td>
<td>GAT GCG TTC CAG TTC GAG TA</td>
<td>GGT CTT CCG GTG GTC TTG TA</td>
<td>59</td>
</tr>
<tr>
<td>Collagen III</td>
<td>TTA TAA ACC AAC CTC TTC CT</td>
<td>TAT TAT AGC ACC ATT GAG AC</td>
<td>55</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>CGG CAG CTG TAC ATT GAC TT</td>
<td>AGC GCA CGA TCA TGT TGG AC</td>
<td>55</td>
</tr>
<tr>
<td>IGF-1</td>
<td>AGC TGG TGG ATG CTC TTC AGT T</td>
<td>GAA GCA GCA CTC ATC CAC GAT</td>
<td>56</td>
</tr>
<tr>
<td>MuRF1</td>
<td>AGT GTG TCT TCT CTC TGC TC</td>
<td>CAA TCG CTG GTC ACA CAA TG</td>
<td>55</td>
</tr>
</tbody>
</table>

All sets conformed to qPCR requirements for amplification characteristics

### 6.2.6 Data analysis

Knee extensor strength (femoral nerve stimulation) and muscle mass of BTX-A injected groups were expressed as a percentage of the values obtained from control group rabbits.
Reduction in knee extensor strength will hereafter be referred to as muscle weakness, while the loss of muscle mass will be referred to as muscle atrophy. Knee extensor strength for direct muscle stimulation of BTX-A injected rabbits was expressed as the difference in strength relative to the corresponding values obtained from femoral nerve stimulation. The amount of contractile material for each muscle is given as the percentage area of the contractile material relative to the total cross-sectional area of the analyzed sections.

A one-way ANOVA was used to assess muscle weakness, atrophy, contractile material, and mRNA profiles with the main factor groups (control, 1-BTX-A, 2-BTX-A, and 3-BTA). A post-hoc Tukey test was performed if indicated. A paired $t$-test was used for comparisons between femoral nerve vs. direct muscle stimulation in BTX-A experimental group rabbits. The level of significance was chosen as $\alpha=0.05$ a priori.

6.3 Results

The first BTX-A injection induced loss of body weight and lack of appetite, however the subsequent injections were well tolerated by all rabbits. Additionally, body weight recovered to before injection values for 1, 2, and 3 BTX-A group rabbits followed by a 6 months recovery time period (appendix B-3). Data were analyzed from all animals, except for the percentage of contractile material from the rectus femoris of the control rabbits, as these samples were of bad quality.
6.3.1 Muscle weakness

Knee extensor strength was significantly reduced to 45% at six months following a single BTX-A injection compared to control group rabbits (100% - dark bar, p<0.05 Figure 6-1). There was no additional loss in muscle strength for repeat BTX-A injections (2/3-BTX-A group rabbits).

Figure 6-1 Mean muscle strength (±1 SE) normalized to control group rabbits (100%) six months after the last BTX-A injection in rabbits receiving 0 (control), 1 (1-BTX-A), 2 (2-BTX-A), and 3 (3-BTX-A) BTX-A injections with a three months interval between injections. Knee extensor strength in the quadriceps femoris of all experimental group rabbits was reduced compared to control group rabbits, but was the same among the three BTX-A experimental groups. * compared to control group rabbits. For absolute values see appendix C-8.
6.3.2 Muscle atrophy

Muscle mass in all experimental BTX-A group rabbits was unaltered compared to control rabbits at six months following the last BTX-A injections (Figure 6-2).

![Figure 6-2 Mean muscle mass (±1 SE) normalized to the values of control group rabbits (100%) six months following the last BTX-A injection in rabbits receiving 0 (control), 1 (1-BTX-A), 2 (2-BTX-A), and 3 (3-BTX-A) BTX-A injection with a three months interval between injections. Muscle mass in all experimental BTX-A group rabbits was unaltered compared to control group rabbits. For absolute values see appendix C-9.](image-url)
6.3.3 Contractile material

The percentage of contractile material for control rabbit was 96.9 ±2.0%. Following a single BTX-A injection, the contractile material was reduced to 59.2 ±6.0% at six months following the injection ($p<0.05$). There was no additional loss in contractile material at six months following the last injection for 2-BTX-A (62.5 ±6.1%) or 3-BTX-A (59.9 ±11.8%) group rabbits (Figure 6-3).

