Functional Assessment and Potential Therapeutic Role of Carbon Monoxide Releasing Molecule-3 in a Rodent Model of Compartment Syndrome

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery
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Functional Assessment and Potential Therapeutic Role of Carbon Monoxide Releasing Molecule-3 in a Rodent Model of Compartment Syndrome

(Thesis format: Integrated-Article)

by

Al Walid Hamam

Graduate Program in Surgery

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Surgery

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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Abstract

Compartment syndrome (CS) is a life and limb threatening condition resulting in long term morbidity. Gold standard treatment of CS is surgical fasciotomy. Long-term morbidity is common post fasciotomy. We tested a gait analysis system (CatWalk™) to see if we could detect functional effects of CS in our rodent model. We also investigated the effects of carbon monoxide releasing molecule-3 (CORM-3) on the function of gait in rodents post CS.

The CatWalkTM system was able to detect abnormalities in a rodent’s gait post CS. CORM-3 was also found to alleviate the functional deficits following CS. Multiple dose but not single dose CORM-3 has a potential to become a therapeutic agent to be used as an adjunct to surgical fasciotomy for CS to help decrease the long-term morbidity associated with the disease.

Keywords: compartment syndrome, limb function, gait analysis, CatWalk™, carbon monoxide, CORM-3.
Co-Authorship

Each of the co-authors listed below made important contributions to this work. I performed the experiments, data collection and analysis. I have written the manuscripts presented in this thesis with consultation, assistance and critical review by the co-authors.

AbdelRahman Lawendy, PhD, MD, FRCSC and Dave Sanders, M.Sc., MD, FRCSC, in their roles as my joint supervisors have given me direction, advice, and encouragement throughout this project. They also assisted with the study design and data analysis.

Aurelia Bihari, MSc, assisted with the animal preparation, study design, data collection and analysis, and manuscript editing.

Hussein Abdo, BSc, assisted with animal preparation and data acquisition.

Brian Fraser, MD, FRCSC, assisted with animal preparation, histological data collection and analysis.
Dedications

I dedicate this work to my parents. They started their lives as refugees with nothing of material value. They fought a long and hard battle to ensure that my sisters and I have every opportunity possible in this world. They have been a constant source of support and encouragement throughout the years. They have never wavered despite the many years that my education has kept me away from them. I will always be indebted to them for all the love and support that they have provided.

To my dear and loving wife Lina, who left her family, friends, and life to be by my side when I was still a university student at the Faculty of Science. For over 10 years she has helped me through medical school, residency, fellowship and finally this master’s degree. She encouraged me when I doubted myself, and shared with me my joys and sorrows over the years. Thank-you.

To my two children Khaled and Tala. You always meet me with smiles, hugs and kisses. Your unconditional love is my source of joy and motivation.

To the ONE, without who’s guidance and protection I would not have made it this far.
Acknowledgements

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Mrs. Aurelia Bihari for teaching me the techniques, and animal handling/preparation required for this project. Also for helping me perform my experiments, and editing the manuscript. Your support was pivotal for the success of this work.

Hussein Abdo, BSc for help in animal preparation, handling and testing. Also for brainstorming sessions when we encountered difficulties along the way.

Lee Ang, MD for preparing and scoring samples for histological examination.

Gediminas Cepinskas, DVM, PhD for providing CORM-3.

Ms. Jude Hamam for her help with illustrating some figures.
Table of Contents

CERTIFICATE OF EXAMINATION .................................. Error! Bookmark not defined.

Abstract ........................................................................................................................................ ii

Co-Authorship .............................................................................................................................. iii

Dedications ........................................................................................................................................ iv

Acknowledgements ....................................................................................................................... v

List of Tables ................................................................................................................................ x

List of Figures ............................................................................................................................... xi

List of Abbreviations, Symbols, Nomenclature .......................................................................... xii

Chapter 1 - Introduction/Literature Review ................................................................................. 1

1.1 Compartment Syndrome ......................................................................................................... 2

1.1.1 Definition ........................................................................................................................... 2

1.1.2 Etiology .............................................................................................................................. 2

1.1.3 Pathophysiology of CS ........................................................................................................ 3

1.1.4 Diagnosis ............................................................................................................................ 6

1.1.5 Treatment .......................................................................................................................... 10

1.1.6 Long Term Sequelae .......................................................................................................... 10

1.2 Potential Medical Treatments ................................................................................................ 12

1.2.1 L-Ascorbic Acid ............................................................................................................... 12

1.2.2 NSAIDS ............................................................................................................................ 13
1.2.3 N-Acetylcysteine ........................................................................................................ 14

1.3 Carbon Monoxide ........................................................................................................ 15
  1.3.1 “The Silent Killer” .................................................................................................. 15
  1.3.2 Heme Oxygenase System .................................................................................... 16
  1.3.3 Biological Activity of Heme-Oxygenase Products .................................................. 19
  1.3.4 Mechanisms of Action of Carbon Monoxide .......................................................... 19

1.4 Carbon Monoxide Releasing Molecules (CORM) ....................................................... 22
  1.4.1 CORM-3 ............................................................................................................... 25

1.5 Functional Testing ...................................................................................................... 31
  1.5.1 CatWalk™ ............................................................................................................. 32

1.6 Thesis Rationale and Hypothesis .................................................................................. 36

1.7 References .................................................................................................................. 37

Chapter 2 Functional Evaluation in a Rodent Model of Compartment Syndrome

2.1 Introduction .................................................................................................................. 60

2.2 Materials and Methods .............................................................................................. 61
  2.2.1 Experimental Setup .............................................................................................. 62
  2.2.2 Animal Preparation ............................................................................................... 62
  2.2.3 Functional Testing ................................................................................................. 63
  2.2.4 Histologic Testing .................................................................................................. 63
  2.2.5 Statistical Analysis ................................................................................................. 64

2.3 Results ......................................................................................................................... 66
  2.3.1 Functional Testing ................................................................................................. 66

2.4 Discussion ..................................................................................................................... 81
2.4.1 Functional Testing ........................................................................................................ 82
2.4.2 Histological Assessment .............................................................................................. 83
2.5 References ..................................................................................................................... 84

Chapter 3 Functional Effect of CORM-3 Post Compartment Syndrome .......................... 89
3.1 Introduction ..................................................................................................................... 90
3.2 Materials and Methods ................................................................................................. 92
  3.2.1 Animal Preparation .................................................................................................... 92
  3.2.2 Compartment Syndrome ......................................................................................... 92
  3.2.3 CatWalk™ ............................................................................................................... 93
  3.2.4 Functional Testing .................................................................................................. 93
  3.2.5 Statistical Analysis ................................................................................................ 94
3.3 Results .......................................................................................................................... 94
  3.3.1 Static Gait Parameters ......................................................................................... 95
  3.3.2 Dynamic Gait Parameters ................................................................................... 100
3.4 Discussion .................................................................................................................... 103
3.5 Conclusion .................................................................................................................... 105
3.6 References .................................................................................................................... 106

Chapter 4 Summary and Discussion .............................................................................. 114
4.1 Overview of Results ...................................................................................................... 115
  4.1.1 CatWalk™ as a Measure of Function ..................................................................... 115
  4.1.2 Effect of CORM-3 on Function .......................................................................... 116
4.2 Limitations and Future Directions .............................................................................. 116
  4.2.1 CatWalk™ system ............................................................................................... 116
  4.2.2 Animal Model ..................................................................................................... 118

viii
List of Tables

TABLE 1-1 UNDERLYING CONDITIONS CAUSING CS. .................................................................................................................. 5
TABLE 1-2 SUMMARY OF THE EFFECTS OF CO ON THE IMMUNE SYSTEM ........................................................................... 21
TABLE 3-1 BASELINE ANIMAL CHARACTERISTICS .............................................................................................................. 97
List of Figures

Figure 1-1 Pathophysiology of CS................................................................. 7
Figure 1-2 Compartment Syndrome induced microvascular dysfunction.......................... 8
Figure 1-3 Heme Degradation Pathway.................................................................. 18
Figure 1-4 Alternative pathway for CO delivery...................................................... 23
Figure 1-5 Molecular structure of common CORM’s................................................. 24
Figure 1-6 Potential anti-inflammatory effects of CORMs........................................ 30
Figure 1-7 The glass walkway of the CatWalk system.............................................. 34
Figure 1-8 The CatWalk setup used in our lab......................................................... 35
Figure 2-1 Experimental Set-up ............................................................................ 65
Figure 2-2 Static Gait Parameters CORM-3 vs iCORM-3....................................... 69
Figure 2-3 Dynamic gait parameters: CORM-3 v.s. iCORM-3................................ 70
Figure 2-4 Effect of CS on Static Gait Parameters................................................... 71
Figure 2-5 Effect of CS on Dynamic Gait Parameters.............................................. 72
Figure 2-6 Static Gait Parameters in Splayed Foot Response Animals....................... 73
Figure 2-7 Static Gait Parameters in Antalgic Response Animals............................. 74
Figure 2-8 Dynamic Gait Parameters in Splayed Foot Response Animals................ 75
Figure 2-9 Dynamic Gait Parameters in Antalgic Response Animals........................ 76
Figure 2-10 Static Gait Parameters for Control Group.............................................. 77
Figure 2-11 Run Duration ................................................................................... 78
Figure 2-12 Histologic Testing ............................................................................. 80
Figure 3-1 Static Gait Parameters of Splayed Foot Response Animals....................... 98
Figure 3-2 RH-LH Static Gait Parameters................................................................ 99
Figure 3-3 RH - LH Dynamic Gait Parameters..................................................... 102
### List of Abbreviations, Symbols, Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>Acute compartment syndrome</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia-mutated</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3',5'-Monophosphate a</td>
</tr>
<tr>
<td>CO-Hb</td>
<td>Carboxyhemoglobin</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-Oxygenase</td>
</tr>
<tr>
<td>CS</td>
<td>Compartment Syndrome</td>
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<tr>
<td>CORM</td>
<td>Carbon Monoxide Releasing Molecule</td>
</tr>
<tr>
<td>iCORM</td>
<td>Inactivated CORM</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cells</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelial Derived Relaxing Factor</td>
</tr>
<tr>
<td>HO</td>
<td>Heme Oxygenase enzyme</td>
</tr>
<tr>
<td>HRQOL</td>
<td>Health Related Quality of Life</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IMP</td>
<td>Intramuscular Pressure</td>
</tr>
<tr>
<td>IP</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>LH</td>
<td>Left Hind limb</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-Activated Protein</td>
</tr>
<tr>
<td>MC</td>
<td>Perivascular Mast Cells</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
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<tr>
<td>NSAIDs</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen Gas</td>
</tr>
<tr>
<td>PAEC</td>
<td>Porcine Aortic Endothelial Cells</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Mononuclear Cells</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear granulocytes</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts Per Million</td>
</tr>
<tr>
<td>RH</td>
<td>Right Hind limb</td>
</tr>
<tr>
<td>$T_{\text{reg}}$</td>
<td>Regulatory T-Cell</td>
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Chapter 1 - Introduction/Literature Review
1.1 Compartment Syndrome

1.1.1 Definition

Compartment syndrome (CS) is defined as an increased pressure within myofascial compartments that produces a reduced-flow ischemic state (Lawendy et al. 2014). It is a surgical emergency, with fasciotomy being the only known effective treatment (Tzioupis, Cox, and Giannoudis 2011). Compartment syndrome results in profound long-term morbidity, and is even associated with 3% mortality acutely (Fry et al. 2013). The resulting systemic inflammatory response leads to remote organ dysfunction; this further exacerbates the morbidity of this condition.

1.1.2 Etiology

Compartment syndrome is caused by swelling of the contents of a myofascial compartment in response to trauma (Harvey et al. 2012). Edema is usually proportional to the amount of energy imparted to the contents of the compartment (Whitesides and Heckman 1996). This is further exacerbated by bleeding and haematoma formation. Since the capacity to increase the volume of the compartment is limited due to the unyielding fascial enclosure, the increased volume results in increased intra-compartmental pressures. As the intra-compartmental pressure approaches the diastolic blood pressure, vascular supply to the compartment is compromised, resulting in a reduced flow ischemic state. The oxidative stress caused by the ongoing ischemia/re-perfusion cycle leads to further inflammation, which, in turn, increases the swelling/edema (Whitesides and Heckman 1996; Lawendy et al. 2011; Giannoudis, Tzioupis, and Pape 2009).
Orthopaedic trauma is the most common cause for compartment syndrome. Other causes include: vascular (ischaemia/re-perfusion injury, haemorrhage, or phlegmasia caerulea dolens), iatrogenic (vascular puncture in anticoagulated patients/haemophiliacs, intravenous/intra-arterial drug injection), or soft tissue injury (prolonged limb compression, crush injury, or burns) (Tiwari et al. 2002).

Of the orthopaedic causes, tibial diaphyseal fractures are associated with the highest rate of CS; almost 6% of patients under the age or 35 with a tibial diaphyseal fracture sustain a compartment syndrome (Table 1.1) (McQueen and Gaston 2000).

1.1.3 Pathophysiology of CS

The pathophysiology of CS involves a complex interplay of hypo-perfusion, inflammation and oxidation, which results in muscle death and remote organ injury (Harvey et al. 2012). Increased pressure in the compartment can result from increased contents (hemorrhage, edema) or decreased space (constrictive dressings or casts) (Prasarn and Ouellette 2011).

There have been several theories to try to explain this phenomenon including the “microvascular occlusion theory” (Hargens et al. 1978), the “critical closing pressure theory”(Ashton 1975), and the “arterio-venous gradient theory”(Matsen 1980; Vollmar, Westermann, and Menger 1999; Matsen and Krugmire 1978).

The microvascular occlusion theory proposed that, as tissue pressure increased past a critical pressure, the capillaries collapsed leading to decreased blood flow resulting in tissue ischemia (Hargens et al. 1978). In 1998, Hartstock et al
designed an experiment to test Hargens’ theory by studying the capillary blood flow in rodent cremasteric muscle under varying amounts of external pressure. Even though they observed decreased perfusion, no arteriolar collapse was observed.