Figure 6-3 Exemplar histological cross-sectional images showing the percentage of muscle contractile material (H&E – red staining) and non-contractile material (white color –
primarily fat and connective tissue). The amount of contractile material for control group rabbits was 96.9 ±2.0% (top left). Following a single BTX-A injection, there was a significant reduction of contractile material for 1-BTX-A group rabbits (top right – 59.2 ±6.0%). There was no additional loss of contractile material for rabbits that received two injections (2-BTX-A; bottom left – 62.5 ±6.1%) and three injections (3-BTX-A; bottom right – 59.9 ±11.8%) group rabbits, respectively.

6.3.4 Femoral nerve vs. direct muscle stimulation in the 3-BTX-A group rabbits

Direct muscle stimulation of the injected quadriceps of 3-BTX-A group rabbits produced 15% greater knee extensor strength than femoral nerve stimulation ($p<0.05$; Figure 6-4)

![Figure 6-4 Knee extensor strength obtained with femoral nerve (dark bar) and by direct muscle stimulation (light bar) for the injected quadriceps femoris musculature of 3-BTX-A](image-url)
group rabbits. Knee extensor strength was normalized relative to control group values (100%, not shown). Direct muscle stimulation produced significantly greater muscle forces than femoral nerve stimulation. This result suggests that despite a six months recovery period following the last BTX-A injection, there is a persistent blockage of acetylcholine release at the neuromuscular junction. * compared to 3-BTX-A femoral nerve stimulation.

6.3.5 mRNA expression profiles

Experimental BTX-A group rabbits (1-BTX-A, 2-BTX-A, and 3-BTX-A) showed significantly elevated mRNA levels for fibrotic scar tissue and muscle inflammatory markers ($p<0.05$; Figure 6-5). Specifically, mRNA expression levels for the matrix molecules collagen I and III, the anabolic growth factors IGF-1 and TGFβ, and muscle-specific atrophy marker MuRF1 were significantly elevated in the BTX-A injected animals compared to control group values. There were no statistically significant differences in mRNA profiles between the three experimental BTX-A group rabbits.
Figure 6-5 mRNA expression normalized to the values of control group rabbits (dark bars – 100%). Gene expression of Collagen I, Collagen III, IGF-1, TGFβ, and MuRF1 were significantly increased compared to control group rabbits. Similar to the mechanical and histological data, there was no difference in mRNA expression between the three BTX-A experimental groups. * compared to control group rabbits.
6.4 Discussion

The primary results of this study were that BTX-A injections resulted in muscle weakness, structural damage, and altered mRNA profiles for a select subset of relevant molecules in the rabbit quadriceps muscles six months following a clinically relevant treatment protocol. Furthermore, and somewhat surprisingly, these adverse effects were not exacerbated by multiple injections of BTX-A.

Knee extensor strength was significantly decreased despite the six months recovery period following the last BTX-A injection (Figure 6-1). The persistence of muscle weakness and contractile material loss suggests that BTX-A injections produce effects that last much longer than previously thought. Currently, a 3-6 months interval between BTX-A injections is recommended to prevent antibody responses against the toxin and to avoid cumulative damage of the target muscles (Graham et al., 2000; Heinen et al., 2010). In a recent paper, it had been shown that a single BTX-A injection into a healthy human muscle caused a loss in muscle volume one year following the injection (Schroeder et al., 2009). Furthermore, patients suffering from anterior knee pain who received BTX-A injections into the vastus lateralis musculature reported improved function and reduced knee pain up to 2 years following treatment (Dunne et al., 2010; Silbert et al., 2012). BTX-A treatment protocols, thus, may produce effects that are much longer lasting than previously thought (Barber et al., 2013; Kaňovský et al., 2009). Therefore, it might be prudent to carefully evaluate the frequency of BTX-A injections, especially in patients who already have a weakened musculature, such as children with cerebral palsy (Damiano, Martellotta, Quinlivan, & Abel, 2001; Stackhouse et al., 2005).
Muscle strength did not recover to control values following a six months recovery period from the last BTX-A injection. This result is likely caused by the reduced amount of contractile material following the recovery period (Figure 6-3), and the remnant blockage of acetylcholine at the neuromuscular junction, as evidenced by the greater quadriceps femoris forces obtained with direct muscle stimulation compared to femoral nerve stimulation (Figure 6-4). Direct muscle stimulation increased peak quadriceps forces across all experimental groups by 15%, on average. Combined with the 34% average loss of contractile material, which would cause at least a 34% in force loss, the average loss of muscle strength of 45% across all experimental group rabbits is well explained by the remnant BTX-A effect and loss of contractile material (Figure 6-6). From the results of this study, it is not clear how long it might take for the contractile material, and thereby muscle strength, to return to control values. It is perceivable that BTX-A alters muscle repair mechanisms permanently, thereby reaching a new homeostatic state, as evidenced by the altered mRNA expression six months following the last injection. Studies using a one or two year recovery period should be performed to evaluate if muscles ever return to normal control values following BTX-A treatment.
Figure 6-6 Average muscle strength in BTX-A group rabbits averaged across all three experimental groups (light gray bar), and estimated loss in strength associated with reduction in contractile material (dark gray bar), and the remnant effects associated with BTX-A blockage (shaded bar). Six months following the last BTX-A injection, muscle strength was still significantly reduced to 55% of the strength in control group rabbits (white bar). The loss in strength associated with the loss in contractile material was obtained by assuming a linear relationship between the amount of contractile material and strength. This assumption likely underestimates the real loss in strength associated with the loss of contractile material.
Muscle mass was the same across all experimental and control group rabbits. At first glance, this result may be surprising. However, careful analysis revealed that the loss of contractile material was offset by a gain in non-contractile material, primarily fat, thereby leaving the overall muscle mass, but not the muscle composition, approximately constant. This finding might be of clinical relevance, as muscle volume measured using non-invasive imaging techniques (MRI, ultrasound) are sometimes used to approximate muscle mass in patient populations to determine progression of a disease or success of a treatment intervention (Damiano & Moreau, 2008). Structural integrity and functional properties of muscles, rather than muscle mass or volume, might be more appropriate outcome measures to determine disease progression or intervention effects.

Increasing the number of injections did not produce additional loss in muscle strength and contractile material, as one might have suspected, suggesting that most of the adverse effects of BTX-A injection into muscles are caused by the first injection, or that recovery period between injections, was sufficient for partial recovery, thereby masking the actual damage induced by subsequent injections. Antibody responses following first BTX-A exposure may prevent muscles from additional damage to subsequent exposures to the toxin, and such responses should be measured in future experiments.

Our findings agree with those of Barber et al. (2013) who found no differences in muscle volume, fascicle length, and physiological cross-sectional area in patients with spastic cerebral palsy exposed to single and repeat BTX-A injections (Barber et al., 2013). Children with spastic cerebral palsy already have a weakened musculature and a loss of muscle mass (Damiano &
Abel, 1998; Moreau, Falvo, & Damiano, 2012; Moreau, Simpson, Teefey, & Damiano, 2010), therefore submitting them to BTX-A treatments has to be evaluated carefully using a risk and benefit assessment, and relieve from spasticity and increased joint range of motion in the short term, might be offset by a loss in muscle mass and strength in the long term.

The temporary blockage effects of BTX-A treatment are supposed to offer a “window of opportunity” to re-train target muscles and induce improved motor control and muscle coordination. Therefore, BTX-A treatments should always be combined with an exercise protocol aimed at taking full advantage of the temporary relaxation of the spastic musculature and limiting muscle atrophy and weakness to the greatest possible extent. It has been shown that a direct muscle stimulation protocol in conjunction with BTX-A treatment limits the loss of contractile material and retains strength and functionality of muscles by circumventing the loss of voluntary muscle activation in the presence of BTX-A induced nerve ablation (Fortuna et al., 2013; Williams et al., 2012). Nevertheless, our findings suggest that multiple injections do not produce additional adverse effects in muscle structure, composition and function, but multiple injections will prolong the period of improved joint function and reduced spasticity which might help delay invasive surgical interventions and might provide increased independence of patients with cerebral palsy.