The critical closing pressure theory was proposed by Ashton et al. in 1975. It suggested that a drop in the transmural pressure triggered an active closure, or spasm of arterioles. Closure of the arterioles would decrease blood flow to target tissues leading to tissue ischemia. In 1999, Vollmar, Westermann, and Menger performed an experiment in an animal model to assess the vascular response of varying transmural pressure; they failed to show any arterial spasm or collapse.

Finally, the arterio-venous gradient theory proposed that increasing compartment pressures would result in a decreased arteriovenous pressure gradient (Elliott and Johnstone 2003). Flow of blood from the high-pressure arteriole to the low-pressure venules is dependent on maintaining this pressure gradient. As the interstitial pressure increases, venules will collapse due to the lack of muscle in their walls (Prasarn and Ouellette 2011). Arterioles, however, will remain patent, leading to increasing tissue edema and a further rise in interstitial pressure. The cycle leads to microvascular dysfunction and areas of ischemia (Lawendy et al. 2011). This in turn, leads to neutrophil infiltration, inflammation, generation of reactive oxygen metabolites, and increased osmotic load, all of which further increase tissue edema and compartment pressures (Figure 1.1) (Lawendy et al. 2011).
<table>
<thead>
<tr>
<th>Underlying Condition</th>
<th>Number of Patients</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibial diaphyseal fracture</td>
<td>59</td>
<td>36.0</td>
</tr>
<tr>
<td>Soft-tissue injury</td>
<td>38</td>
<td>23.2</td>
</tr>
<tr>
<td>Distal radial fracture</td>
<td>16</td>
<td>9.8</td>
</tr>
<tr>
<td>Crush syndrome</td>
<td>13</td>
<td>7.9</td>
</tr>
<tr>
<td>Diaphyseal fracture of the Radius or ulna</td>
<td>13</td>
<td>7.9</td>
</tr>
<tr>
<td>Femoral fracture</td>
<td>5</td>
<td>3.0</td>
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<tr>
<td>Tibial plateau fracture</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>Hand fractures</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Tibial pilon fractures</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Foot fractures</td>
<td>3</td>
<td>1.8</td>
</tr>
<tr>
<td>Ankle fracture</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Elbow fracture-dislocation</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Pelvic fracture</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Fracture of the humerus</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>164</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Table 1-1 Underlying conditions causing CS.

Adapted from McQueen and Gaston 2000.
1.1.4 Diagnosis

Diagnosis of compartment syndrome has traditionally been mainly clinical. Pain on passive stretch of the muscles within the compartment, as well as pain out of proportion to the injury, and tense compartments are strong indicators of CS. The “5 Ps” currently taught to many medical trainees (Pain, Paraesthesias, Pallor, Paralysis, Pulselessness), are in fact signs of an established compartment syndrome with irreversible damage (Whitesides and Heckman 1996; Harvey et al. 2012; Prasarn and Ouellette 2011; Elliott and Johnstone 2003; Giannoudis, Tzioupis, and Pape 2009). Waiting until all 5 P’s are present for diagnosis would result in poor functional outcomes for patients, and increased morbidity and mortality. Ulmer et al (2002) have shown that the specificity of these clinical signs is 97%, while the sensitivity of each varies between 13-19%. When two clinical signs are present, sensitivity increases to 68%; when three are present, it increases to 93%. It is clear from the analysis of Ulmer et al (2002), that the absence of clinical signs is much more powerful in rejecting a diagnosis of CS, than confirming CS in their presence.

Because of the low sensitivity, and instances where clinical exam is not possible (i.e. decreased level of consciousness), various authors have explored the use of pressure monitoring in the diagnosis of CS (Giannoudis, Tzioupis, and Pape 2009; Cobb, Cooney, and An 1995; McDougall and Johnston 1991; Margaret McQueen and Duckworth 2012; Whitesides, Haney, Morimoto, et al. 1975; Whitesides 1998).
Figure 1-1 Pathophysiology of CS.

Proposed pathophysiology of CS centers on increased intracompartmental pressure leading to increased venous pressure and further tissue edema. *Adapted from Prasarn and Ouellette 2011.*
Figure 1-2 Compartment syndrome induced microvascular dysfunction

A combination of a reduced flow state, coupled with activation of the inflammatory apparatus is responsible for microvascular dysfunction found in CS. Illustrated by Jude Hamam; Modified from Lawendy et al (2014).
Several protocols have been studied, including using an absolute intramuscular pressure (IMP) of 30 mmHg (Mubarak et al. 1978) or 45 mmHg (Matsen, Winquist, and Krugmire 1980). Whitesides introduced the idea of pressure difference between diastolic blood pressure and IMP (Whitesides, Haney, Harada, et al. 1975; Whitesides and Heckman 1996; Whitesides, Haney, Morimoto, et al. 1975; Whitesides, Hirada, and Morimoto 1971), based on canine studies. He proposed using a diagnostic trigger of $\Delta P$ of 10-20 mmHg for fasciotomy. McQueen and Court-Brown (1996) performed a prospective study of 116 patients with tibial diaphyseal fractures. They found that when a criterion of $\Delta P$ of 30 mmHg was used, only 3 patients required fasciotomies (2.6%), while using absolute value of 30 mmHg would have led to 43% of patients requiring fasciotomies. A cutoff of 40 mmHg would have led to 23% of patients requiring fasciotomies. No patients in the non-fasciotomy group developed late sequelae of CS.

Routine IMP measurements are problematic as there are multiple myo-fascial compartments in each limb; these have to be measured independently. The location of the measurement within the compartment is also important. Whitesides and Heckman (1996) have shown that measuring IMP 5 cm from an injury will give significantly lower pressure readings. It has also been demonstrated that continuous IMP monitoring was not better than close clinical observation, in terms of time to diagnosis and long-term complications (Al-Dadah et al. 2008). Routine continuous pressure monitoring is still a controversial topic, but is not routinely implemented in Canadian institutions.
1.1.5 Treatment

Compartment syndrome is a surgical emergency. It is widely accepted that fasciotomy is the gold standard treatment. The surgical procedure involves opening or releasing the tough fibrous tissue enveloping the concerned compartment. This allows the contents of the compartment to expand, thereby decreasing the IMP.

Fasciotomy wounds are often left open for 48-72 hours, then closed either primarily or with skin graft. Several methods for performing fasciotomies exist (Appendix A).

The outcome of surgical decompression is directly related to the duration of the compartment syndrome. It has been shown that irreversible muscle necrosis occurs at 4-6 hours after onset of ischemia (Blaisdell 2002). Vaillancourt et al (2004) have shown that in the setting of acute compartment syndrome, 37% of all cases of ACS may develop muscle necrosis within 3 hours of the injury.

Medical treatment of CS is limited to an adjunct to surgical decompression. The aim is to prolong the window between the onset of CS and the time when irreversible changes occur, as well as a decrease in complications/long-term morbidity, and the prevention of the onset of CS in high-risk injuries.

1.1.6 Long Term Sequelae

Undiagnosed or missed compartment syndrome presents with varying degrees of neurological deficits, necrosis, contracture and remote organ damage such as renal failure (Elliott and Johnstone 2003; Whitesides and Heckman 1996).

Infection in missed CS is uncommon, unless an attempt at decompressing the compartment is made after total necrosis of the muscle. In such cases, complete
excision of necrotic tissue or amputation is indicated, in order to avoid sepsis and systemic inflammatory response, both of which may be fatal.

Several authors have reported on the outcomes after fasciotomy (Dover et al. 2012; Hayakawa, Aldington, and Moore 2009; Giannoudis, Tzioupis, and Pape 2009; Fitzgerald et al. 2000). Despite being life- and limb-saving, the procedure is associated with significant long-term morbidity/disability. In a systematic review of 55 studies (reporting on 1920 cases), Hayakawa, Aldington, and Moore (2009) found that 18% of patients had an unacceptable outcome. This was defined as a residual motor or sensory deficit, renal failure, infection, chronic osteomyelitis, rhabdomyolysis, or contracture. They also reported that 5.5% of patients underwent a CS-related amputation, and there was a rate of 3.3% CS associated mortality. They also found that of those patients undergoing fasciotomy in under 6 hours, 88% had acceptable results whereby the delay of more than 12 hours resulted in only 15% acceptable results.

In a retrospective study of 60 patients, Dover et al (2012) found that 70% of patients had persistent symptoms (62% weakness, 66% paraesthesia, 71% dysesthesia). Of those that were symptomatic, 24% had mild, 28.5% had moderate, and 47.5% had severe symptoms. In another retrospective study, Frink et al (2009) found that 15.4% of all patients reported pain at rest, and 26.9% had pain on exertion. Furthermore, 53% of patients had sensory deficits, and approximately 70% had limitations in range of motion. Peak torque for ankle dorsiflexion and plantar flexion was also reduced compared to uninjured side.
Giannoudis et al (2002) reported on the impact of CS on health related quality of life (HRQOL). They administered the EQ-5D (a standardized measure of health related quality of life based on five dimensions i.e. self-care, pain/discomfort, mobility, usual activities and anxiety/depression) to 30 patients at a mean of 15 months post fasciotomy. Compartment syndrome patients reported significantly worse scores on the pain and mobility, compared to a control group with tibia fractures without CS.

It is clear from the above that even though fasciotomy is the only gold-standard treatment for CS, it is associated with significant long-term morbidity. Thus, any adjunct treatment that may decrease the need for fasciotomy, or increase the window of opportunity before muscle necrosis develops has the potential to significantly decrease the societal burden associated with compartment syndrome.

1.2 Potential Medical Treatments

Several authors have investigated various potential medical treatments/adjuncts for CS, due to the significant morbidity associated with CS and fasciotomy. Given that the pathophysiology of CS involves inflammation, oxidative stress and ischemia, any substance that can reduce one (or more) of these pathways may potentially be beneficial. Thus, L-ascorbic acid (Vitamin C), n-acetylcysteine (NAC), and non-steroidal anti-inflammatory drugs (NSAIDs) have been investigated.

1.2.1 L-Ascorbic Acid

L-ascorbic acid, or vitamin C, is a naturally occurring essential nutrient produced by all plants and most mammals. The exception to this is humans,
primates, guinea pigs, and the Indian fruit bat. Vitamin C is necessary in the production of collagen, and is a potent anti-oxidant. It has been shown to be concentrated in leukocytes, eyes, adrenals, pituitary, and brain (Levine and Rumsey 1998; Jacob and Sotoudeh 2002). Vitamin C has also been shown to play an important role in immune function, largely owing to its anti-oxidant properties (Jacob and Sotoudeh 2002).

The work of Kearns et al (2004; 2001) has demonstrated that vitamin C can preserve muscle function, decrease neutrophil myeloperoxidase (MPO) activity, and decrease tissue edema in models of ischemia/re-perfusion as well as in compartment syndrome.

1.2.2 NSAIDS

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of drugs that inhibit the activity of the enzyme cyclo-oxygenase (COX). The membrane-bound enzyme has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed, and leads to the production of prostacyclin from an arachidonic acid metabolite. In the endothelium, prostacyclin has anti-thrombotic properties, while in the gastric mucosa it exhibits cytoprotective functions (Write 2002). COX-2 is an inducible isoform; its expression is increased in response to inflammatory stimuli and cytokines (Write 2002). It is the inhibition of COX-2 that is thought to be the mechanism of action of NSAIDs (Vane and Botting 1998).

Inflammation appears to be responsible for at least some of the muscle necrosis seen CS (Harvey et al. 2012). As such, anti-inflammatory medications may
prove to be of benefit in the setting of CS. Manjoo et al (2010) has demonstrated that administration of indomethacin, an NSAID, improved perfusion and tissue viability in a rodent model of CS.

1.2.3 N-Acetylcysteine

N-Acetylcysteine (NAC), a precursor of glutathione, and a donor of nitric oxide (NO) is a potent anti-oxidant. NAC has been used in a wide variety of clinical applications (Schaser et al. 2005; Aruoma et al. 1989). These include reduction of hepatocyte injury after acetaminophen overdoses, protection of kidneys prior to administration of radiographic dye, protection of lung epithelial cells from injury caused by activated neutrophils, reduction of oxidative burst activity of neutrophils while enhancing phagocytosis, in both rodents and humans (Kearns et al. 2010; Zafarullah et al. 2003).

While NAC is an especially potent anti-oxidant, it also works to replenish intracellular glutathione stores. In fact, it appears that glutathione is one of the most important components of the endogenous cellular anti-oxidant defense (Schaser et al. 2005).

Using their rodent CS model, Kearns et al (Kearns et al. 2010) showed that treatment with NAC either before, or after inducing CS preserved muscle contractility when compared to control. Myeloperoxidase activity and respiratory burst activity in stimulated neutrophils were also attenuated by administration of NAC.
1.3 Carbon Monoxide

Recently, another potential medical therapy for CS, has received an increased attention: carbon monoxide (CO). CO is known to be a toxic gas, responsible for many deaths every year around the world (Gorman et al. 2003).

1.3.1 “The Silent Killer”

Carbon monoxide (CO) has traditionally been considered a poison. Public health campaigns have labeled CO as “The Silent Killer”. In fact, it is classified under WHIMIS as: class D: poisonous material-division 1: Materials Causing Immediate and Serious Toxic Effect("WHMIS - Classification : OSH Answers" 2011). CO is a common cause of injury world wide, and the leading cause of poisoning death in the United States (Prockop and Chichkova 2007). Its toxicity is related to its high affinity for hemoglobin, estimated to be 210-300 times that of oxygen (Burg; Prockop and Chichkova 2007; Ryter and Otterbein 2004; Gorman et al. 2003). Not only does CO compete with oxygen for binding to hemoglobin, but it also causes a shift in the oxygen dissociation curve to the left; this makes it harder for O2 to dissociate from hemoglobin at target tissues, leading to hypoxia (Lippi et al. 2012; Ryter and Otterbein 2004).

Other toxic effects of CO have been proposed based on the ability of CO to bind to other hemoproteins such as cytochrome P-450 and cytochrome-c oxidase (Ryter and Otterbein 2004). At concentrations above 88nM CO may also directly activate caspase-1, a pro-apoptotic enzyme. CO has also been shown to deplete mitochondrial glutathione stores, enhance leukocyte sequestration, promote
oxidation of plasma low-density lipoproteins, and increase free-radical production (Lippi et al. 2012; Gorman et al. 2003).