Following BTX-A injections, there is a persistent mRNA elevation for fibrotic response molecules. While we did not detect overt fibrosis in our study, a persistent low grade fibrotic response may interfere with normal muscle repair, consistent with the idea of compromised
muscle function. Future investigations should focus on elucidating the mechanisms of repair in muscles exposed to BTX-A.

6.5 Conclusions

We conclude from the results of this study that muscle strength and contractile material do not fully recover from BTX-A exposure within a six months recovery period. Furthermore, increased frequency of BTX-A exposure was not associated with increased adverse effects in the target muscles.
Chapter Seven: **Summary and conclusion**

### 7.1 Summary

Muscle spasticity is the most prevalent muscle tone abnormality affecting 70-80% of children with cerebral palsy (Love et al., 2010). Currently, there is no cure for muscle spasticity. Therefore, interventions have been focussed on reducing muscle tone in order to prevent the progression of muscle spasticity and the development of joint contractures. Since the early 1990s, BTX-A has been used as a temporary chemical muscle denervation agent aimed at decreasing muscle spasticity in children with cerebral palsy in an attempt to increase range of motion and joint function (Koman et al., 1993). However, there have been no systematic studies aimed at evaluating the effects of the BTX-A on skeletal muscle structure and function. The specific purpose of this research were:

1. To quantify the changes in muscle strength, mass, and contractile material following 1, 3, and 6 monthly BTX-A injections in the injected and contralateral non-injected quadriceps femoris muscles of NZW rabbits.
2. To determine the effects of a unilateral electrical stimulation training protocol exercise on strength, mass, and contractile material after a six-months BTX-A injection protocol in the injected quadriceps femoris muscles of NZW rabbits, and to quantify the effects of BTX-A injections and electrical stimulation exercise on the contralateral non-injected and non-exercised musculature.
3. To determine the rate of recovery of muscle strength, mass, and contractile material following a six-monthly BTX-A injection protocol in the injected and contralateral non-injected quadriceps femoris musculature.
4. To evaluate muscle strength, mass, contractile material, and selected mRNA expression profiles six months following a single or repeat BTX-A injection (2 and 3) into the injected quadriceps femoris of NZW rabbits. An interval of three months between repeat injections was chosen to approximate clinically accepted practices.

In chapter 3, we found that repeat BTX-A injections cause substantial muscle weakness, atrophy, and grave structural changes in the contractile material on the injected musculature. Surprisingly, we reported that chronic exposure to the BTX-A also affected the strength, muscle mass, and contractile material in the contralateral non-target musculature.

In chapter 4, we showed that a direct muscle stimulation training protocol alleviates some of the BTX-A induced adverse effects in the injected target musculature and prevented all adverse side effects in the contralateral non-injected and untrained musculature. Taken together, these results suggest that direct muscle stimulation provided some protection against the adverse side effects seen in target and non-target muscles following a BTX-A treatment protocol.

In chapter 5, we found that neither the injected nor the contralateral non-injected muscles fully recovered within a six-month recovery period following repeat BTX-A injections, and concluded that clinical studies evaluating muscle structure and recovery should be pursued.

In chapter 6, we found that muscle strength and contractile material do not fully recover within a six months recovery period following clinically relevant BTX-A treatment protocols. Additionally, repeat BTX-A injection exposures were not associated with increased adverse
effects in the target musculature. These results suggest that cerebral palsy children can benefit from repeat BTX-A injections without additional adverse effects induced by BTX-A when a three months interval between injections is respected.

7.2 Conclusions

Based on the findings of this work, we concluded that either a single or repeat BTX-A injections caused substantial muscle weakness, atrophy, and contractile material loss in injected and contralateral non-injected quadriceps femoris muscles of NWZ rabbits. An electrical stimulation training protocol helped alleviate some of the adverse effects induced by the toxin. Lastly, when a three months interval between BTX-A injection is respected, there is no additional adverse effects of repeat injections on muscle strength, mass, and contractile material.

7.3 Future Directions

The results of this thesis have shown that BTX-A injections can cause significant changes in muscle structure and function in injected target muscles and in contralateral non-injected non-target muscles. Changes induced by BTX-A treatments should be considered when planning treatments for children with spastic cerebral palsy, as BTX-A injections may lead to progressive muscle weakness in already compromised muscles. The consistent results found here and the association of BTX-A injections with adverse effects raises questions regarding the application and safety of BTX-A treatment. For example, it seems essential that the following aspects of TX-A treatments are better understood: what is the proper injection dosage, what are the best sites of injection, what dilution is optimal for specific needs, and what is the appropriate duration of
treatment, the best number of repeat injections, as well as the optimal time intervals between
injections.

BTX-A has been used previously as an animal model for muscle weakness (Longino et al.,
2005) and has been linked to a possible systemic spread of BTX-A following injection, thus
affecting distant non-target musculature (Fortuna et al., 2011). We showed indirectly that BTX-
A affects the neuromuscular junction of contralateral non-target muscles in a manner that is not
fully understood (Fortuna et al., 2013). It would be interesting to label and track the BTX-A
using monoclonal antibodies (Marinelli et al., 2012; Meunier, Lisk, Sesardic, & Dolly, 2003) or
by radio activate toxin (Habermann, 1974; Wiegand et al., 1976) in order to understand not only
the pattern of toxin spread, but also the timing of spread and the associated sequence of paralysis
on muscles. Moreover, such findings would provide better insights into the treatment efficacy of
BTX-A by reducing the toxin to an optimal dosage, minimizing toxin spread, and reducing the
adverse effects.

Even though BTX-A caused substantial muscle weakness and grave structural changes,
animals used their injected hind limbs normally with a minimum effect on gait (Longino et al.,
2005). However, repeat BTX-A injections may reduce the animal’s daily life activity levels and
might create a sedentary/inactive animal model. Therefore, it would be ideal to monitor daily life
activity of animals during the BTX-A treatment.

The results of our study showed that muscle strength and structure do not fully recover in a
six-month recovery period. Future studies should provide recovery periods of one to two years to
investigate if muscles ever recover from BTX-A treatment protocols and if so, how long it takes for a full recovery of muscle structure and function.

Lastly, I believe it is important to highlight that the results reported here are based on a healthy animal model. Thus, there exists the possibility that the effects of BTX-A injections might differ if these studies were repeated in an animal model of spastic cerebral palsy with a musculature that is compromised prior to treatment onset. Hence, future studies should focus on understanding the effects of BTX-A on muscle structure and function in compromised and spastic muscles of a cerebral palsy model. Additionally, systematic clinical studies in children with cerebral palsy should be pursued much more aggressively and over much longer follow up times than has been done to date.
REFERENCES


120


Frasson, E., Dall’Ora, E., Bordignon, M., Brigo, F., Tocco, P., Primon, D., … Bertolasi, L. (2012). Spread of Botulinum Neurotoxin Type A at Standard Doses Is Inherent to the Successful Treatment of Spastic Equinus Foot in Cerebral Palsy: Short-Term


APPENDIX A – BTX-A injection protocol timeline

A-1 Injection protocol for 1 (top), 3 (middle), and 6 (bottom) BTX-A month groups.
A-2 Injection protocol for 6 BTX-A without (top) and with direct electrical muscle stimulation (bottom) month groups.
A-3 Injection protocol for BTX-A+0, 1, 3, and 6 months recovery following BTX-A injection protocol.
A-4 Injection protocol for 1 (top), 2 (middle), and 3 (bottom) BTX-A groups followed by a 6 months recovery time period.
B-1 Body weight average (± 1 SE) for BTX-A+ES group rabbits during the BTX-A injection protocol.
B-2 Body weight average (± 1 SE) during the BTX-A injection protocol (up to 6 months) and for BTX-A+1M (7 months), BTX-A+3M (9 months), and BTX-A+6M (12 months) recovery time period.
B-3 Body weight average (± 1 SE) during the BTX-A injection protocol for 1, 2, and 3 BTX-A group rabbits followed by 6 months recovery period.
APPENDIX C – Absolute values of strength and muscle mass