Symptoms from CO poisoning start to appear when the carboxyhemoglobin (COHb) levels reach around 20% (normal baseline level is 1%). These include headache, nausea, vomiting, impaired memory, emotional instability, dizziness, dimness of vision, and confusion. Coma and death occur when COHb levels reach 70-80% (Burg, Ryter and Otterbein 2004; Gorman et al. 2003).

Recently, the understanding of the biological role of CO has undergone a change of paradigm, similar to that of another toxic gas, nitric oxide (NO). In 1988 NO was discovered to be, in fact, endothelial derived relaxing factor (EDRF), a gaseous intracellular signaling molecule (Marks et al. 1991). This discovery prompted researchers to investigate the potential role of CO in intra-cellular signaling. It was already known that CO acted as an activator of guanylyl cyclase (Furchgott and Jothianandan 1991); in 1993, Varma et al published an article in Science showing that CO had a role as a potent regulator of guanosine 3',5'-monophosphate (cGMP) in rat olfactory neurons. Once the role of CO as a messenger was shown, a research field to elucidate the physiological functions of CO was established (Ryter and Otterbein 2004).

1.3.2 Heme Oxygenase System

Endogenous production of CO is accomplished by heme-oxygenase enzymes (HO). Heme oxygenase catalyses the breakdown of heme into CO, iron and biliveridin (Figure 1.3). Three isoforms of this enzyme exist, HO-1, HO-2 and HO-3.
HO-1 is an inducible form that is up-regulated in response to cellular stress such as hypoxia, oxidative stress, heavy metals, thiol-reactive substances, and bacterial lipopolysaccharide (Ryter et al. 2002; Ryter and Otterbein 2004; Maines 1997). One theory on the action of HO-1 is that it acts as a common pathway for the actions of mediators such as Interleukin-10, Prostaglandin J2, and NO (Otterbein et al. 2003). It is said that there is no known enzyme that is affected by as many diverse stimuli as HO-1 (Maines 1997). HO-1 is found ubiquitously in all tissues, but it is only constitutively expressed in the spleen, where it functions as part of the hemoglobin recycling system (Maines 1997). Heme oxygenase 2 (HO-2) is constitutively expressed in endothelial, heart, kidney, liver, testicular, and neuronal cells (Rochette et al. 2013; Maines 1997). Its reaction rate is limited by the availability of heme (Morse et al. 2009). Its function appears to control the basal level of heme in tissues from the degradation of heme-dependent proteins or retiring red blood cells (Motterlini, Mann, Johnson, Clark, Foresti, and Green 2003a).

Heme oxygenase 3 has a structure similar to HO-2. It has been identified in rat brain tissues, but its function is poorly understood (Rochette et al. 2013).
Figure 1-3 Heme Degradation Pathway.

Heme is broken down into biliverdin, iron (Fe2+), and carbon monoxide (CO). All three products are potential signaling molecules. *Used with permission from Ryter and Otterbein 2004.*
1.3.3 Biological Activity of Heme-Oxygenase Products

There is evidence that all byproducts of the HO pathway are biologically active (Maines 1997; Otterbein et al. 2003). Biliverdin is converted to bilirubin by biliverdin reductase. Both biliverdin and bilirubin have been shown to possess powerful anti-oxidant properties (Baranano et al. 2002). Ferrous iron (Fe$^{2+}$) induces expression of transferrin, which chelates Fe$^{2+}$ and prevents formation of iron free radicals. It also upregulates transmembrane iron transport proteins that increase the efflux of iron from the cells. These two mechanisms have been shown to contribute to the cytoprotective function of HO-1 (Ferris et al. 1999). Despite the biological activity of all three products of HO, many authors (Lee and Chau 2002; Otterbein et al. 2000; Otterbein et al. 2003; Lee et al. 2007; Ryter et al. 2002) believe that CO is the main player responsible for the cytoprotective effects of HO-1.

1.3.4 Mechanisms of Action of Carbon Monoxide

CO has been shown to possess several functions, including neurotransmission, control of vessel tone, anti-inflammatory actions, inhibition of platelet aggregation, neuroendocrine functions, activation of ion channels, oxygen sensing, control of cell proliferation, and anti-apoptotic effects (Motterlini et al 2003a). These are achieved via different signaling pathways, depending on the tissue CO is active in (Otterbein et al. 2000; Otterbein et al. 2011; Lee and Chau 2002; Motterlini and Otterbein 2010). The first demonstration of CO biological function came from Verma et al (1993) and Morita et al (1995). They were able to demonstrate that CO acted via a cGMP pathway to effect change in neuronal signaling and vascular tone respectively.
Morita et al. also showed that CO acted in a negative feedback loop to downregulate hypoxic HO-1 expression. More recently, it has been shown that HO-1, or exogenously applied CO, reduced smooth muscle cell proliferation in a cGMP dependent pathway (Durante 2011).

Another pathway through which CO has been found to exert its effect is the mitogen-activated protein kinase (MAP kinase) system (Otterbein et al. 2000). CO was found to produce a strong anti-inflammatory effect in macrophages stimulated with LPS; CO specifically decreased production of TNF-α, interleukin-1β, and macrophage inflammatory protein-1β, while increasing the anti-inflammatory IL-10 both in vitro and in vivo, mediated by MAP kinase, independent of the cGMP pathway. In another study, Lee et al. (2002) demonstrated that the anti-inflammatory actions of IL-10 were mediated by HO-1 and CO, via p38 MAP kinase.

CO has also been shown to induce DNA repair pathways (Otterbein et al. 2011). HO-1 expression and exogenous CO were shown to act via ataxia telangiectasia-mutated (ATM) to signal DNA damage, and recruit repair machinery. Rodents with Hmox1/- mutation were unable to repair broken DNA efficiently, and had shorter lifespans; application of exogenous CO reversed these effects (Otterbein et al. 2011).

Finally, CO has been shown to increase activity of regulatory T-Cells (Treg) through activation of the FoxP3 transcription factor (S. S. Lee et al. 2007). This effect increased survival of transplanted islet cells in recipient animals (Table 1.2).
Table 1-2 Summary of the effects of CO on the immune system.

*Used with permission from Wegiel, Hanto, and Otterbein (2013)*
1.4 Carbon Monoxide Releasing Molecules (CORM)

Several studies, registered on clinicaltrials.gov, investigate the effect of inhaled CO on various conditions, such as pulmonary fibrosis, intestinal paralysis, pulmonary inflammation, and mitochondrial biogenesis (Home-ClinicalTrials.Gov 2014). Despite gaseous CO being used in clinical trials, several concerns exist with regards to its safe implementation. CO has an affinity to hemoglobin that is 220-fold higher than that of oxygen (Motterlini 2007). Thus, CO has the ability to compromise oxygen delivery to tissues, resulting in hypoxia. Given the toxic potential of CO, its administration would require hospital admission and observation. In addition, other questions also exist regarding inhaled CO: even though COHb level may be maintained below the toxic 15%, it is not certain that it is the optimal measure of toxicity. It is not known what exposure times and COHb levels are most efficient in achieving therapeutic results; whether different pathological states require different concentrations; what the effect of long term inhalation of low concentration CO gas would be. These have pushed the need to develop an alternative therapeutic substance that can deliver exogenous CO in an oral or parenteral route (Motterlini and Otterbein 2010).

A novel class of drugs has been created to safely deliver CO to target tissues: carbon monoxide releasing molecules (CO-RMs). These are based on transition metals, and have the ability to reversibly bind CO (Figure 1.4). The ability of CO to reversibly bind iron in hemoglobin, to form a stable iron carbonyl, was used as the basis for the development of this class of compounds (Figure 1.5) (Motterlini and Otterbein 2010).
Figure 1-4 Alternative pathway for CO delivery.

Used with permission (Romão et al. 2012)
Figure 1-5 Molecular structure of common CORM's.

CORM-3 has a single Ru core, with three CO ligands, one chlorine ligand, and one glycine ligand. *Adapted from Motterlini and Otterbein 2010.*
1.4.1 CORM-3

Several CO-RMs have been developed, and are in various stages of testing. The general formula for most CO-RMs is $M(CO)_xL_y$ (Santos-Silva et al 2011b), where “$M$” is a transition metal, “$x$” is the number of CO ligands and “$y$” is the number of other ligands. The first CO-RMs (CORM-1, CORM-2) to be produced were lipid-soluble, requiring DMSO/ethanol to dissolve them. This made their development as drugs more challenging as researchers had to overcome several obstacles including toxicity, and the ability of the metal carbonyl to release CO when in an aqueous solution (Motterlini et al 2005b). This limitation led to the development of water soluble CO-RM, CORM-3. CORM-3 (molecular formula $[Ru(CO)_3Cl(\text{glycinate})]$) is based on the transition metal ruthenium. Water solubility was conferred by the addition of the amino acid glycine to the metal carbonyl. It has a short half-life, releasing CO in about 1 minute in physiological conditions (i.e. $37^\circ C$ and pH7.4) (Motterlini, et al 2005b). Proof of concept testing by Motterlini et al (2005b) has shown CORM-3 to exert a profound vasodilatory effect on preconditioned isolated rat aorta, and produced significant hypotension after administration. Using a negative control (inactivated CORM-3, iCORM-3), no vasodilation or hypotension was observed, indicating that CO was responsible for these effects. Furthermore, CORM-3 was found to be non-toxic to cells at concentrations up to 500µM. Thus, Motterlini et al (2005) have shown that CORM-3 met the criteria for a viable pharmacologically active CO carrier.
1.4.1.1 Evidence

The effects of CORM-3 have been investigated by several authors (Maicas et al. 2010; Soni, Jain, and Mehta 2011; Guo et al. 2004; Masini et al. 2008; Bani-Hani et al. 2006a; Bani-Hani et al Motterlini 2006b; Desmard et al. 2009; Filippo et al. 2012; Kramkowski et al. 2012; Varadi et al. 2007; Davidge et al. 2009; Mizuguchi et al. 2010; Sawle et al. 2005; Urquhart et al. 2007; Lancel et al. 2009; Lawendy et al. 2014). The following have been investigated: anti-thrombotic effects, anti-hypertensive effects, vascular relaxation effects, down regulation of the inflammatory response, cardio-protective effects in models of myocardial infarct, and anti-rejection effects in allografts (Appendix C).

1.4.1.1.1 Safety of CORM-3

Before its potential for pharmaceutical application can be considered, it is important to elucidate the biological activity, metabolism and toxicology of the CO carrier. CORM-3 has been investigated extensively with that respect. Characterization of CORM-3’s interaction in aqueous solution with plasma proteins was carried out using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), X-ray crystallography, Liquid-Chromatography Mass Spectrometry (LC-MS) and infrared (IR or FTIR) spectroscopy (Santos-Silva et al. 2011a). The studies have revealed that upon administration, the chlorine and glycine ligands on CORM-3 are lost or replaced by plasma proteins. They also showed that only one of the three CO ligands is lost to plasma proteins (myoglobin/hemoglobin). CO is released rapidly after administration ($t_{1/2}$ in vivo is $\sim$3.6 min). The residual metal carbonyl
takes on the form Ru (CO)₂ which is bound to protein. Though it has not been shown definitively yet, it has been suggested that the remaining two CO ligands slowly release from the Ru-protein adduct, giving rise to a more sustained slow release of CO. This theory was supported by the finding of Wang et al. (2010), where mice were given a daily dose of CORM-3. The mice maintained a constant level of COHb of 6%. This evidence appears to support the idea of a slow sustained release of CO rather than an immediate rapid release. In the first paper characterizing CORM-3, Motterlini et al (2003b) also noted that using multiple doses of iCORM-3 did elicit a vasodilatory effect on an isolated aortic ring preparation, suggesting that in the presence of a cellular system, the 2nd and 3rd CO ligands may be liberated.

Cell viability in the presence of CORM-3 was examined by Vadori et al (2009a), using porcine aortic endothelial cells (PAEC) and primate peripheral blood mononuclear cells (PBMC). Cell preparations were incubated with CORM-3 or iCORM-3 at various concentrations ranging from 0.02 mM to 1mM for 72 hrs. Sustained concentrations of CORM-3 of 0.5 and 1mM were found to effect PAEC viability within 24 hours, while 0.3mM CORM-3 only affected PAEC viability after 72 hours. All other concentrations tested did not affect PAEC viability. Primate PBMC viability was only affected at concentrations of 0.5 and 1 mM. Apoptosis in primate PBMC was only seen when incubated at concentrations of 1mM (Vadori et al 2009a).

In the study of the effects of CORM-3 on the inflammatory response following LPS administration, Sawle et al (2005) determined that CORM-3 or iCORM-3 did not affect the viability of RAW264.7 macrophages at concentrations of 10-100 µM.
Similarly, the viability of rat aortic smooth muscle cells was found to be affected by concentrations of CORM-3 of 1mM (Motterlini, Mann, and Foresti 2005a).

In summary, the above-mentioned studies aimed to characterize the interaction of CORM-3 in physiologic solutions, and to determine its toxic limit. They have shown the toxic limit of CORM-3 to both small and large animal cell culture is above 500 µM. They have also shown that only one CO ligand is released rapidly in plasma, though there is still some evidence that late liberation of the remaining two CO ligands is possible.

1.4.1.1.2 Anti-inflammatory

Based on the evidence that HO-1 and CO exhibit strong anti-inflammatory properties, several authors have examined the potential anti-inflammatory effects of CORM-3 (Sawle et al. 2005; Vadori et al 2009b; Yabluchanskiy 2012; Vannacci et al. 2006; Bani-Hani et al 2006c). Sawle et al (2005) found that CORM-3 significantly reduced nitrite production in LPS-stimulated murine macrophages in a dose dependent manner – an effect not seen with iCORM-3, suggesting that it was the action of CO that was responsible. Next, effect of CORM-3 on TNF-α, a powerful pro-inflammatory cytokine, was assessed. Release of TNF-α was completely abolished with the addition of just 10 µM of CORM-3 (Sawle et al 2005). Similarly, Bani-Hani et al (2006c) showed that CORM-3, but not iCORM-3, was able to reduce nitrite and TNF-α production in microglial cells, in a dose dependent fashion, independent of soluble guanylate cyclase or nitric oxide synthase. Rather, the anti-inflammatory effect of CORM-3 was dependent on MAP kinase (Bani-Hani et al 2006c).
Masini et al (2008) examined the effect of CORM-3 on activated human polymorphonuclear granulocytes (PMN), the interaction between PMNs and endothelial cells (EC), and that of perivascular mast cells (MC). CORM-3 was found to significantly decrease the generation of the superoxide anion (O2-) and the expression of β2 integrin (an adhesion molecule in activated human PMNs). CORM-3 also decreased the expression of CD54 (EC surface adhesion molecule) in EC cells, and CD203c in MC co-incubated with activated PMNs. Histamine release from the MC cells was also decreased (Masini et al 2008).

Based on these studies, it is clear that a role for CORM-3 as a therapeutic agent in inflammatory disorders exist, perhaps targeting different parts of the inflammatory cascade (Figure 1.6).
Figure 1-6 Potential anti-inflammatory effects of CORMs

Adapted from Motterlini, Haas, and Foresti (2012).
1.5 Functional Testing

Function in rodents can be assessed using two general methods. The first is electrophysiologic testing. The second is based on free locomotion. In electrophysiologic testing, muscles are isolated and stimulated with electrodes. Isometric contraction is measured in response to stimulation frequency, and maximum contractile force with tetanic activation (Hill 1972; Taylor and Fowler 1976). These measures isolate the muscle, and make it possible to measure the effect of certain conditions on the muscle itself, but do not necessarily reflect the function of the animal. Function is a product of muscle function, inter-limb coordination, and neurologic function. Free locomotion provides better overall reflection of function in a rodent.

Several methods of free locomotion assessments have been devised over the years. Open field locomotion was first developed by Tarlov and Klinger (1954). It involved placing the test subject in a test area with raised boarders, and observing various activities. The method was modified by Basso et al (1995) to what has become known as the Basso, Beattie and Bresnahan (BBB) locomotor rating scale. The BBB scale is the most widely used open field functional test (Koopmans et al. 2005). However, the BBB scale has it’s limitations, including not being able to assess the dynamics of locomotion such as inter- and intra- limb coordination, degree of weight bearing, duration of swing, and stance phases, as well as it being extremely time consuming (F. Hamers, Lankhorst, and van Laar 2001). Other methods were devised to address these concerns such as Afelt’s electric grid method (1983), Cheng’s glass plate method (1997), and paper paw print method (de
Medinaceli, Freed, and Wyatt 1982). Each of these methods has its drawbacks such as being too time consuming, not being able to measure paw area, pressure, contact area at maximal pressure, or swing and contact duration.

1.5.1 CatWalk™

In the past, functional analysis in rodent models required analysis of foot prints made by walking rats through ink, and then onto a paper roll (de Medinaceli, Freed, and Wyatt 1982). This technique was very labour/time intensive. Then, with the advent of computer technology, a new way of gait analysis was designed – the CatWalk™.

The CatWalk™ method was first developed by Hamer et al (2001). In this system, an animal walks along a glass walkway approximately 8 cm wide and 1 metre long. A light source is attached such that all the light is internally refracted in the glass walkway. When a paw makes contact the glass, the light scatters and is captured by a digital video camera connected to a computer. Software then calculates many static and dynamic gait parameters (Appendix B) from the digital images after paw classification. In the original description of the CatWalk (Hamers, Lankhorst, and van Laar 2001) the system was found to be a fast and reliable tool; it was sensitive enough to differentiate between 2 different spinal cord injuries.

Since its introduction in 2001, the CatWalk™ system has been validated as a measure of function in models of spinal cord injury (Hamers, Koopmans, and Joosten 2006; Hendriks et al. 2006; Koopmans et al. 2005; Bozkurt et al. 2011), sciatic nerve injury (Bozkurt et al. 2008; Deumens, Jaken, and Marcus 2007),
movement disorders (Vandeputte et al. 2010), allodynia (Gabriel et al. 2009), and arthritis (Ferreira-Gomes, Adães, and Castro-Lopes 2008; Angeby-Möller, Berge, and Hamers 2008).

The CatWalk™ system requires animals to be trained to run from one end of the walkway to the other, in un-interrupted runs. The speed at which they run across the runway can also significantly affect most parameters. Batka et al (2014) were able to show that around 96% of the parameters produced by the CatWalk™ were affected by the velocity of the traversing animal. It is therefore imperative that animals are trained well and are comfortable with the apparatus prior to initiating the testing process. The weight of the animal can also affect some parameters; it has been demonstrated that a weight gain of more than 40% will significantly change the results (Gabriel et al. 2007).
Figure 1-7 The glass walkway of the CatWalk system.

Metal grates on either end of walkway prevent rats from escaping. Animals walk back and forth along the walkway and paw prints are captured by camera positioned below (Figure 1.8).
Figure 1-8 The CatWalk setup used in our lab.

Digital video camera is mounted below the glass walkway to capture footprints. This video is sent to the CatWalk software to be analyzed.
1.6 Thesis Rationale and Hypothesis

From the review of the literature, it becomes apparent that CS is a morbid condition and its treatment is urgent surgical decompression. This leads to a high level of associated long-term morbidity. The pathophysiology of CS has been shown to involve low-flow vascular dysfunction, inflammation, and oxidative stress, leading to muscle necrosis in as little as 3 hours. Carbon monoxide has been shown to be directly or indirectly involved in multiple signaling pathways involving modulation of vascular tone, immune response and inflammation. These properties would make CO a very good target to pursue in the search for novel medical treatments of compartment syndrome.

The measure of success of any medical adjunct to surgical decompression will ultimately be based on the potential functional improvement. Functional testing using gait analysis is the most comprehensive method, as it combines all factors associated with limb function including pain, paraesthesias, coordination, strength, and endurance.

Therefore, the aim of this thesis was to investigate whether CORM-3 could improve limb function following CS, using the CatWalk™ gait analysis system.

We hypothesized that (A) The CatWalk™ system is a sensitive and specific functional assessment tool of function in rats following CS; (B) administration of CORM-3 will decrease the severity or duration of functional deficits following CS.
1.7 References


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Chapter 2 Functional Evaluation in a Rodent Model of Compartment Syndrome
2.1 Introduction

Compartment Syndrome (CS) is a devastating complication of limb trauma (Tiwari et al. 2002; McQueen and Gaston 2000). The underlying pathology involves hypo-perfusion of a muscular compartment due to increased intra-compartmental pressures, along with the inflammatory response associated with the ischemia/reperfusion cycle (Giannoudis, Tzioupis, and Pape 2009; Lawendy et al. 2011). Combined, these two mechanisms result in local tissue necrosis, along with remote organ dysfunction. CS constitutes a surgical emergency (Harvey, Bernstein, et al. 2012) (Harvey, Sanders, et al. 2012); muscle necrosis can develop as early as 3 hours post-initiation of CS (Vaillancourt et al. 2004). Current gold standard treatment is surgical decompression of involved compartments by fasciotomy. Both CS and fasciotomy are associated with prolonged morbidity, and in some cases, mortality (Dover et al. 2012; Fitzgerald et al. 2000; Giannoudis et al. 2002).

Historically, functional testing post CS has been accomplished by measuring muscle twitch and tetanic activity both in vitro and in vivo (Kearns et al. 2010; Kearns et al. 2004; Criswell et al. 2012; Taylor and Fowler 1976; Hill 1972; Lomo, Westgaard, and Dahl 1974). This involves direct muscle stimulation with electrodes, and does not take into the account other variables that may play a role in animal function, such as neuronal injury, pain inhibition, chronic ischemia, or loss of fine motor coordination. In models of rodent sciatic nerve injury, histologic, morphologic and electrophysiological testing has not correlated with actual functional recovery (Kanaya, Firrell, and Breidenbach 1996; Koka and Hadlock 2001; Varejão et al. 2001; Walker et al. 1994; Munro et al. 1998; Jabaley et al. 1976; J. M. Shenaq,
Normal gait requires cortical coordination between both motor and sensory input (Walker et al. 1994), and as such is not adequately assessed by electrophysiological testing.

While gait analysis has been validated as a measure of function in the sciatic nerve/spinal cord injury models (Hamers, Koopmans, and Joosten 2006; Hendriks et al. 2006; Koopmans et al. 2005; Bozkurt et al. 2011), it has not been assessed in models of compartment syndrome previously. Using our rodent model of CS, we expect dysfunction and fibrosis in both the anterior and posterior compartment muscles, as previous work in our lab has confirmed that both compartments’ pressure is uniformly elevated with this technique. We expect this to manifest itself in abnormality in both static and dynamic gait parameters.

The purpose of this study was to validate an automated gait analysis as a measure of function in a rodent model of CS. Histologic and morphologic data, as well as static and dynamic measures of gait from the CatWalk™ gait analysis system were correlated, in order to quantify functional deficits and recovery post CS. Additionally, the effect of a single-dose of CORM-3 on the severity of CS was investigated.

2.2 Materials and Methods

All protocols and experiments were conducted in agreement with the Canadian Council on Animal Care, and were approved by the Animal Use Subcommittee at the University of Western Ontario. All animals were kept on a 12 hour light/12 hour dark cycle, and fed standard rat chow, with access to water ad
libitum. Animals used for CatWalk™ analysis were trained for one week prior to experiments, to run uninterrupted over the glass walkway in 1-3 seconds.

FruitLoops™ cereal was used as an incentive during the training and testing process.

2.2.1 Experimental Setup

Twenty-four male Wistar rats, body weight 190-230g, were randomized into two groups: control (n=4) and CS (n=20), just after training. The CS group was further divided into CORM-3 (n=10) and iCORM-3 (n=10) groups. All animals received a single intraperitoneal (IP) dose of CORM-3 or its inactive compound, iCORM-3, at a dose of 10mg/kg at the conclusion of CS.

2.2.2 Animal Preparation

Hind limb CS was intitated, as described by Lawendy et al (2011) (Figure 2.1). Briefly, rats were anesthetized with inhalational isoflurane (5% induction, 2% maintenance) in 1:1 mixture of N\textsubscript{2}/O\textsubscript{2}. CS was generated by infusion of normal saline into the right anterior leg compartment through a 24-gauge needle, to maintain an intracompartamental pressure of 40-60 mmHg for 180 minutes (Figure 2.1). A pressure transducer (Synthes, USA) was introduced into the posterior compartment via a 14-gauge angiocatheter, to allow for monitoring of compartment pressures. Control animals underwent the same preparation, but no saline was infused into the anterior compartment.

Following CS, all animals were allowed to recover, with pain management as needed (0.05mg/kg buprenorphine, SC).
2.2.3 Functional Testing

All animals were tested on the CatWalk™ system at 24 hours post CS, and then daily, until 14 days post CS. Primary outcome measures were static and dynamic gait parameters, including contact area (mm²), print length (mm), print intensity, print area (mm²), step regularity index (%), duty cycle (%) stride length (mm) and swing speed (mm/sec).

2.2.4 Histologic Testing

Nineteen rats were used for this part of the study. Animals were euthanized at 24hrs (n=4), 48hrs (n=4), 72hrs (n=4) and 7days (n=5) following CS. The extensor digitorum longus (EDL) muscle was harvested, fixed in 10% formalin; following fixation, 5μm paraffin-embedded sections were prepared (longitudinal and cross-sectional). All samples were then stained using a standard hematoxylin and eosin stain. Mid-muscle sections were analyzed (by a blinded senior pathologist) for cellular infiltration, tissue necrosis and regeneration. Cellular infiltration was defined as any infiltrate of neutrophils, macrophages, or other lymphocytes post-CS. Necrosis appeared as a loss of cross striation of muscles and breakdown of muscle fibers, as well as macrophage infiltration.

Regenerative changes were characterized as cells displaying more translucent nuclei and prominent nucleoli, with sarcoplasm becoming more basophilic. An index of inflammation, necrosis and regeneration was determined by estimating the percentage of each, in relation to the total area in both cross-
sectional and longitudinal specimens, and was reported on a scale of none, mild, moderate, and severe, corresponding to <20%, 20-50%, and >50%, respectively.

2.2.5 Statistical Analysis

Data was analyzed using statistical software (Prism 5, Graphpad Software Inc). Repeated measures one-way ANOVA, with Tukey’s post-hoc testing, was used to determine significant changes within groups. Repeat measures two-way ANOVA with Bonferroni post-hoc test, was used to compare the between-group differences. A p<0.05 was considered statistically significant.
Figure 2-1 Experimental Set-up.

Rodent model of CS. Rat is anesthetized with isoflurane. 24G needle was inserted into anterior compartment, and 14G catheter into posterior compartment.
2.3 Results

2.3.1 Functional Testing

None of the 20 experimental rats died during the experiment. All rats had similar baseline characteristics including weight 256±12g (mean ± SD) at time of CS.

2.3.1.1 - CORM-3 v.s. iCORM-3

Analysis of the static and dynamic gait parameters using 2 Way ANOVA (time and treatment) did not show any difference in response between CORM-3 and iCORM-3 groups in either static or dynamic parameters (Figure 2.2 and 2.3). Mean ± 95% confidence interval (CI) was plotted. This shows the two populations have the same means and can be combined to look at the effect of CS on function.

2.3.1.2 Effect of CS on Static Parameters

Combining CORM-3 and iCORM-3 groups together (n=20) we analyzed the effect of CS on both static and dynamic gait parameters. Maximum contact area increased from 45±2.9mm² on day 0 to 60±5mm² (p>0.05) on day 1 post CS. The Max contact area remained significantly elevated on day 1-3 when compared to day 7; 60.0±5.1mm², 59.6±4.0mm², and 55.9±3.0mm² v.s. 35.6±2.9mm² (p<0.05). A similar result was seen in paw print area. Day 1 and day 2 were significantly elevated compared to day 7; 79.4±6.9 mm² and 82.7±5.0 mm² v.s. 42.8±2.8 mm² (p<0.05). Print intensity increased non-significantly from 187.0±6.1% on day 0 to 204.2±5.0% on day 2 (p>0.05) followed by a statistically significant decrease to 157.7±7.1% on day 7 (p<0.05 v.s. Day 0 and day 2) (Figure 2.4).
2.3.1.3 Effect of CS on Dynamic Parameters

Only run duration showed a statistically significant effect from CS (Figure 2.11). All other dynamic parameters did not show any significant evidence of injury (Figure 2.5). There was a significant increase in swing speed over the course of the experiment becoming significant at day 8; 1464±48.4 mm/s on day 0 to 1698±31.1 mm/s on day 8 (p<0.05). This trend was also seen in stride length 153.0±4.6 mm to 179.4±3.5 mm/s; day 0 v.s. Day 8 (p<0.05). Run duration showed a significant increase from baseline on day 1; 1.24±0.05 s on day 0 to 1.49±0.06 s on day 1 (p<0.01). This returned to normal by day 2 (1.28±0.06 s). A trend towards decreasing run duration was observed and became significant on days 10-12 (p<0.05) and days 13,14 (p<0.01).

2.3.1.4 Effect of CS in Splayed Foot Injury Pattern

Data was divided into 2 injury patterns observed. First is the Splayed foot (n=14) and the second is the antalgic gait (n=6). In the splayed foot pattern, we could see a significant increase in maximum contact area; 43.02±3.5 mm2 on day 0 to 70.17±3.7 mm2 on day 1 (p<0.001). Paw print area showed a similar pattern; 63.0±5.7 mm2 on day 0 to 95.1±4 mm2 on day 1 (p<0.001) and 88.6±7.9 mm2 on day 2 (p<0.05). Print intensity and paw print length showed a similar trend, but this did not reach statistical significance (Figure 2.6).

No injury pattern could be seen in the dynamic parameters of the splayed foot group (Figure 2.8). A trend of increasing swing speed and stride length was observed and became significant on day 8 (p<0.05).
2.3.1.5 Effect of CS in Antalgic Foot Injury Pattern

Injury pattern in this group (n=6) was the opposite that observed in the splayed foot group. Static parameters decreased following injury (Figure 2.7). Paw print area decreased from 83.4±6.7mm$^2$ on day 0 to 42.6±10.6 mm$^2$ on day 1 (p<0.05) before recovering on day 2. Paw print length had a similar result, decreasing from 18.9±0.728mm on day 0 to 13.1±2.1mm on day 1 (p<0.05). Maximum contact area and pint intensity both showed similar trends, but did not reach statistical significance. In all the static parameters, recovery was evident by day 2. No significant injury pattern was evident in the dynamic parameters, though once again a trend towards increasing swing speed and stride length became significant after day 10 (Figure 2.9).

2.3.1.6 Control Group

Control group (n=4) did show an increase of static parameters with time. This trend became significant (p<0.05) in maximum contact area, paw print area, and print intensity (Figure 2.10).
Figure 2-2 Static Gait Parameters CORM-3 vs iCORM-3

No difference was observed between CORM-3 and iCORM-3. Mean±95%CI plotted. (A) Max Contact Area (mm²); (B) Paw Print Length (mm); (C) Print Area (mm²); (D) Print intensity. Two way ANOVA at α=0.05 did not show any difference between CORM-3 and iCORM-3. No difference in either group was seen between baseline and day 1 post CS (P>0.05).
Figure 2-3  Dynamic gait parameters: CORM-3 v.s. iCORM-3.

Mean±95%CI (A) Regularity index; (B) Right hind limb duty cycle; (C) Right hind limb stride length; (D) Right hind limb swing speed. Two-way ANOVA at α=0.05 did not show any difference between CORM-3 and iCORM-3.
Figure 2-4  Effect of CS on Static Gait Parameters.

(A) Max Contact Area (mm²); (B) Paw Print Length (mm); (C) Print Area (mm²); (D) Print intensity. These graphs combine data from CORM-3 and iCORM-3 groups. One-way ANOVA with Tukey's post-hoc test at $\alpha=0.05$ did not find a significant effect from CS, though a trend towards increasing parameters following injury is seen.
Figure 2-5 Effect of CS on Dynamic Gait Parameters

(A) Regularity index; (B) Right hind limb duty cycle; (C) Right hind limb stride length; (D) Right hind limb swing speed. One way ANOVA with Tukey’s post-hoc test at α=0.05 did not show an effect of CS on dynamic gait parameters, though Stride length (C) and Swing speed (D) did increase throughout experiment.
Figure 2-6  Static Gait Parameters in Splayed Foot Response Animals

(A) Maximum contact area; (B) Paw Print Area; (C) Paw Print Length; (D) Print Intensity; only animals displaying a splayed foot response were included in this analysis (n=14). Max contact area as well as print length were significantly increased post CS (p<0.05). Print length and intensity had similar trends but were not significant.
Figure 2-7 Static Gait Parameters in Antalgic Response Animals

(A) Maximum Contact Area; (B) Paw Print Area; (C) Paw Print Length; (D) Print Intensity; Only animals displaying an antalgic response (n=6) were included in this analysis. Paw print area and paw print length showed a significant (p<0.05) decrease after CS. Print intensity and maximum contact area showed similar trends but did not reach significance.
Figure 2-8  Dynamic Gait Parameters in Splayed Foot Response Animals

(A) Regularity index - measures inter-limb coordination.  (B) Duty Cycle.  (C) Stride length.  (D) Swing Speed. Only Animals displaying a splayed foot response were included in this analysis (n=14). No significant injury could be detected. Stride length and swing speed increased over the course of the experiment and became significant (p<0.05) at day 8.
Figure 2-9 Dynamic Gait Parameters in Antalgic Response Animals

(A) Regularity index - measures inter-limb coordination.  (B) Duty Cycle.  (C) Stride length.  (D) Swing Speed. Only animals displaying an antalgic response were included in this analysis.
Control animals were run on the CatWalk™ for 11 days. No interventions were performed on these animals. A trend of increasing parameters can be seen in all graphs. This became significantly different from baseline around day 10.
Figure 2-11 Run Duration

All animals included in this analysis (n=20). The average duration of runs increased post injury, returning to normal by day 2, and then continues to decrease. Run duration becomes significantly (p<0.05) shorter than baseline at day 10.
2.3.2 Histologic Testing

Muscle necrosis was evident in 100% of samples at 24 hrs (50% mild, 25% moderate and 25% severe). At 48 again 100% of samples had necrosis (75% severe, 25% moderate). By 72 hours, only 75% of samples had necrosis (50% moderate; 25% severe). Finally by 7 days there was no evidence of necrosis.

Cellular infiltration was seen as early as 24hrs post compartment syndrome (25% mild, 50% moderate and 25% severe). Cellular infiltration peaked by 48hrs (50% moderate; 50% severe). At 72 hours post CS, only 75% of samples had cellular infiltration (25% mild; 50% severe). Cellular infiltration normalized by 7 days (25% mild).

Regenerative changes were seen in 25% of samples at 24 hours; 0% at 48 hours; 50% at 72 hours (50% of samples had moderate changes) and 80% at 7 days (20% mild; 20% moderate; 40% severe) (Figure 2.9).
Figure 2-12 Histologic Testing

(A) Muscle necrosis. (B) Cellular infiltration (C) Regeneration. Muscle necrosis was present in all samples as early as 24 hours, but increased in severity until 72hrs. A similar trend was found in cellular infiltration. Cellular regeneration was present at mild levels in 25% of samples at 24 hour. 80% of samples had evidence of regeneration at 7 days.
2.4 Discussion

Analysis of the data showed that there is no difference in any parameter between the CORM-3 and iCORM-3 groups (Figures 2.2 and 2.3). Given the almost exact replication of data in both groups, we decided to combine them to evaluate the effect of CS on the gait parameters. None of the static or dynamic gait parameters showed a definitive injury pattern, especially when compared to pre-injury measurements (Day 0) (Figures 2.4 and 2.5). There was a trend towards increased static parameters followed by a decrease to a low on day 7. It became evident that there were two different gait patterns post CS. The first involving an antalgic response with decreased static gait parameters (n=6). The second with a splayed foot resulted in increased static gait parameters (n=14) (Figures 2.6 and 2.7). Separating the animals based on the post-injury gait pattern revealed a statistically significant (p<0.05) increase or decrease in static gait parameters in the splayed foot and antalgic gait animals respectively. This returned to normal by day seven. A somewhat unexpected increase in these parameters was observed after day 7. A similar increase was observed in control animals (Figure 2.7) and is likely related to weight gain. No statistically significant injury pattern was observed in dynamic gait analysis even after separating the 2 gait patterns. There was a gradual and significant (p<0.05) increase in swing speed and stride length as well as a trend towards decreased duty cycle over the course of the experiment in both antalgic and splayed foot animals (Figure 2.6). A significant increase in run duration was observed on day 1 post injury, followed by a gradual decrease. The run duration was statistically faster in the last week of testing compared to baseline (Figure 2.8).
2.4.1 Functional Testing

To our knowledge, this was the first time that the CatWalk™ system had been used to assess function following CS. We managed to further characterize our rodent model of CS, both functionally and histologically.

Administration of a single dose of CORM-3 did not appear to have an effect on any gait parameters. This was somewhat expected, as the in vivo half-life of CORM-3 is 1-3 minutes (Motterlini, et al 2005). Based on our previous work, we understand that an administration of iCORM-3 does not have any effect on pathophysiology of CS. Given that both CORM-3 and iCORM-3 groups had extremely similar results (there were no statistical differences between them), we combined them to analyze the gait disturbance following CS.

It was interesting to find that, despite the exact replication of CS by saline infusion, we had two very different injury patterns emerge; we termed these “splayed foot” and “antalgic”. The splayed foot pattern was much more common, with a ratio of 7:3 splayed versus antalgic. Given that the two injury patterns exhibit the opposite effects on static gait parameters, it was imperative to separate these two groups during analysis. The static gait parameters were most sensitive to the injury in both groups. Unfortunately, statistical significance was only present on the first day post injury. These results are similar to those by Gabriel et al (2009) using the CatWalk™ method compared to von Frey testing for the assessment of mechanical allodynia.
2.4.2 Histological Assessment

The histology results support the findings of the functional testing. Once the two gait patterns were separated, it became apparent that the gait defects all normalized by 7 days post CS. Histologically, all cellular infiltration and muscle necrosis had also resolved by 7 days, and the maximum regeneration was observed on day 7 post CS. These results are in agreement with those of Criswell et al (2012), who found that the majority of muscle fibers had shown evidence of regeneration by seven days post injury, in their reduced-flow CS model. In addition, they reported that the in vitro function also improved from day 4 through to 35 post injury.

This study was not without limitations: fruitloops were used as incentive for running on the CatWalk™, resulting in weight gain of approximately 75-100% over the course of the experiment; this may explain the increase in print size and intensity found after 7 days post CS. Gabriel et al (2009) also found that an increase in animal weight of more than 40% lead to statistically increased gait parameters. In addition, the effect of the animal’s speed on dynamic gait parameters has to also be taken into the account: run duration decreased significantly over the course of the experiment, resulting in a decrease in duty cycle, as well as an increase of swing speed and stride length. Finally, the age of the animals should also be considered: young rats were used, which may have contributed not only to the observed weight gain, but also to the resilience and rapid recovery post CS.

Based on our results, we determined that our rat model of CS is valid, and displays all the hallmarks of acute compartment syndrome, including inflammation
and muscle necrosis. We found the CatWalk™ system a valid functional assessment tool, although our testing protocols may have to be adjusted to increase sensitivity.

2.5 References


Chapter 3 Functional Effect of CORM-3 Post Compartment Syndrome
3.1 Introduction

Compartment syndrome (CS) is a life and limb threatening condition associated with 5% risk of amputation and 3.3% risk of death (Hayakawa, Aldington, and Moore 2009). The most common causes of CS are trauma, ischemia/reperfusion injury, prolonged limb compression, vascular puncture/hemorrhage and constrictive dressings (McQueen and Gaston 2000; Tiwari et al. 2002). Muscles and the accompanying neurovascular structures are enclosed within compartments surrounded by an unyielding fibrous tissue, fascia. The myofascial compartments normally have a resting pressure of 0-4 mmHg. Compartment syndrome occurs when the pressure within the closed myofascial compartment is elevated, resulting in ischemic/inflammatory changes within the affected compartment (Lawendy et al. 2011).

CS is a surgical emergency. Gold standard treatment consists of releasing the fascial compartments involved, thereby relieving the pressure, and re-establishing normal perfusion to the compartment (Harvey et al. 2012). Delay of the treatment will lead to muscle necrosis, systemic inflammation and remote organ failure (Harvey et al. 2012; BD et al. 2012). It is well established that irreversible muscle damage occurs within 6 hours of onset; Vaillancourt et al. (2004) have shown that muscle necrosis can happen in as little as 3 hours after the onset of symptoms. Despite the emergent necessity of fasciotomy, the long-term outcomes are profound: as many as 70% of patients with CS/fasciotomy are left with long-term pain, paraesthesias/dysesthesias, and weakness in the affected limb (Hayakawa et al. 2009; Dover et al. 2012; Fitzgerald et al. 2000; Giannoudis et al. 2002; Frink et al.
Given the emergent nature of this condition, and the long-term consequences of its treatment, it is prudent to find medical adjuncts that would 1) increase the window of muscle survival and 2) decrease long-term morbidity. It has been shown that the underlying pathophysiology of CS involves a complex interplay of inflammation, ischemia/reperfusion, and oxidative stress (Lawendy et al. 2014). Thus, any medical intervention that can interrupt the process of ischemia, inflammation, and oxidative stress would be a good candidate to examine as a medical adjunct. Several substances have been investigated with some limited success, including vitamin C (Kearns et al. 2001; Kearns et al. 2004), indomethacin (Manjoo et al. 2010), and N-acetylcystine (Kearns et al. 2010; Schaser et al. 2005).

The use of carbon monoxide (CO) has been explored in a wide variety of pathologic conditions, including ischemia/reperfusion, organ transplant and sepsis (Ryter, Alam, and Choi 2006). Unfortunately, the therapeutic potential of CO is limited by its toxicity when administered exogenously. In order to maximize the benefit of CO, it became imperative to develop a carrier that could deliver CO to target tissues; one such substance is carbon monoxide releasing molecule-3 (CORM-3). CORM-3 is a water-soluble, ruthenium-based compound able to release CO to target tissues without altering levels of carboxyhemoglobin (Lawendy et al 2014). Lawendy et al (2014) have demonstrated that CORM-3 can improve microvascular perfusion, decrease inflammation and muscle necrosis when administered in a rodent model of CS.
Therefore, the aim of this study was to investigate whether the previously demonstrated histopathologic protective effects of CORM-3 could translate to a functional difference in a rodent model of CS.

3.2 Materials and Methods

3.2.1 Animal Preparation

All protocols and experiments were conducted in agreement with the Canadian Council on Animal Care, and were approved by the Animal Use Subcommittee at the University of Western Ontario. All animals were kept on a 12 hour light/12 hour dark cycle, and fed standard rat chow, with access to water ad libitum. Animals used for CatWalk™ analysis were trained for one week prior to experiments, to run uninterrupted over the glass walkway in 1-3 seconds. FruitLoops™ cereal was used as an incentive during the training and testing process. In order to limit animals’ weight gain, rats were limited to ½-1 piece per animal per day.

3.2.2 Compartment Syndrome

Hind limb CS was initiated, as described by Lawendy et al (2011) (Figure 2.1). Briefly, rats were anesthetized with inhalational isoflurane (5% induction, 2% maintenance) in 1:1 mixture of N2/O2. CS was generated by infusion of normal saline into the right anterior leg compartment through a 24-gauge needle, to maintain an intracompartmental pressure of 40-60 mmHg for 180 minutes (Figure 2.1). A pressure transducer (Synthes, USA) was introduced into the posterior compartment via a 14-gauge angiocatheter, to allow for continuous monitoring of
compartment pressures. Control animals underwent the same preparation, but no saline was infused into the anterior compartment.

Following CS, all animals were allowed to recover, with pain management as needed (0.05mg/kg buprenorphine, SC).

3.2.3 CatWalk™

Animals were trained on the CatWalk for 5-9 days prior to experiments. The goal was to reliably achieve un-interrupted walks across the platform within 1-2 seconds. Once animals achieved the training goal, a baseline (Time 0) recording was taken. Each animal had to achieve at least 3 compliant runs (un-interrupted run of 1-2 second duration and less than 40% variability) at each time-point. Animals were run on the CatWalk 4 hours post CS, then daily for 14 days. CatWalk 7.1 software (Noldus Information Technology, Wageningen, The Netherlands) was used to analyze the runs, using both static (paw length, max contact area, print area, and print intensity) and dynamic (duty cycle, regularity index, standing mean, and swing speed) gait parameters.

3.2.4 Functional Testing

Twenty-two male Wistar rats were used. Rats were obtained from a commercial breeder at approximately 175g. All rats weighed ~200g at the time of experimentation. Animals were randomly assigned into three groups: sham (n=6), CORM-3 (n=7) and iCORM-3 (n=9) groups. Sham animals underwent all procedures as the experimental animals, but no saline was infused into the anterior compartment. At the conclusion of 180 minutes of CS, CORM-3 and iCORM-3
animals received the first intraperitoneal (IP) injection of 10mg/kg CORM-3 or iCORM-3; this was followed by additional injections at 12hr, 24hr, 36hr, and 48hrs post CS. Animals were recovered as per the approved protocols and tested on the CatWalk as stated previously.

Data was analyzed with *a priori* knowledge that there would be 2 different gait patterns, as was observed in chapter 2 (Figure 2-6). To control for these two differing data patterns, we calculated right hind (RH) minus left hind (LH) limb values. This calculation also controlled for the increasing dynamic parameters that were observed due to increasing speed of the animals traversing the CatWalk, also observed in chapter 2 (Figure 2-8).

### 3.2.5 Statistical Analysis

Data was analyzed using statistical software (Prism 5, GraphPad Software Inc). One-way ANOVA with Tukey’s post-hoc testing was used to determine significant changes within groups. Two-way ANOVA with Bonferroni post-hoc test was used to compare between-group differences. Significance was set at p<0.05.

### 3.3 Results

The summary of all animal characteristics is shown in Table 1. Two injury patterns were identified, and were present in both CORM-3 and iCORM-3 groups. The first was a splayed foot with increased static gait parameters; the second was an antalgic gait with decreased static gait parameters. The relative proportion of splayed foot to antalgic was 4:3 in CORM-3 group and 5:4 in the iCORM-3 group.
3.3.1 Static Gait Parameters

Static gait parameters measured were maximum contact area, paw print area, paw print length, and print intensity. Initially, only the animals in the splayed foot response were examined. A significant injury was observed in max contact area, print area, and print intensity at the 4-hour mark (Figure 3.2); max contact area increased from $25\pm5.5$ mm$^2$ in sham to $68.5\pm9.4$mm$^2$ in CORM-3 ($p<0.01$) and $86.1\pm13.3$mm$^2$ in iCORM-3 ($p<0.001$). The difference between CORM-3 and iCORM-3 was not significant ($p=0.12$). Paw print area increased from $35.8\pm7.8$ mm$^2$ in sham to $90.2\pm9.8$ mm$^2$ in CORM-3 ($p<0.01$) and $110.9\pm14.5$ mm$^2$ in iCORM-3 group ($p<0.001$). Again, the difference between CORM-3 and iCORM-3 did not reach significance ($p=0.15$). Print intensity increased from $143.1\pm16.2\%$ in sham to $204.9\pm4.8\%$ in CORM-3 ($p>0.05$) and $215.2\pm8.9\%$ in iCORM-3 ($p<0.05$). Animals in iCORM-3 group had significantly different print intensity from sham at 4 hrs post CS. There was a trend towards a more serious injury in the iCORM-3 group versus the CORM-3 group, but this was not significant. Given a large amount of variability in baseline values (i.e. time=0) an analysis of the difference of right hind (RH) and left hind (LH) limbs was also included (Figure 3.3). The data from all animals was used (both splayed and antalgic response). The maximum contact area between CORM-3 (-31.6±20 mm$^2$) and iCORM-3 (-29±8.0 mm$^2$) was significantly different from sham (-0.4±2.6 mm$^2$) at 4hrs ($p<0.05$). The paw print area exhibited a significant difference from sham (-0.32±0.59 mm$^2$) only in iCORM-3 group (-6.6±1.8 mm$^2$) ($p<0.05$). The paw print area showed that iCORM-3 (-49.3±11.0) was different from
sham (-1.7±3.6) (p<0.01); this was also demonstrated for the CORM-3 (-45.8±24.5) group (p<0.05) at 4 hrs. No significant differences were found in print intensity.
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</table>

Table 3-1 Baseline Animal Characteristics

22 Animals were included in the study. No difference was found in any of these parameters.
Figure 3-1 Static Gait Parameters of Splayed Foot Response Animals

(A) Maximum contact area; (B) Paw print area; (C) Paw print length; (D) Print intensity. Antalgic gait pattern animals were excluded (3 from CORM and 4 from iCORM excluded); iCORM-3 was significantly (p<0.05) different from sham in A, B, and C. CORM-3 was only different from sham in A and B.
Figure 3-2  RH-LH Static Gait Parameters

(A) Maximum contact area; (B) Paw print area; (C) Paw print length; (D) Print intensity. 22 animals were randomized to Sham n=6, CORM-3 n=7 and iCORM-3 n=8. No animals were excluded for this analysis (antalgic + splayed). Both iCORM-3 and CORM-3 were significantly different from sham in A and B. Only iCORM-3 was different from sham in (C). No Significant difference was observed in print intensity.
3.3.2 Dynamic Gait Parameters

Dynamic gait parameters appeared to be less affected by the pattern of disability. They were much more sensitive to detecting a difference between CORM-3 and iCORM-3 (Figure 3.4). iCORM-3, but not CORM-3, had a significant injury compared to sham animals in the categories of regularity index (97.8±1.2%, 74.0±12.3% and 83.9±14% for sham, iCORM-3 and CORM-3 groups, respectively, p<0.05) and swing speed (37±80 mm/s, -624±164mm/s, -498 ±169mm/s in sham, iCORM-3 and CORM-3 groups, respectively, p<0.01 in sham versus iCORM-3; p>0.05 in sham versus CORM-3). The CORM-3 group was significantly different from sham in the stance mean parameter at time 4hrs (0.006±0.007s, -0.07±0.03s, -0.19±0.04s in sham, CORM-3 and iCORM-3, respectively, p<0.05 versus CORM-3; p<0.001 versus iCORM-3). iCORM-3 was also significantly different from sham on day 1 (-0.017±0.007s versus -0.08±0.03s in iCORM-3, p<0.05). The trend of more severe disruption of gait with iCORM-3 versus CORM-3, as well as a faster return to baseline in CORM-3 group was evident, but did not reach statistical significance. Duty cycle showed a strong trend, but did not achieve significance.

It was noted that the CatWalk system was not calibrated properly at the start of the experiment. This was corrected after the first group of animals (i.e. 2 in sham, 2 in CORM-3 and 1 in iCORM-3 group) had undergone testing for the following time-points: baseline, 4hrs, and day 1. The values for these time-points were excluded in the analysis of static gait parameters, as the calibration directly affected the software's ability to determine print size and intensity. Dynamic parameters were
relatively immune to the effects of the calibration, and hence all available data points were used in analysis.
Figure 3-3 RH – LH Dynamic Gait Parameters

(A) Regularity index, (B) Stance phase, (C) Swing speed, (D) Duty Cycle. All data points included in calculation of dynamic parameters. We can see that at the 4hr mark, iCORM-3 is significantly different from sham in A, B, and C, where as CORM-3 is only different from sham in B. iCORM-3 was also different from sham in B at day 1. A trend of more severe, or prolonged deficits in iCORM-3 versus CORM-3 were observed, but were not significant.
3.4 Discussion

Compartment syndrome resulted in profound and long-term decrease in function. It has been demonstrated that CORM-3 does decrease injury at the histopathological level (Lawendy et al. 2014). We set out to examine if CORM-3 would affect function in our rodent CS model. We were able to show some evidence that CORM-3 does, in fact, affect function in our model of CS. A strong trend towards less severe injury was found in the CORM-3 group versus the iCORM-3 group, especially in dynamic gait parameters.

To our knowledge, this is the first study to examine the effect of CORM-3 on physiological function post CS. Other studies have used electrophysiologic techniques to measure function (Kearns et al. 2010; Kearns et al. 2004). The merits of physiologic functional testing include reproducibility of the results and elimination of confounding factors such as pain, loss of co-ordination, and paraesthesias. From a purely scientific perspective, the isolation of muscle function from the other confounding variables may have some merit. From a clinical perspective, though, the end goal is physiological function, a product of all the above-mentioned confounding factors.

The pathophysiology of CS has been shown to involve microvascular dysfunction, resulting in hypo-perfusion/ischemia, inflammation, and oxidative stress. Foresti et al (2004) have demonstrated that CORM-3 produced a dose-dependent vasodilatory effect mediated by the cGMP pathway. The cardioprotective properties of CORM-3 in the setting of hypoxia/reperfusion injury to cardiac tissue have also been studied by several authors (Clark et al. 2003; Vannacci et al. 2006;

Finally the anti-inflammatory effects of CORM-3 have been shown in various models, including arthritis (Ibáñez et al. 2012; Ferrandiz et al. 2007), hemorrhagic stroke (Yabluchanskiy et al. 2012), sepsis (Mizuguchi et al. 2009) (Bani-Hani et al. 2006), organ transplant, (Pizarro et al. 2009; Vadori et al. 2009), and others (Song et al. 2009; Masini et al. 2008). Our study adds to the evidence that CORM-3 has protective functions in ischemia/re-perfusion and inflammatory conditions, given that both of these mechanisms are involved in the pathology of CS.

The only time point in which we could detect a difference between CORM-3 and iCORM-3 was the 4-hour post CS. This is likely related to the short half-life of CORM-3. The 4-hour test was performed 4 hours post CS and CORM-3/iCORM-3 injection. For all the other time points, testing was at least 12 hours after the previous administration of the drug, as testing was done before injection. Also, the inflammatory response continued to escalate for several hours after the termination of CS. Again, since CORM-3 has a short half-life, the inflammatory process would have run un-impeled for almost 12 hours before the second dose of drug was administered, at which point significant damage may have already been done to the limb.

In the process of analyzing the data, we observed two different injury patterns. First, the splay limb, which was characterized by increased print dimensions and intensity; second, the antalgic limb, characterized by the opposite, namely decreased print dimensions and intensity. Combining data from both injury patterns resulted in a net cancellation of their effect. The relative frequency of splay
limb to antalgic limb was 9:7. We aimed to minimize the effect of the different gait patterns by calculating the difference between RH and LH limbs, which created a more homogeneous data set that permitted the inclusion of all data points with regards to the gait pattern observed.

A confounding factor was the animals’ weight gain over the course of the experiment. Others (Gabriel et al. 2009) have noted that a weight gain of more than 40% resulted in statistically significant changes in the static gait parameters. In our experiments, we limited the amount of Fruitloops used as incentive to ½-1 pieces a day, thus keeping the average weight gain to 38.8-44.4%. It was interesting to note that the sham animals gained the most weight (44.4%), followed by the CORM-3 group (39.5%). The iCORM-3 animals gained only 38.6%, suggesting that CORM-3 may have a somewhat protective effect with this regard.

Another limitation of this study was the use of iCORM-3 as a control. Two studies have been done suggesting that iCORM-3 may be able to deliver low levels of sustained CO release in vivo (Wang et al 2010; Motterlini 2003b). A better control in future studies would be ruthenium chloride, which is a salt of ruthenium devoid of any CO ligands.

3.5 Conclusion

We have demonstrated that CORM-3 does have a protective effect in our rodent CS model. This was seen most significantly in the dynamic gait parameters. All abnormalities in gait (both static and dynamic) normalized by 7 days, regardless
of intervention. Further study is needed to elucidate optimal dose and frequency of administration of CORM-3 to achieve optimal results.

3.6 References


Chapter 4 Summary and Discussion
4.1 Overview of Results

4.1.1 CatWalk™ as a Measure of Function

The purpose of our experiments was to determine if the CatWalk™ system is sensitive enough to detect perturbations in function following CS. Results from Chapter 2 showed that the CatWalk was able to detect an injury following CS. The CatWalk™ system needs to be accurately calibrated in order to detect subtle differences in gait parameters. We feel that the calibration of the CatWalk™ was improved in Chapter 3.

We were able to detect a change in both static and dynamic gait parameters post CS. Static parameters normalized rapidly after the injury, but there was a trend to prolonged (up to 7 days) dysfunction in the dynamic parameters. When calibrated accurately, the CatWalk™ system could be a valuable tool to assess function post CS in our rodent model. Both static and dynamic parameters may be used, but there is a trend towards improved specificity with dynamic parameters. In Chapter 2 we found that the increasing weight of the animals significantly increased all static gait parameters, while their increasing speed across the walkway affected the dynamic gait parameters. In order to avert these, we limited the amount of incentive treats used, which limited weight gain to about 40% in Chapter 3. As for the increasing speed, we chose to calculate the difference of the right hind limb minus the left hind limb. Using longer training period may have diminished this effect.
4.1.2 Effect of CORM-3 on Function

CORM-3 has been shown to have anti-inflammatory, anti-apoptotic, and vasodilatory effects both in vivo and in vitro. We explored whether these effects would affect the function post CS. In Chapter 2 we saw that a single dose of CORM-3 had no effect on function. The possible explanation is the short half-life of CORM-3 in vivo (t1/2=1-3 minutes). It had been demonstrated that TNF-α continues to rise for at least 45 min following the termination of CS (Lawendy 2014). Given the short duration of action of a single dose, it is reasonable to expect that it would have minimal effect. Therefore, in Chapter 3 we administered 5 doses of CORM-3 or iCORM-3 to animals post CS. This was accomplished at the end of CS, then every 12 hours for 48 hours. We found a strong trend towards decreased injury in animals receiving CORM-3 relative to iCORM-3. The trend was the strongest in dynamic gait parameters, where only iCORM-3 was significantly different from sham animals in several parameters. The trend in the dynamic parameters was a faster recovery to baseline in the CORM-3 compared to the iCORM-3 groups.

4.2 Limitations and Future Directions

4.2.1 CatWalk™ system

Our aim was to assess function post CS. To do this we used an optical gait analysis tool (CatWalk™). Many factors can affect the results of this assessment. We have demonstrated that weight gain by the animal can significantly increase static gait parameters. In Chapter 2 we did not take this into account, while our animals gained 75-100% of their starting weight. The control group in Chapter 2 clearly
shows that even in the absence of any intervention, static gait parameters are significantly affected by weight gain. In Chapter 3 we made an effort to limit weight gain to close to 40%, as this was the limit above which other authors have demonstrated significant changes in gait parameters (Gabriel et al. 2009). Our sham groups in Chapter 3 did not have a significant increase in gait parameters and only gained 44% of their starting weight.

Another factor that affected the CatWalk™ results is the speed at which the animal traversed the platform. We trained the animals for 4-9 days prior to initiating CS. Our goal was to achieve un-interrupted runs for 1-3 seconds consistently. Unfortunately as the animals became more comfortable running across the platform, they started running faster. This was observed in Chapter 2 when we saw that run duration was significantly shortened compared to baseline after 10 days. In Chapter 3 we attempted to negate this effect by calculating the RH-LH values for dynamic parameters. We found that there was no increase in dynamic parameters in the sham group with time (Figure 3.4).

The CatWalk system relies on light refraction in glass walkway. When an animal’s paw touches the glass walkway, light is reflected from the glass and is captured by a camera below. Any moisture on the walkway will increase the amount of light reflected from the walkway, thereby altering gait parameters calculated by the software. This effect was observed in the time immediately post CS testing (4 hr) as well as occasionally at other time points. All efforts to ensure only runs recorded with a dry walkway were taken, but this was not always possible due to fluid leaking from the CS site, as well as excessive urination following CS.
At times, the animals would drag their underside on the walkway, resulting in excessive noise being picked up by the camera. In these situations, the software was unable to determine the paw prints, which had to be done manually. This introduces a source of error, as each paw print was selected in every frame manually, with no definite differentiation of the print from background noise.

Finally, Noldus, the manufacturer of CatWalk™ system, has developed more advanced software to analyze footprints. This was not available to us in these experiments. The new feature of the software is the ability to measure toe spread, as well as other parameters related to the paw-print. These have been shown to be useful in measuring function post sciatic nerve or spinal cord injury models, and may increase the sensitivity and specificity of the CatWalk™ system to the effects of CS.

4.2.2. Animal Model

Young rats were used as the model for these experiments. Animals were obtained at a weight of 175g from the supplier, although we ensured that they were at least 200g prior to undergoing CS. The animals proved to be incredibly resilient to the effects of CS. Any deficits were normalized by 7 days post injury. Compared to humans, deficits from CS would not be expected to resolve before at least 6-12 months post injury, and the majority of patients will have prolonged morbidity (as discussed in Section 1.1.6). From that respect, young rats may not be an ideal subject to test deficits in voluntary function. Older animals may be less resilient, and less likely to continue to gain weight over the course of the experiment.
Another concern regarding the animal model is the method of inducing CS. In humans, direct tissue trauma such as crush injury or fracture produces a high level on underlying inflammation, which then precipitates the CS. In our rodent model, the inflammation is caused by the high compartment pressures, not vice versa. The effects of CORM-3 may be more pronounced in conditions that have a higher level of underlying inflammation.

4.2.3 Experimental Design

The experiments in Chapter 3 were designed with the assumption that only one CO ligand is released from CORM-3, and none from i-CORM-3. This was confirmed by several authors, as well as our lab (Lawendy et al 2014). There is some evidence, however, (as discussed in Section 1.4.1.1.1) that the two remaining CO ligands may dissociate with time when in physiologic solutions. If that were the case, then our iCORM-3 control may have been delivering smaller, but more sustained levels of CO, especially with the multiple dose administration. This phenomenon would essentially decrease the magnitude of the injury in the iCORM-3 and potentially mask any beneficial effects of CORM-3. In order to control for this, a negative control group needs to be done in which the animals undergo CS, but receive ruthenium chloride as a control. This is a salt of ruthenium that has not been combined with any CO ligands.

4.3 Future Directions

In the future, optimization of CORM-3 dosing of should be undertaken. Either continuous infusion of CORM-3 in the early post CS period, or multiple
injections within the first 12 hours may have more significant findings, as it would offer a more sustained protective effects. This can, at first, be examined using the histologic and IVVM methods described by Lawendy et al (2014) at varying time points after CS, followed by a Catwalk functional assessment after the appropriate dosing regiment is determined.

As discussed in section 1.2, there are several other potential medical treatments for CS that have been investigated. Each of these has shown some promise in improving outcomes. Combining these treatments may produce synergistic effects, greatly decreasing the disability associated with CS.

4.4 References


Appendix A – Fasciotomies

Fig. 3

Technique for decompression of the volar compartments of the forearm. A. The incision extends from the medial epicondyle to the ulnarmost extent of the flexor crease of the wrist. B. The lacertus fibrosus and fascia overlying the flexor carpi ulnaris (FCU) are opened. C. The flexor carpi ulnaris is retracted ulnarily and the flexor digitorum superficialis (FDS) is retracted radially to permit opening of the fascia of the deep volar compartment. Care is taken to avoid the ulnar artery and nerve (U. a. & n.).

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Fig. 4
Technique of four-compartment paratibial decompression of the leg. A. An incision is made from the fibular neck to the lateral malleolus. B. The lateral compartment (LC) is opened. C. Retracting the anterior skin exposes the fascia of the anterior compartment (AC), which is opened, with care being taken to avoid the superficial peroneal nerve (SPn). D. The posterior skin is retracted to expose the fascia of the superficial posterior compartment (SPC), which is opened. E. The lateral compartment is retracted anteriorly. The soleus is released from the fibular shaft and is retracted posteriorly, exposing the fascia of the deep posterior compartment (DPC), which is opened.

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Appendix B - Definition of gait analysis parameters.

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**Paw**

The Paw label.

**Initial Contact At (s)**

Initial Contact At (s) is the time in seconds since the start of the run at which a paw makes contact with the glass plate.

**Stand (s) (or Stance) (s)**

Stand (s) or Stance phase is the duration in seconds of contact of a paw with the glass plate. Kloos et al. (2005) showed that rats with a moderate spinal cord injury showed an increase in stand duration of the hind legs compared to pre-operative values.

**Stand Index**

Stand Index is a measure for the speed at which the paw loses contact with the glass plate. The formula for Stand Index is:

\[
Stand \ Index = \frac{a}{X_0} \times \text{frame rate}
\]

where \( a \) is derived from \( y = ax + b \), that describes a line that best fits through the data points at Max Contact (see below) at and the 90% percentile; \( X_0 \) is the max contact area.

Stand Index should only be used for smoothly moving animals. Stand Index is only calculated when Stand consists of at least 5 frames and there are at least 3 data points between \( t(\text{Max area}) \) and the 90% percentile.

Stand Index is not calculated when two stances were combined (see “combining stances” on page 70) and the Max Contact at point was in the first stance.

**Max Contact At (s)**

Max Contact At (s) is the time in seconds since the start of the run that a paw makes maximum contact with the glass plate. It can be regarded as the point at which the braking phase turns into the propulsion phase during Stand.

**Max Contact At (%)**

Max Contact At (%) is Max Contact At (s) relative to Stand of a paw.

The formula for Max Contact At (%) is:

\[
\text{Max Contact At} = \frac{\text{Max Contact at (s)} - \text{Initial Contact}}{\text{Stand}} \times 100\%
\]
Max Contact At (%) is used in research on spinal cord injury. For example, Hamers et al. (2001) found an increase in Max Contact At (%) for both front and hind paws in rats. This increase was more marked following a contusion injury compared to a transection injury.

**Max Contact Area**

Max Contact Area is the maximum area of a paw that comes into contact with the glass plate. In other words, it is the Print Area at Max Contact at (s).

**Max Contact Max Intensity**

This is the maximum Intensity at Max Contact of a paw. Intensity ranges from 0 to 255. The intensity of a print depends on the degree of contact between a paw and the glass plate and increases with increasing weight. Therefore, Intensity is a measure of weight put on the glass plate.

The Intensity parameter is used to assess the effects of neuropathic pain, including mechanical alldynia (Vrinten and Hamers, 2003). They found that after a chronic constriction injury, which causes neuropathic pain, Intensity was reduced two weeks after surgery and gradually returned to pre-operative values. This change in Intensity showed a high degree of correlation with Von Frey thresholds.

**Max Contact Mean Intensity**

This is the mean Intensity of a paw at Max Contact. Intensity ranges from 0 to 255. See also Max Contact Max Intensity.

**X position**

X is the position of the center of the paw print at Max Contact in the horizontal (walking) direction. X is the distance in the Distance Unit from the left side of the walkway to the center of the print.

**Y position**

Y is the position of the center of the paw print at Max Contact in vertical direction. Y is the distance in the Distance Unit from the top of the walkway to the center of the print.

**Print Length**

Print length is the length (horizontal direction) of the complete print. The complete print is the sum of all contacts with the glass plate, as if the animal’s paw would have been inked (see picture below). Print Length is displayed in the Distance Unit you selected in the Preferences (see page 32).
Print Width

Print width is the width (vertical direction) of the complete paw print. Print Width is displayed in the Distance Unit you selected in the Preferences (see page 32).

Print Area

Print area is the surface area (in the Distance Unit you selected in the Preferences) of the complete print (indicated by the hashed area in the figure below). The Print area is by definition at least as large as Max Contact Area.

Max Intensity At (s)

Max Intensity At (s) is the time in seconds since the start of the run that the maximum Intensity is measured.

Max Intensity At (%)

Max Intensity At (%) is Max Intensity At (s) relative to Stand.
The formula for Max Intensity At (%) is:

\[ \text{Max Intensity at} = \frac{\text{Max intensity At (s) — Initial Contact}}{\text{Stand}} \times 100 \%
\]

**Max Intensity**

Max Intensity is the maximum Intensity of the complete paw.

**Min Intensity**

Min Intensity is the minimum Intensity of the complete paw.

**Mean Intensity**

Mean Intensity is the mean Intensity of the complete paw.

**Mean Intensity of the 15 Most Intense Pixels**

This is the mean Intensity of the 15 pixels of a paw with the highest intensity.

**Swing (s)**

Swing (s) or Swing Phase is the duration in seconds of no contact of a paw with the glass plate.

**Swing Speed**

Swing Speed is the speed (Distance Unit/second) of the paw during Swing.

The formula of Swing Speed is:

\[ \text{Swing Speed} = \frac{\text{Stride Length}}{\text{Swing}} \]

**Stride Length**

Stride Length is the distance (in Distance Units) between successive placements of the same paw.

Calculation of Stride Length is based on the X-coordinates of the center of the paw print of two consecutive placements of the same paw during Max contact and taking into account Pythagoras' theorem. The Stride Length is the line indicated by the arrow in the figure below.
Step Cycle (s)
Step Cycle is the time in seconds between two consecutive Initial Contacts of the same paw:
\[ \text{Step Cycle} = \text{Stand} + \text{Swing} \]

The figure below graphically depicts Step Cycle, Stand and Swing.

Duty Cycle (%)  
Duty Cycle (%) expressed Stand as a percentage of Step Cycle:

\[ \text{Duty Cycle} = \frac{\text{Stand}}{\text{Stand} + \text{Swing}} \times 100 \%
\]

Toe Spread  
For more information on (Intermediate) Toe Spread, Manual Print Length and Paw Angle, see also “Classifying toes” on page 81.

Toe Spread is the distance (in Distance Units) between the center of the first and fifth toe of a hind paw.

Intermediate Toe Spread  
Intermediate Toe Spread is the distance (in Distance Units) between the center of the second and fourth toe of a hind paw (see also “Classifying toes” on page 81).
Manual Print Length

Manual Print Length is the distance (in Distance Units) between the center of the third toe and the heel of a paw (see also “Classifying toes” on page 81).

Paw Angle Body Axis

Paw Angle Body Axis is the smallest angle (in degrees) between the Manual Print Length line and the line representing the orientation of the body axis (see also “Classifying toes” on page 81).

Paw Angle Movement Vector

Paw Angle Movement Vector is the smallest angle (in degrees) between the Manual Print Length line and the line representing the direction of movement of the animal’s body (see also “Classifying toes” on page 81).

Single Stance

Single Stance is the duration (in seconds) of ground contact for a single hind paw (Coulthard et al., 2002, 2003). It is used for gait analysis in pain models.

In CatWalk XT, Single Stance is the part in the step cycle of a hind paw where the contralateral hind paw does not touch the glass plate (see ‘3’ in Figure 6.7).

Initial Dual Stance

Dual Stance is the duration (in seconds) of ground contact for both hind paws simultaneously (Coulthard et al., 2002, 2003). Dual Stance is used for gait analysis in pain models.

In CatWalk XT, a distinction is made between Initial Dual Stance and Terminal Dual Stance. Initial Dual Stance is the first time in a step cycle of a hind paw that the contralateral hind paw also makes contact with the glass plate (see ‘2’ in Figure 6.7).

Terminal Dual Stance

In CatWalk XT, Terminal Dual Stance is the second step in a step cycle of a hind paw that the contralateral hind paw also makes contact with the glass plate (see ‘4’ in Figure 6.7).

Single and Dual Stance values are only calculated if the contralateral hind paw has been placed on the glass plate before and after a step cycle of the other hind paw.
Figure 6.7 Timing View with a graphical depiction of Single and Dual Stance. 1 - Step cycle of the right hind paw, 2 - Initial Dual Stance of RH and LH, 3 - Single Stance of RH, 4 - Terminal Dual Stance of RH and LH.

Please note that the Terminal Dual Stance for a hind paw is the Initial Dual Stance for the successive step cycle of the contralateral paw.

**Body Speed**

The Body Speed of a step cycle of a specific paw is calculated by dividing the distance that the animal's body traveled from one initial contact of that paw to the next by the time to travel that distance.

**Body Speed Variation**

Body Speed Variation (%) is calculated by dividing the absolute difference between the Body speed and the Average Speed of a run by the Average Speed.

**STEP SEQUENCE**

The Step Sequence sheet contains information on the order in which the four paws are placed. The following parameters are displayed in the Step Sequence sheet.

In the Step Sequence sheet, when you click a Code:

- The Print View highlights the corresponding print.
- The Timing View shows the corresponding Stand of that paw.
- The Sub Print View shows the individual prints of the Stand.

**Step sequence**

The Step Sequence lists the order in which the paws were placed on the glass plate. This order determines the footfall pattern that can assigned.
**Codes**

Each Code corresponds to a particular paw: 1 = left hind, 2 = left front, 3 = right hind, 4 = right front. The Codes are used to create the Footfall Patterns graph.

**Patterns**

Here the abbreviations of the assigned footfall patterns are shown (see also Figure 6.7). The colors of the cells correspond to the colors of dots in the Footfall Patterns graph.

**Normal Patterns**

This is the number of Step Cycles that fall within one of the footfall pattern categories (see Figure 6.7 on page 104).

**Accounted Steps**

This is the number of steps that was taken into account to determine the footfall patterns.

**Regularity Index (%)**

The Regularity Index expresses the number of normal step sequence patterns relative to the total number of paw placements.

The formula of Regularity Index is:

\[
\text{Regularity Index} = \frac{\text{NSSP} \times 4}{\text{PP}} \times 100\%
\]

where NSSP represents the number of normal step sequence patterns and PP the total number of paw placements (see Figure 6.8).

If the footfall pattern changes during a run (for example, from Aa to Ab), the Regularity Index is not affected.

![Figure 6.8 A Footfall Patterns diagram. In this example, the animal had 4 normal footfall patterns. All footfall patterns start with the right front paw (RF: blue dot). The first paw print and the last three are not taken into account. The number of accounted steps is '46'. Regularity Index is '100%'](image-url)
The Regularity Index (RI) is used in research on spinal cord injury. It is a fractional measure of inter-paw coordination. In healthy, fully coordinated animals its value is 100%. For example, one week after rats were subjected to a transection injury, the RI decreased from 100% (pre-treatment) to approximately 80%. The RI again increased to 90% after 4 weeks (Hamers et al., 2001. See also Figure 6.8).

**BASE OF SUPPORT**

Base of Support (BOS) is the average width between either the front paws or the hind paws. In the Base of Support sheet the following variables/parameters are displayed:

- **Paw** – The label of the paw.
- **N** – The number of times each paw was placed.
- **Mean ([Distance Unit])** – The mean value for the Y position for each paw.
- **SEM (%)** – This is the Standard Error of the Mean, expressed as a percentage of the mean Base of Support.

At the bottom:

- **Paws** – Front paws or Hind paws.
- **N** – The total number of times the paw combinations were placed.
- **Base of Support ([Distance Unit])** – The mean distance between either front paws or hind paws. The Base of Support is calculated as follows:

  \[
  \text{Base of Support Front paws} = (\bar{Y}_{RF} - \bar{Y}_{LF}) \quad \text{Base of Support Hind paws} = (\bar{Y}_{RH} - \bar{Y}_{LH})
  \]

The Base of Support parameter is used in research on spinal cord injury. It has been found that as a result of trauma to the spinal cord (either as a result of a transection or contusion), the Base of Support (BOS) of the front paws is unaffected but the BOS of the hind paws showed an increase (Hamers et al., 2001).
Appendix C - Summary of evidence for CORM

Adapted from Motterlini et al.: Emerging concepts on the antiinflammatory actions of carbon monoxide-releasing molecules (CORMs). Medical Gas Research 2012 2:28

<table>
<thead>
<tr>
<th>Type of CO-RM</th>
<th>Inflammatory disease models in vitro and in vivo</th>
<th>Effect on inflammatory markers/Overall outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CORM-3</td>
<td>Vascular thrombosis in rats</td>
<td>↓ fibrinogen and fibrin ↑ pro-thrombin</td>
<td>[18]</td>
</tr>
<tr>
<td>CORM-3</td>
<td>Hemorrhagic stroke induced by collagene in rats</td>
<td>↓ TNF-α production ↓ brain injury</td>
<td>[19]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Polymicrobial sepsis induced by cecal ligation and perforation (CLP) in mice</td>
<td>↑ protein C system ↓ plasma thrombomodulin ↓ number of thrombi in liver, kidney and lung</td>
<td>[20]</td>
</tr>
<tr>
<td>CORM-3</td>
<td>Postmenopausal rheumatoid arthritis osteoporosis in mice</td>
<td>↓ cellular infiltration and cartilage degradation ↓ TNF-α production ↓ Serum levels of IL-6, alkaline phosphatase and MMP-3</td>
<td>[21]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Coagulation and fibrinolytic markers in human umbilical vein endothelial cells (HUVEC)</td>
<td>↓ tissue factor ↓ plasminogen activator inhibitor type 1 (PAI-1)</td>
<td>[22]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Neuropathic pain and microglia activation in mice induced by nerve injury</td>
<td>↓ mechanical allodynia ↓ thermal hyperalgesia ↓ nNOS and iNOS expression</td>
<td>[23]</td>
</tr>
<tr>
<td>CORM-3</td>
<td>Vascular inflammation in human umbilical vein endothelial cells (HUVEC)</td>
<td>↓ VCAM-1 and ECAM expression ↓ NF-κB and p38-MAPK expression ↓ mitochondrial respiration ↓ NF-κB and iNOS expression</td>
<td>[24-26]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Ischemia-reperfusion injury after kidney transplantation in rats</td>
<td>↑ survival rate</td>
<td>[27]</td>
</tr>
<tr>
<td>CORM-3</td>
<td>Colitis induced by dextran sodium sulfate in mice</td>
<td>↓ activity index ↓ myeloperoxidase (MPO) activity ↓ TNF-α production</td>
<td>[28]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Endoplasmic reticulum (ER) stress induced by thapsigargin in human hepatocytes and mice</td>
<td>↑ C-reactive protein (CRP) ↓ serum amyloid P component (SAP)</td>
<td>[29]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Cutaneous wound healing in rats</td>
<td>↑ cell proliferation and wound contraction ↑ collagen synthesis ↓ TNF-α production and ICAM-1 expression ↑ IL-10 production</td>
<td>[30]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Acute hepatic ischemia-reperfusion injury in rats</td>
<td>↑ anti-apoptotic protein Bcl2 ↓ markers of hepatic damage (AST/ALT) ↓ serum levels of TNF-α and IL-6</td>
<td>[31]</td>
</tr>
<tr>
<td>CORM-2</td>
<td>Acute pancreatitis in rats</td>
<td>↓ serum levels of TNF-α and IL-1β ↑ NF-κB expression and MPO activity ↑ IL-10 production</td>
<td>[32]</td>
</tr>
<tr>
<td>Table 1 Effect of CO-RMs on <em>in vitro</em> and <em>in vivo</em> inflammatory disease models (Continued)</td>
<td></td>
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<tr>
<td><strong>CORM-3</strong> Rheumatoid arthritis induced by K/BxN serum transfer in mice</td>
<td>↑ serum osteocalcin and HO-1 expression</td>
<td></td>
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<tr>
<td></td>
<td>↓ MMP-9, MMP-13 and IL-1β expression</td>
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<td></td>
<td>↓ high mobility group box 1 (HMGB1)</td>
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<td></td>
<td>↓ Receptor activator of nuclear factor κB ligand (RANKL)</td>
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<tr>
<td><strong>CORM-2</strong> Ischemia-reperfusion induced inflammation of small intestine in mice</td>
<td>↓ TNF-α and ICAM expression</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>↓ leukocytes rolling and adhesion</td>
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<tr>
<td></td>
<td>↓ NF-κB expression and MPO activity</td>
<td></td>
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<tr>
<td><strong>CORM-2</strong> Inflammatory response induced by lipopolysaccharide in RAW 264.7 macrophages and BV-2 microglia</td>
<td>↓ INOS mRNA and protein expression</td>
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<tr>
<td></td>
<td>↑ COX-2 expression and NF-κB expression</td>
<td></td>
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<tr>
<td></td>
<td>↑ PGE2 levels</td>
<td></td>
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<tr>
<td></td>
<td>↑ phosphorylation of Akt and MAPKs</td>
<td></td>
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<tr>
<td><strong>CORM-2</strong> Sepsis induced by cecal ligation and puncture (CLP) in mice</td>
<td>↓ neutrophil infiltration in bronchoalveolar lavage and liver</td>
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<tr>
<td></td>
<td>↓ MPO activity in lung and NF-κB in liver</td>
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<tr>
<td></td>
<td>↓ serum TNF-α and high mobility group box 1 (HMGB1)</td>
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<tr>
<td></td>
<td>↓ nitrite/nitrate plasma levels</td>
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<tr>
<td></td>
<td>↑ cardiac PGC-1α and mitochondrial biogenesis</td>
<td></td>
<td></td>
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<tr>
<td><strong>CORM-A1</strong> Inflammatory response induced by TNF-α in brain vascular endothelial cells</td>
<td>↓ NADPH oxidase activity</td>
<td></td>
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To: Al Walid Hamam <

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Pauline Spiess, MSc.
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From: Al Walid Hamam
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<td>Functional Assessment in a Rodent Model of Compartment Syndrome and Potential Therapeutic Role of Carbon Monoxide Releasing Agent 3</td>
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<tr>
<td><strong>Expected completion date</strong></td>
<td>Aug 2014</td>
</tr>
<tr>
<td><strong>Estimated size(pages)</strong></td>
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<td><strong>Billing Type</strong></td>
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**Total** 0.00 USD
You may publish figures – not to be resold or republished other than for your thesis.

Sincerely,

Program Manager Journals
CMA Publications
Canadian Medical Association

From: Al Walid Hamam
Sent: July-23-14 5:19 PM
To: Permissions
Subject: Compartment syndrome–induced microvascular dysfunction: an experimental rodent model

Hello,


I am writing my master's of surgery thesis titled "Functional Assessment in a Rodent Model of Compartment Syndrome and Potential Therapeutic Role of Carbon Monoxide Releasing Agent 3" and would like to use Figure 4.
Appendix E – Animal Protocol Approval

*This is the original approval for this protocol*
*A full protocol submission will be required in 2017*

Dear Dr. Lawendy:

Your animal use protocol form entitled:

Direct and Remote Organ Injury Following Hind Limb Compartment Syndrome

Funding agency Orthopaedic Trauma Association – Direct and Remote Organ Injury Following Hind Limb
Compartment Syndrome – Grant #R4889A04 has been approved by the University Council on Animal Care.

This approval is valid from 11.01.13 to 11.30.17 with yearly renewal required.

The protocol number for this project is 2009-083.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

**ANIMALS APPROVED FOR 4 YEARS**

<table>
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<tr>
<th>Species</th>
<th>Strain</th>
<th>Other Detail</th>
<th>Pain Level</th>
<th>Animal # Total for 4 years</th>
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<tbody>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>150-350 g</td>
<td>C</td>
<td>680</td>
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<tr>
<td>Pig</td>
<td>Yorkshire-Landrace</td>
<td>50-60 kg</td>
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**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. R Bih
## Curriculum Vitae

<table>
<thead>
<tr>
<th>Name:</th>
<th>Al Walid Hamam</th>
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<tbody>
<tr>
<td><strong>Post-secondary Education and Degrees:</strong></td>
<td></td>
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<tr>
<td>University of Ottawa</td>
<td></td>
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<tr>
<td>Ottawa, Ontario, Canada</td>
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<tr>
<td>1999 – 2003, BSc</td>
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<td>University of Ottawa</td>
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<tr>
<td>Ottawa, Ontario, Canada</td>
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<tr>
<td>2003 – 2007, MD</td>
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<tr>
<td>University of Manitoba</td>
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<tr>
<td>Winnipeg, Ontario, Canada</td>
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<td>2007 – 2012, FRCSC</td>
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<tr>
<td>University of Western Ontario</td>
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<tr>
<td>London, Ontario, Canada</td>
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<tr>
<td>2013 – 2014, MSc</td>
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<tr>
<td><strong>Honours and Awards:</strong></td>
<td>NSERC Summer Studentship 2003</td>
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<td>Ivan Smith Studentship (Oncology) 2005</td>
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<tr>
<td><strong>Related Work Experience:</strong></td>
<td>Student R&amp;D Scientist</td>
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<td></td>
<td>iSTAT Canada Ltd, Kanata, ON 2001</td>
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<td></td>
<td>Student Researcher</td>
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<td></td>
<td>OHRI, Ottawa, ON 2003</td>
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