C-1 Absolute values of muscle strength (± 1 SE) following 1, 3, and 6 months BTX-A injections for injected (dark bars) and contralateral non-injected hind limbs (light bars).
C-2 Absolute values of muscle mass (± 1 SE) following 1, 3, and 6 months BTX-A injections for injected (dark bars) and contralateral non-injected hind limbs (light bars).
C-3 Absolute values of muscle mass (± 1 SE) following 1, 3, and 6 months BTX-A injections for the different portions of the quadriceps femoris (VL, RF, and VM).
C-4 Absolute values of strength (± 1 SE) for Control, BTX-A, and BTX-A+ES group rabbits.
C-5 Absolute values of muscle mass (± 1 SE) for Control, BTX-A, and BTX-A+ES group rabbits.
C-6 Absolute values of strength (± 1 SE) for Control, BTX-A+0M, 1M, 3M, and 6M recovery group rabbits.
C-7 Absolute values of muscle mass (± 1 SE) for Control, BTX-A+0M, 1M, 3M, and 6M recovery group rabbits.
C-8 Absolute values of strength (± 1 SE) for the injected Control, 1, 2, and 3 BTX-A group rabbits.
C-9 Absolute values of muscle mass (± 1 SE) for the injected Control, 1, 2, and 3 BTX-A group rabbits.
APPENDIX D – Copyrights

Title: Changes in contractile properties of muscles receiving repeat injections of botulinum toxin (Botox)

Author: Rafael Fortuna, Marco Aurélio Vaz, Aliaa Rehan Youssef, David Longino, Walter Herzog

Publication: Journal of Biomechanics

Publisher: Elsevier

Date: 4 January 2011

Copyright © 2010 Elsevier Ltd. All rights reserved.

Order Completed

Thank you very much for your order.

This is a License Agreement between Rafael Fortuna ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Get the printable license.

License Number 3718971340689
License date Sep 30, 2015
Licensed content publisher Elsevier
Licensed content publication Journal of Biomechanics
Licensed content title Changes in contractile properties of muscles receiving repeat injections of botulinum toxin (Botox)
Licensed content author Rafael Fortuna, Marco Aurélio Vaz, Aliaa Rehan Youssef, David Longino, Walter Herzog
Licensed content date 4 January 2011
Licensed content volume number 44
Licensed content issue number 1
Number of pages 6
Type of Use reuse in a thesis/dissertation
Portion full article
Format both print and electronic
Are you the author of this Elsevier article? Yes
Will you be translating? No
Title of your thesis/dissertation Acute and Chronic Effects of Botulinum Toxin Type-A on the Structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits
Expected completion date Sep 2015
Estimated size (number of pages) 171
Elsevier VAT number GB 494 6272 12
Customer Tax ID BR00946236003
Permissions price 0.00 USD
VAT/Local Sales Tax 0.00 USD / 0.00 GBP
Title: The effects of electrical stimulation exercise on muscles injected with botulinum toxin type-A (botox)

Author: Rafael Fortuna, Monika Horisberger, Marco Aurélio Vaz, Robert Van der Marel, Walter Herzog

Publication: Journal of Biomechanics

Publisher: Elsevier

Date: 4 January 2013

Copyright © 2012 Elsevier Ltd. All rights reserved.

Order Completed

Thank you very much for your order.

This is a License Agreement between Rafael Fortuna ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Get the printable license.

<table>
<thead>
<tr>
<th>License Number</th>
<th>3718971142839</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Sep 30, 2015</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Journal of Biomechanics</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>The effects of electrical stimulation exercise on muscles injected with botulinum toxin type-A (botox)</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Rafael Fortuna, Monika Horisberger, Marco Aurélio Vaz, Robert Van der Marel, Walter Herzog</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>4 January 2013</td>
</tr>
<tr>
<td>Licensed content volume number</td>
<td>46</td>
</tr>
<tr>
<td>Licensed content issue number</td>
<td>1</td>
</tr>
<tr>
<td>Number of pages</td>
<td>7</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>full article</td>
</tr>
<tr>
<td>Format</td>
<td>both print and electronic</td>
</tr>
<tr>
<td>Are you the author of this Elsevier article?</td>
<td>Yes</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Title of your thesis/dissertation</td>
<td>Acute and Chronic Effects of Botulinum Toxin Type-A on the Structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Sep 2015</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>171</td>
</tr>
<tr>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------</td>
</tr>
<tr>
<td><strong>Customer Tax ID</strong></td>
<td>BR00946236003</td>
</tr>
<tr>
<td><strong>Permissions price</strong></td>
<td>0.00 USD</td>
</tr>
<tr>
<td><strong>VAT/Local Sales Tax</strong></td>
<td>0.00 USD / 0.00 GBP</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>
Title: Do skeletal muscle properties recover following repeat onabotulinum toxin A injections?

Author: Rafael Fortuna, Monika Horisberger, Marco Aurélio Vaz, Walter Herzog

Publication: Journal of Biomechanics

Publisher: Elsevier

Date: 27 September 2013

Order Completed

Thank you very much for your order.

This is a License Agreement between Rafael Fortuna ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Get the printable license.

<table>
<thead>
<tr>
<th>License Number</th>
<th>3718970838945</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Sep 30, 2015</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Journal of Biomechanics</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Do skeletal muscle properties recover following repeat onabotulinum toxin A injections?</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Rafael Fortuna, Monika Horisberger, Marco Aurélio Vaz, Walter Herzog</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>27 September 2013</td>
</tr>
<tr>
<td>Licensed content volume number</td>
<td>46</td>
</tr>
<tr>
<td>License content issue number</td>
<td>14</td>
</tr>
<tr>
<td>Number of pages</td>
<td>8</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>full article</td>
</tr>
<tr>
<td>Format</td>
<td>both print and electronic</td>
</tr>
<tr>
<td>Are you the author of this Elsevier article?</td>
<td>Yes</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Title of your thesis/dissertation</td>
<td>Acute and Chronic Effects of Botulinum Toxin Type-A on the Structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Sep 2015</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>171</td>
</tr>
<tr>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td>Customer Tax ID</td>
<td>BR00946236003</td>
</tr>
<tr>
<td>Permissions price</td>
<td>0.00 CAD</td>
</tr>
<tr>
<td>VAT/Local Sales Tax</td>
<td>0.00 CAD / 0.00 GBP</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 CAD</td>
</tr>
</tbody>
</table>
This is a License Agreement between Rafael Fortuna ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions. All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier Elsevier Limited
The Boulevard, Langford Lane
Kidlington, Oxford, OX5 1GB, UK
Registered Company Number 1982084
Customer name Rafael Fortuna
Customer address 2500 university drive NW
Calgary, AB T2L 0V5
License number 3718971340689
License date Sep 30, 2015
Licensed content publisher Elsevier
Licensed content publication Journal of Biomechanics
Licensed content title Changes in contractile properties of muscles receiving repeat injections of botulinum toxin (Botox)
Licensed content author Rafael Fortuna, Marco Aurélio Vaz, Aliaa Rehan Youssef, David Longino, Walter Herzog
Licensed content date 4 January 2011
Licensed content volume number 44
Number of pages 6
Start Page 39
End Page 44
Type of Use reuse in a thesis/dissertation
Intended publisher of new work other
Portion full article
Format both print and electronic
Are you the author of this Elsevier article? Yes
Will you be translating? No
Title of your Acute and Chronic Effects of Botulinum Toxin TypeA on the
9/30/2015 RightsLink Printable License
https://s100.copyright.com/App/PrintableLicenseFrame.jsp?publisherID=70&publisherName=ELS&publication=00219290&publicationID=12527&rightID=1&ty... 2/6
thesis/dissertation Structure and Function of the Quadriceps Femoris Muscles of New Zealand White Rabbits
Expected completion date Sep 2015
Estimated size (number of
INTRODUCTION
1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS
2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
   "Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the
combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment
terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions