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Gait Analysis and Therapeutic Application of Carbon Monoxide in a Rodent Model of Complex Regional Pain Syndrome Type-1

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Medical Biophysics

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**GAIT ANALYSIS AND THERAPEUTIC APPLICATION OF
CARBON MONOXIDE IN A RODENT MODEL OF COMPLEX
REGIONAL PAIN SYNDROME TYPE-1**

(Thesis Format: Integrated Article)

by

Hussein Abdo

Graduate Program in Medical Biophysics

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

The School of Graduate and Postdoctoral Studies
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ABSTRACT

Complex regional pain syndrome type-I (CRPS-I) is a debilitating pain disorder often occurring secondary to distal extremity trauma. Its pathophysiology is not well understood; however, microvascular dysfunction is proposed as an important factor in its development and maintenance. Using a rodent model, we tested an automated gait analysis system (CatWalk™) to examine functional changes. In addition, the use of carbon monoxide releasing molecule-3 (CORM-3), a compound known to be a potent vasodilator and anti-inflammatory agent, was also tested as a treatment of CRPS-I-like symptoms. Using the CatWalk™ system, we observed significant changes in gait parameters post-injury, several of which persisted throughout the 14-day experiment. CORM-3 administration significantly reduced mechanical allodynia symptoms, as demonstrated through the restoration of withdrawal thresholds during mechanical stimulation testing. Functional deficits were not restored after CORM-3 application; however, trends for improvement were observed. CORM-3 has relevance as a potential therapy to alleviate symptoms associated with CRPS-I.

Keywords: complex regional pain syndrome type-I, CORM-3, microcirculation, gait analysis, CatWalk™, carbon monoxide, chronic pain

CO-AUTHORSHIP

Although each of the co-authors listed below made significant contributions to this work, I was the primary author and performed the vast majority of the experimental data collection and statistical analysis. This manuscript was entirely written and prepared by me, with consultation from co-authors.

Dr. David W. Sanders, MD, FRCSC, provided much leadership over the course of my project and his ideas helped to direct the project towards the end product.

Dr. Gediminas Cepinskas, DVM, PhD, provided leadership over the course of my studies and insight into CORM-3 mechanisms and uses. He also helped to critically review this work.

Dr. Abdel-Rahman Lawendy, MD, PhD, FRCSC, also provided invaluable leadership and offered guidance and support on data interpretation. His work with ischemia-reperfusion and his clinical background offered significant help in this work.

Aurelia Bihari, MSc, taught me all of the experimental techniques used in this project and helped in the collection and analysis of experimental data. She provided technical support and guidance in all aspects of my laboratory work, including writing.

Dr. Geoff A. Bellingham, MD, FRCPC, offered knowledge into the current understanding and treatments of complex regional pain syndrome. He also provided insight during data analysis.

DEDICATION

I would like to dedicate this thesis:

To my mother, words cannot justify the support she has given me. As both a child and a young man, her love and unyielding motivation have provided me with inspiration through the most difficult of times. Heaven is truly at the feet of our mothers.

To my father, whose kindness, love and sense of principle have made me the man I am today. A role model, his character is one of modesty, intellect and humour. His friendship is one to be cherished. I hope I have made him proud of his title as “Abu Hussein.”

ACKNOWLEDGEMENTS

First, I'd like to acknowledge the ONE (may He be Glorified and Exalted), for granting me the health and knowledge to complete this work.

I acknowledge my supervisors, Drs. Sanders and Cepinskas, for their mentorship encouragement over the course of my studies and beyond.

I acknowledge Dr. Lawendy for his mentorship and patience, and for the use of his laboratory space and equipment for my project.

I also acknowledge Dr. Bellingham for his advice and knowledge as a member my advisory committee.

Mrs. Aurelia Bihari taught me the techniques for animal preparation, anaesthesia, experimental techniques and computer software analysis necessary for the completion of this project. I acknowledge her for this and for being a patient tutor through this project.

I would also like to acknowledge the other members of the research team (Dr. Jennifer Urquhart, Nathan Bedard, Calvin Poon, Dr. Erin Donohue, Dr. Moustafa Haddara and Dr. Al Walid Hamam) for making my time at the lab an enjoyable one.

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LIST OF ABBREVIATIONS

- ANOVA, Analysis Of Variance
- BB, Bisbenzimidazole
- cGMP, Cyclic Guanosine Monophosphate
- CGRP, Calcitonin Gene-Related Peptide
- CPIP, Chronic Post-Ischemia Pain
- CO, Carbon Monoxide
- CO-RM, Carbon Monoxide Releasing Molecule
- iCORM, Inactivated Carbon Monoxide Releasing Molecule
- CRPS, Complex Regional Pain Syndrome
- DMSO, Dimethyl Sulfoxide
- EB, Ethidium Bromide
- EC, Endothelial Cell
- EDL, Extensor Digitorum Longus
- HbCO, Carboxyhemoglobin
- HBO, Hyperbaric Oxygen
- HLA, Human Leukocyte Antigen
- HO, Heme Oxygenase
- iCORM-3, inactive Carbon Monoxide Releasing Molecule 3
- IASP, International Association for the Study of Pain
- IL, Interleukin
- IP, Intra-peritoneal
- IR, Ischemia-Reperfusion

ITDD, Intrathecal Drug Delivery
IVRA, Intravenous Regional Anesthesia
IVVM, Intravital Video Microscopy
MAPK, Mitogen Activated Protein Kinase
NF κ B, Nuclear Factor κ B
NK-1, Neurokinin-1
NMDA, N-Methyl-D-Aspartate
NO, Nitric Oxide
PDN, Painful Diabetic Neuropathy
PHN, Post-Herpetic Neuralgia
PNS, Peripheral Nerve Stimulation
PWT, Paw Withdrawal Threshold
RCT, Randomized Controlled Trial
RSD, Reflex Sympathetic Dystrophy
SC, Spinal Cord
SCS, Spinal Cord Stimulation
sGC, Soluble Guanylate Cyclase
SIP, Sympathetically Independent Pain
SMP, Sympathetically Maintained Pain
SNS, Sympathetic Nervous System
TCA, Tricyclic Antidepressant
TNF- α , Tumor Necrosis Factor- α

CHAPTER 1
INTRODUCTION AND HISTORICAL REVIEW

CHAPTER 1: INTRODUCTION AND HISTORICAL REVIEW

1.1 COMPLEX REGIONAL PAIN SYNDROME

In the clinic, persistent and chronic pain is the most common reason for patient presentation (Von Korff et al., 1988; Von Korff et al., 2008). Our current understanding of pain sensation and pain mechanisms in both normal and pathological conditions is continuously expanding and helping physicians to better cope with the increasing load of chronic pain patients. Headway has been made to recognize pain as a disease in its own right, as several studies show that over 1 in 5 Canadians are affected by chronic pain. According to the Chronic Pain Association of Canada, “The cost of chronic pain for Canadians is well over 40 billion dollars in direct and indirect costs. This is more than cancer, HIV, and heart disease combined.” (Lynch, 2011) One such chronic pain condition, Complex Regional Pain Syndrome, has both significant direct and indirect costs on our society, and its peoples.

Complex Regional Pain Syndrome (CRPS) describes a range of painful conditions and is characterized by a continuing regional pain – either spontaneous or induced – that is disproportionate to the inciting event or the usual course of a known trauma, in both time and degree. It typically occurs secondary to an extremity trauma and has a distal presence of abnormal sudomotor, vasomotor, sensory, motor and/or trophic signs (Bean et al., 2014; Bruehl et al., 2002; Harden, Bruehl, Perez, Birklein, Marinus, Maihofner, Lubenow, Buvanendran, Mackey, Graciosa, Mogilevski, Ramsden, Schlereth, et al., 2010). Typical symptoms include a constant burning pain, mechanical and cold allodynia, swelling, temperature changes and

limited active range of motion of the limb; exacerbation of symptoms is often observed upon exertion and exercise as well (M. Stanton-Hicks et al., 1998). Other considerable symptoms include severe motor dysfunction (dystonia, tremors, weakness, incoordination), dystrophy, psychiatric co-morbidities, sweating, bone changes, and changes in hair and nails. Anxiety and depression are common to this disorder, as patients often do not respond to any available treatment. Current therapeutic options include pharmacological, surgical, psychological and physical therapy (Harden, 2000; M. D. Stanton-Hicks et al., 2002). As a result of often failing treatment attempts, there is a push towards translational research in the study of CRPS. Although the triggers of the disorder are known (soft tissue injuries, fractures, sprains, crush injuries, surgery, spinal cord disorders and infections), CRPS sometimes occurs spontaneously, making it extremely difficult to diagnose (Wasner et al., 2001; Wilson et al., 2005). Diagnostic criteria are constantly evolving, as the consequences of a misdiagnosis can have multiple implications in patient quality-of-life, employment status, and healthcare costs. As suggested by Harold Merskey's quote below, pain is devastating to a person's livelihood:

“If I have matters right, the consequences of pain will include direct physical distress, unemployment, financial difficulties, marital disharmony, and difficulties in concentration and attention...” (Merskey, 2000).

In order to help better understand the complex mechanisms underlying the pathophysiology of CRPS, and to develop accurate assessments for patients, both basic science and clinical research modalities are jointly needed.

1.2 HISTORY OF CRPS

The history of CRPS traces back to 16th century European battles, where severe pain symptomology from trauma, induced by lance (and later, bullet) injuries, was reported (Bonica, 1953). Potts, a famous British surgeon, reported in the 18th century that trauma of the extremities can result in pain and atrophy (Hooshmand, 1993). One of the first amputations was actually performed by a surgeon named Denmark on sailors whose musket bullet injuries resulted in severe burning pain with inflammatory symptoms; this condition was termed “*tic douloureux*” at the time (Ley, 1835). Throughout the next several hundred years, the nomenclature and definition of the disorder has seen many changes; however, no concrete description of the pathophysiology has been accepted.

In the 19th century and the American Civil War, a physician named Wier Mitchell is accredited with describing the burning pain soldiers suffered from gunshot wounds and attributing it to a specific condition. He called the condition ‘causalgia’, a name originating from the greek word *kausos* for fever (from *kaiein* for “to burn”) (Mitchell, 1867). Together, Mitchell and William Keen (another doctor serving the Turner Lane Hospital in the Civil War) studied several nerve injuries, and eventually published “Gunshot Wounds and Other Injuries of the Nerves and Reflex Paralysis”, which described causalgia and secondary paralysis (Mitchell, 1864). In their publications, Mitchell, Keen and others described the severe burning pain, abnormal skin colour and temperature, sweating, muscle weakness, osteoporosis and involuntary movements that many soldiers experienced; effectively, well describing the disease (Mitchell, 1864, 1867). It wasn’t until 1916 that a proposal for the

mechanisms of causalgia was suggested by Leriche, who proposed that the sympathetic nervous system had an important role (Leriche, 1916).

Suggesting a much different view on the source of pain, Paul Sudeck, a German physician, theorized that the bone atrophy and other symptoms observed were part of an inflammatory reaction after trauma. The syndrome was then referred to as Sudeck's atrophy (Sudeck, 1902; van der Laan et al., 1998; Veldman et al., 1993).

William Livingston, an American surgeon during the Second World War, described a "vicious cycle" used to explain CRPS symptoms. The cycle explains how afferent input and reflex vasoconstriction could trigger pain, limb disuse and atrophy with even the most minor of nerve injuries (Livingston, 1948). From his hypothesis, Livingston performed many sympathectomies on suffering soldiers, some of which experiencing transient relief of pain symptoms. The findings resulted in Livingston suggesting an important role of the sympathetic nervous system in causalgia (Livingston, 1948).

Around the same time Livingston was performing sympathectomies on injured soldiers, a surgeon by the name of Philip Foisie was hypothesizing the roles of arterial vasospasm and ischemia, from arterial and soft tissue injury, in causalgia (Foisie, 1947). Until then, vasospasms and ischemia had been largely ignored as potential factors contributing to CRPS.

Formally called minor causalgia, the term "Reflex Sympathetic Dystrophy" (RSD) was introduced by John Evans in 1946, and referred to as causalgia without a visible major nerve injury (Evans, 1946, 1947). In 1986, the International Association

for the Study of Pain (IASP) defined RSD as “continuous pain in a portion of an extremity after trauma, which may include fracture but does not involve a major nerve, associated with sympathetic hyperactivity.” An additional definition, proposed by an Ad Hoc committee of the American Association of Hand Surgery, called RSD “a pain syndrome in which the pain is accompanied by loss of function and evidence of autonomic dysfunction.” However, in order to avoid any mechanistic term in its appellation, RSD had been renamed, as many cases did not seem to have sympathetically maintained pain, nor did result in dystrophy. Together, causalgia and RSD have had many different names describing them (Table 1.1).

A discussion at the IASP Task Force on Taxonomy in 1994 resulted in a new umbrella term: Complex Regional Pain Syndrome (CRPS), which is now used instead, as there is “neither clinical nor pathological evidence to suggest that the mechanisms are any different in these two syndromes, and the responses (or lack thereof) to treatments are quite similar” (Wilson et al., 2005). Two types of CRPS are defined: CRPS-I, which encompasses what was formally RSD, and CRPS-II, which is what was formally known as causalgia. Type-I differs from type-II solely on the absence of a distinct nerve injury (Harden et al., 2007).

Table 1.1. Synonyms of Complex Regional Pain Syndrome

Synonyms of Complex Regional Pain Syndrome
Reflex sympathetic dystrophy (RSD)
Causalgia
Sudeck's atrophy
Post-traumatic dystrophy
Shoulder-hand syndrome
Algodystrophy
Algoneurodystrophy
Reflex neurovascular disease
Pourfour du Petit syndrome
Postinfractional sclerodactylia
Fracture disease

1.3 DIAGNOSIS OF CRPS

Sensitivity and accuracy of CRPS diagnosis is imperative to our health care system and patient quality of life. Being diagnosed with CRPS has serious implications to a patient's ability to work and to maintain the livelihood of their families. A study by Kemler and Furnee (2002) concluded that the impact of chronic pain on both patients and their families could be unbearable physically and financially (Kemler & Furnee, 2002). They found that CRPS had a profound effect on employment status, time allocation, additional domestic help and out-of-pocket expenses (Kemler & Furnee, 2002). Therefore, early and accurate diagnosis and prompt management of symptoms are essential in reducing or preventing CRPS from worsening, as well as improving quality of life.

In Budapest, IASP consensus updated the diagnostic criteria for CRPS. Harden et al (2007) summarized the group's revisions to the former diagnostic criteria, which aimed to reduce both over- and under-diagnosis of CRPS while maintaining diagnostic sensitivity (Harden et al., 2007). To make a CRPS diagnosis, the new criteria states that a patient must have:

1. Presence of an initiating noxious event, or a cause of immobilization;
2. Continuous pain, allodynia and/or hyperalgesia in which the pain is disproportionate to the inciting event;
3. Evidence at some time of edema, changes in skin blood flow or abnormal sudomotor activity in the region of pain;
4. This diagnosis is excluded by the existence of other conditions that would otherwise account for the degree of pain and dysfunction.

The diagnosis is further clarified if the patient is seen with or without major nerve damage. Nerve damage would result in a diagnosis of CRPS-II, whereas no nerve damage would garner a diagnosis of CRPS-I. The presence of an initiating noxious event may be unrequired as 5-10% of CRPS patients develop the syndrome spontaneously (Harden et al., 2007).

Considering that CRPS symptoms often vary slightly between patients and over time, probably as a result of a different contribution to symptoms from different pathophysiologic mechanisms, a clinical diagnosis has to meet several criteria. Clinicians examine four categories of symptoms: sensory (hyperesthesia and allodynia), vasomotor (temperature and skin colour changes), sudomotor/edema (edema and sweating changes and asymmetry), and motor/trophic (decreased range of motion; motor dysfunction like weakness, tremor and dystonia; trophic changes like hair, nail, and skin) (Harden et al., 2007). To make a clinical diagnosis of CRPS, the following criteria must be met:

1. Continuing pain disproportionate to the inciting event;
2. Patient reporting at least one symptom in at least three of the four categories listed above (sensory, vasomotor, sudomotor/edema and motor/trophic);
3. Patient must display at least one sign in two or more of the above categories at the time of evaluation;
4. No other diagnosis better explains the signs and symptoms.

Clinically, the above criteria were agreed upon because, compared to the previous IASP diagnostic criteria, they limit false positives. However, depending on the

purpose for which the criteria are intended, the sensitivity and specificity of criteria might need adjusting. For example, in the research context, identifying stringent research samples by minimizing false positives is valuable, compared to clinically identifying as many CRPS cases as possible by minimizing false negatives (Harden et al., 2007). For this reason, diagnostic criteria in research are adjusted. In research, two of four sign categories and four of four symptoms categories must be positive for a CRPS diagnosis (Harden et al., 2007). These result in the greatest probability of CRPS vs non-CRPS according statistical analyses performed by Harden et al (2007).

1.4 THERAPEUTIC APPROACHES AND MANAGEMENT

Over the last few decades, treatment plans for CRPS have adapted to new knowledge; however, the lack of consensus in the pathophysiology of the disorder have proved to make developing appropriate therapies very difficult, to say the least. Early recognition and treatment of CRPS-I are important because CRPS-I patients are at high risk of developing severe disability in the affected limb, potentially compromising future employment and livelihood (Poplawski et al., 1983).

Norman Harden presented a very good summary of why treatment of CRPS is inherently so difficult. It stated:

“The syndrome is inherently complex biomedically, involving both peripheral and central pathophysiology, but it also often has psychosocial features that are critical diagnostic elements (and treatment targets). Successful treatment is further complicated by the diversity of patient presentation and by antecedent pathology. In addition to these clinical challenges, the epidemiology and natural history of CRPS are poorly understood. Even

research data are challenging to interpret, and evidence has been slow to accumulate as to how best to treat CRPS, due in large part to the vagaries of diagnosis (see Chapter 4). How is a specialist to begin treating such a multifaceted condition?" (Wilson et al., 2005)

Indeed, how is a specialist to begin treating such a multifaceted condition? This question was addressed by Harden and others in *CRPS: Current Diagnosis and Therapy* in 2005, after an IASP conference in Budapest. The authors also presented a general treatment algorithm, suggesting an interdisciplinary method for handling CRPS, with a focus on functional restoration. This algorithm recommends the use of conservative care, via physical/occupational therapy and oral medications, when a patient continues to have persistent pain or dysfunction. With regular consultation, if a patient continues to experience CRPS symptoms with conservative care, intermittent regional nerve blocks are then sometimes prescribed. If a failure to progress from nerve blocks were observed, a move to infusion techniques (i.e. epidural infusions) would be recommended. If CRPS symptoms persist, the patient may be a candidate for spinal cord stimulator implantation or intrathecal drug delivery. These options are more invasive and should only be considered if other therapies fail; additionally, the authors suggest intrathecal drug delivery should only be considered if spinal cord stimulation fails to relieve symptoms, or if a patient exhibits a plateau of response (Wilson et al., 2005). Such treatments will be discussed further in the following sections. Of course, CRPS is a very complex condition and treatments cannot be expected to be identical between patients. The basic principle of the functional restoration guidelines is to identify progress through the steps of care, and to intervene with additional treatments if patients do not

progress (Wilson et al., 2005). Treatment guidelines emphasize using a multi-disciplinary approach, to compassionately and methodically help patients regain their quality of life.

The development of additional treatments, targeting potential mechanisms explaining the initiation and maintenance of CRPS, are needed. Patients are often frustrated by the lack of relief pain management regimens provide; the development of adjunct therapies may help both clinic and patient relieve pain and frustration.

1.4.1 Rehabilitation Therapy

In keeping with the focus on functional restoration, rehabilitation therapy is an important part in CRPS treatment. Until recently, rehabilitation has been stated as vital, or at least a supportive complementary approach to CRPS; however, its techniques, frequency and intensity are rarely described in the literature. Instead, writings tend to focus on explanatory hypotheses, medical interventions and adjustments to diagnostic criteria.

Rehabilitation is the culmination of both physical and occupational therapy, with the aim of focusing treatments on the clinical manifestations of CRPS, including edema, restricted range of motion, temperature sensitivity, intolerance of physical activity, reduced muscle strength and, of course, pain. Although physical and occupational therapists differ in their scope of practice, much of their work overlaps in the treatment of a chronic pain syndrome (Wilson et al., 2005).

In the treatment of CRPS, the first steps are often to manage edema and pain. The next goal is the initiation of gentle, active movements, to restore range of

motion. Finally, improvement of muscle strength and function of the extremity are targeted to fulfill the end goal of improving whole body function, to allow patients to be participating members of society (Bengtson, 1997). In a prospective randomized control trial that followed patients over the course of 1 year, physical and occupational therapy modalities led to recovery from CRPS of the upper extremity (Oerlemans et al., 1999).

Reducing edema is often achieved through lymph draining and by active exercises; however, a study by Uher et al (2000) observed no difference between active exercises and active exercises plus lymph draining. This would suggest that physical therapy may be more effective in reducing edema (Uher et al., 2000).

Providing a balanced and varied approach to managing symptoms, physical and occupational therapy as part of the rehabilitation method is currently the pivotal intervention for CRPS, as determined by several large consensus meetings (Harden, Bruehl, Perez, Birklein, Marinus, Maihofner, Lubenow, Buvanendran, Mackey, Graciosa, Mogilevski, Ramsden, Chont, et al., 2010; Harden et al., 2007). As confirmed in rat models, normalization of function may serve to reverse changes observed in CRPS patients (Guo et al., 2004).

1.4.2 Medications and Pharmacotherapy

As a result of a lack of efficacy of rehabilitation therapy for CRPS patients, pharmacotherapy often becomes the major treatment option for those patients whose symptoms do not improve. Of course, used in conjunction with other therapies as directed by pain specialists, medications are prescribed for both early and chronic CRPS, as well as chronic CRPS symptoms other than pain.

Since CRPS is often initiated by trauma or injury, most studies examining treatments and therapeutic approaches have been performed by surgeons, orthopaedists or rehabilitation specialists. As stated previously, acute CRPS is characterized by pain and inflammation; however, it's believed that the final effects on the central nervous system may not have occurred yet, leaving an important window for treatments to potentially affect the disease course (Wilson et al., 2005). The mechanisms and targets of treatment are probably different in acute and chronic CRPS, and there are very few studies investigating the effectiveness of early CRPS treatments on chronic CRPS patient symptoms.

Some evidence-base studies have found that treatments prescribed in early CRPS include: corticosteroids, calcium-regulating drugs, alpha-adrenergic antagonists, and anti-oxidants. A study by Christensen et al (1982) found that systemic corticosteroid (prednisone) treatment reduced entire clinical status by more than 75%; however, corticosteroids are typically not administered in chronic CPRS patients (>6 months) as it has little efficacy (Christensen et al., 1982). Prednisone and methylprednisolone are agents with anti-inflammatory properties, probably affecting neurogenic inflammation and subsequently pain symptoms.

Calcium-regulating drugs like clodronate and alendronate, as well as the hormone calcitonin, have been found to improve swelling, pain, and range of motion in several randomised clinical trials (RCTs) (Braga, 1994; Manicourt et al., 2004; Perez et al., 2001; Varenna et al., 2000; Zyluk, 1998). The mode of action of these treatments is not clear.

Considering the hypothesis that CRPS is caused by oxygen-derived free radical damage that potentiates inflammation and microvascular dysfunction, several studies have investigated antioxidants and free-radical scavengers as possible therapeutics. One such study found that topical dimethylsulfoxide (DMSO) provided alleviating effects (Zuurmond & Perez, 2006); however, a strong garlic odour in exhaled breath was observed as an adverse effect.

Alpha-adrenergic antagonists and vasodilators have also been examined in CRPS patients; although two studies show potential benefits of the alpha-adrenergic antagonist phenoxybenzamine in both early and chronic CRPS (Ghostine et al., 1984; Muizelaar et al., 1997), adverse effects of hypotension from vasodilatory action are very serious.

In Table 1.2 below, the major classes of medications, with documented efficacy, used in the treatment of chronic pain are summarised. Bearing in mind the wide range in CRPS symptom presentation, no single medication is better or worse than others, so clinicians must consider patient symptoms, age, circumstances and personal history when prescribing medications.

Table 1.2. Major Classes of Medications Used in Treating Chronic Neuropathic Pain

Treatment	Medication Name
Topical local anesthetics	<ul style="list-style-type: none"> • Lidocaine 5% patch • Local anesthetic cream, gel, ointment
Tricyclics	<ul style="list-style-type: none"> • Nortriptyline • Desipramine
Antiepileptics	<ul style="list-style-type: none"> • Gabapentin • Carbamazepine (extended release)
Opioids	<ul style="list-style-type: none"> • Tramadol • Oxycodone (extended release) • Morphine (extended release) • Methadone

Adapted from Wilson et al 2005 (Wilson et al., 2005)

With minimal complications to its use, local anesthetics have active ingredients that act locally at the site of application, typically the affected extremity (Wilson et al., 2005). These agents differ from other transdermal medications like fentanyl, which have a systemic distribution once absorbed across the skin. Gels, creams, sprays and patches with active anesthetic ingredients (like lidocaine) are supported by level 2 evidence for relief of allodynia symptoms in CRPS. The 5% lidocaine patch is especially popular since it both covers the skin from potential contact and has active anesthetic to relieve allodynia symptoms (Wilson et al., 2005).

Tricyclics, or tricyclic antidepressants (TCAs), are perhaps the most beneficial pharmacological CRPS therapy in use today (Jensen, 2002). Several trials have found TCAs to be effective in reducing neuropathic pain (Jensen, 2002; Max et al., 1987; Max et al., 1992; Raja et al., 1992; C. P. Watson et al., 1992). They also happen to be one of the most inexpensive therapeutic options, as generic forms of the agent are available. To decrease CRPS symptoms, TCA acts on several mechanisms to alter noradrenergic inhibitory pathways for decreased dorsal horn hyperactivity as well as the depression and psychological changes that accompany the disorder. Use in the elderly is discouraged, however, since some TCAs (such as amitriptyline) may contribute to cardiac arrhythmias.

Currently FDA-approved for use in epilepsy and post-herpetic neuralgia (PHN), some antiepileptic drugs also exhibit antihyperalgesic properties, by decreasing central neuronal hyperexcitability (Wilson et al., 2005). Gabapentin, the best-studied antiepileptic, has been deemed efficacious and safe for CRPS patients

after a study giving patients 600 mg/day (Mellick & Mellick, 1997). Its action is thought to be the binding of alpha-2-delta subunit of voltage-gated calcium channels for the decrease of synthesis and release of excitatory neurotransmitters (Gee et al., 1996). Compared with TCAs, gabapentin has no drug-drug interactions and no serious adverse effects; however, other anticonvulsants and antiepileptics have inconclusive evidence as to their efficacy in pain disorders like CRPS.

The last major class of medication used in the treatment of CRPS patients is the opioid medications. Reluctance to prescribe opioids is common amongst practitioners for several reasons, including a potential for severe substance abuse and addiction, evidence for the cause of diffuse pathological hyperalgesia after long-term opioid use, and physical tolerance at certain doses (applicable to other medications as well) (Wilson et al., 2005). Although there are several consequences of opioid use to consider before prescribing, there is abundant level 2 support for the use of opioids in other painful conditions, including PHN and painful diabetic neuropathy (PDN). Similar to TCAs, opioids do not cause any cognitive deficits as detected by neuropsychological testing (Wilson et al., 2005). One study by Watson and Babul (1998) found that controlled-release oxycodone at 60 mg/day contributed to a 35% reduction in pain in PHN (C. P. Watson & Babul, 1998); other studies show oxycodone ability to reduce pain by 30% in PDN as well (Gimbel et al., 2003; C. P. Watson & Babul, 1998; C. P. Watson et al., 2003). Tramadol is an opioid suggested to have similar effects in both PDN and other painful neuropathies (Sindrup et al., 1999). The data for use in CRPS is inadequate as there are limited RCTs with solid conclusions; however, opioid treatment is currently prescribed.

Thus, pharmacotherapy can reduce symptoms of CRPS; however, there are several drawbacks to the indefinite use of medications: in most patients, only a temporary or partial relief from symptoms is provided, unable to “cure” CRPS. Other treatments (e.g. electrical stimulation) have been found to have disease-modifying effects, such as persistent pain relief even after treatment is complete (Wilson et al., 2005). Another limitation is the length of therapy; the median age of CRPS is approximately 40 years (Wilson et al., 2005), thus these patients may potentially need to take prescription medications for decades. Not only are the long-term effects of prolonged medication use generally unknown, but the long-term use may have significant impacts on social, economic, and medical aspects of patient livelihood.

Currently, much of the evidence-based pharmacotherapy comes from data extrapolated from RCTs for other diseases. Therefore, further work investigating potential disease-modifying or protective agents, as well as performing RCTs specific to CRPS patients, is needed.

1.4.3 Injection Therapy

Traditional interventional therapies also include symptom relief through injection of various compounds. CRPS was formally named reflex sympathetic dystrophy for the implied mechanistic involvement of the sympathetic nervous system; this belief led to various treatments aimed at sympathetic blockade. Although there is indeed sympathetic nervous system involvement (i.e. sympathetically-maintained pain (SMP)), CRPS is a complex disease where sympathetic blockade does not always provide relief. Sympathetic nerve block does, however, provide insight into the diagnosis of pain in CRPS patients as either SIP or

SMP. Common blocks include: sympathetic nerve blocks, intravenous regional anesthesia (IVRA), intravenous infusions and others.

Depending on the location of symptoms, sympathetic nerve blocks are traditionally delivered at the level of stellate ganglion or lumbar sympathetic chain (Wilson et al., 2005). Sometimes, pain relief is evident even after the effects of local anesthesia have expired; in some cases, pain relief may be long-lasting (Burton & Waddell, 1998; Price et al., 1998). In 2002, a systematic review by Cepeda and colleagues of over 79 reports on sympathetic blockade found that most reports had to be rejected as CRPS diagnostic criteria has evolved markedly in the last 100 years (Cepeda et al., 2002). After pooling the data from only 29 remaining studies, Cepeda and colleagues found that only 17% of 454 patients had partial or complete pain relief; the duration of pain relief varied significantly and Cepeda also concluded that it may be inaccurate to pool much of this data together. Overall, there is some evidence for the benefit of classic sympathetic nerve blocks; however, they still remain in most treatment plans as either a test to differentiate SMP from SIP, or to help supplement active rehabilitation/physical therapy regimens.

IVRA is a fairly simple technique used to relieve pain symptoms by delivering anesthetic directly to the affected limb. This is performed by injecting an anesthetic compound, or a mixture of several compounds, into the circulation of the affected limb, while isolating the limb from the rest of circulation with a tight-fitted tourniquet (Davis et al., 2002). The likely mechanism for the block of sympathetic nerves is through vascular beds around peripheral nerves, the vasa nervorum and valveless venules around nerve endings (Wilson et al., 2005); diffusion of the local anesthetic

into local tissues may also be involved. Several high-quality studies have been published comparing local anesthetics delivered by IVRA including guanethidine, lidocaine, bretylium, clonidine, droperidol, ketanserin and reserpine (Forouzanfar et al., 2002; Kingery, 1997; Perez et al., 2001).

Intravenous infusions of phentolamine and lidocaine have also been studied for the relief of pain symptoms. Intravenous infusion involves the controlled injection of fluid into the circulatory system by an infusion pump. Studies differ on the effect of phentolamine; a study by Arner et al (1991) reported an analgesic effect in both adults and children with CRSP-I and II, but Verdugo and Ochoa (1994) found that neither placebo nor phentolamine provided any changes in pain, blood flow or sensory testing after a prospective, single-blinded study (Arner, 1991; Verdugo & Ochoa, 1994). Intravenous infusions are not used as much recently and when used, they often act as a diagnostic tool to differentiate SIP and SMP.

1.4.4 Psychological Interventions

Similar to several other chronic pain disorders, CRPS is a complicated biopsychosocial disorder that requires multidisciplinary treatment in order to improve psychological, social and medical aspects of patient health. Currently, there are very limited controlled studies examining the efficacy of different types of psychological intervention; however, there are several approaches that have shown benefit. These include relaxation training, biofeedback, cognitive intervention and hypnotic imagery (Wilson et al., 2005). As part of a multidisciplinary treatment package, many of the above approaches have yielded significant alleviation of CRPS symptoms. A RCT by

Oerlemans et al (1999 and 2000) of 135 adult CRPS patients concluded that relaxation training and cognitive interventions in conjunction with physical therapy yields a significantly greater improvement in CRPS symptoms than controls at a one-year follow up (Oerlemans et al., 1999; Oerlemans et al., 2000). Several case studies have shown almost complete resolution of symptoms after biofeedback (muscular and thermal), relaxation training and hypnotic imagery (Barowsky et al., 1987; Blanchard, 1979; Gainer, 1992). These approaches often target the learned limb disuse that patients develop. Clinical experiences suggest that using the above techniques in an integrated multidisciplinary context can provide substantial relief of symptoms.

1.4.5 Implanted Therapies

In addition to traditional interventional therapies, advanced pain medicine techniques used in the treatment of CRPS include spinal cord stimulation (SCS), peripheral nerve stimulation (PNS), and intrathecal drug delivery (ITDD). These implantable modalities are more commonly used for CRPS conditions that do not respond appropriately to pharmacotherapy, regional nerve blocks or physical therapy.

1.4.5.1 Spinal Cord Stimulation

Nerve stimulation (either spinal cord or peripheral nerve) was first suggested for use in pain in 1967 (Shealy, Mortimer, et al., 1967; Shealy, Taslitz, et al., 1967). SCS was performed by Cook et al (1976) for pain relief of secondary ischemia to peripheral vascular disease (Cook et al., 1976). It was also found to improve

perfusion after Jacobs et al (1988) showed relief of ischemic leg pain and improvement of ulcer healing (Jacobs et al., 1988). SCS is performed by the implantation (either temporary or permanent) of small, soft wires near the spinal cord. These wires have electrical leads on their tips that pass electrical currents that are produced by a small programmable generator often surgically placed under the skin of the buttocks or abdomen. Neurostimulation in this manner blocks pain signals by applying a mild electrical current to the spinal cord (SC), often resulting in less pain and a tingling feeling in the affected area called paresthesia (Wilson et al., 2005); however, the exact mechanisms by which SCS relieves pain is still not fully understood. It was originally thought to target the dorsal column of the SC but other studies showed that neurostimulation also influenced transmission (affecting sensory dorsal nerves and descending inhibitory pathways in the SC) (Linderoth et al., 1992; Long et al., 1981). It is also thought that the improved peripheral circulation after SC stimulation is the result of autonomic effects as the neurostimulation may modulate efferent impulses that could produce vasodilation in the innervated dermatome (Wilson et al., 2005).

1.4.5.2 Peripheral Nerve Stimulation (PNS)

PNS works in a similar manner to SCS; small electrodes are placed along peripheral nerves in the targeted limb and often cause a reduction in pain, as well as the same paresthesia observed in SCS (Wilson et al., 2005). PNS can be used alone, but it is often used together with SCS. An indication for this type of treatment is pain predominating in a region innervated by only one peripheral nerve. In both

SCS and PNS, nerve stimulation is temporarily tested to allow for patient feedback before any permanent surgical implant is in place (Wilson et al., 2005). Although there are limited randomized studies examining this treatment, pain relief has been demonstrated in several retrospective studies (Ebel et al., 2000; Law et al., 1980).

1.4.5.3 Intrathecal Drug Delivery

ITDD is a complex technique involving the implantation of infusion pumps that deliver pharmacological agents directly to a site on the spinal cord (Wilson et al., 2005). The intrathecal pump, sometimes called the “pain pump”, has been typically used for patients with cancer pain (Onofrio et al., 1981); however, CRPS patients in several case studies have found pain relief from this technique. Medication is delivered via small catheters that originate in a small pump surgically implanted under the skin of the abdomen (Institute, 2013). Typically, morphine is the first-line agent used in ITDD. Morphine administered via intrathecal pump has been reported effective several times, especially in severe cases of CRPS.

There are limitations to ITDD. Similar to other opioid treatments, dose increases are usually required. Complications with the catheter tip exist, where tip masses can form when medication is delivered at high concentration (McMillan et al., 2003). Finally, kinking of the catheter often causes a mild red rash on the skin and jeopardizes the integrity of the catheter (Wilson et al., 2005).

1.4.6 Other Therapeutic Approaches

Several techniques are either not approved or have limited evidence to support their use; a sample of these approaches are briefly addressed in this section below.

In consideration of the tissue hypoxia and acidosis that often accompanies CRPS, Kiralp *et al*, Tuter *et al* and Peach all proposed the use of hyperbaric oxygen (HBO) therapy (Kiralp et al., 2004; Peach, 1995; Tuter et al., 1997). In a randomized, placebo-controlled study, Kiralp demonstrated the use of HBO therapy in markedly attenuating edema and pain in CRPS patients after 15 treatments; these findings lend additional support to warrant further research of HBO therapy and for treatments targeting edema and hypoxia for pain relief.

Traditional Chinese medicine involves qigong, a concept with growing popularity in North America that focuses on balancing one's "life energy". Recently, its use has been examined for therapeutic benefits in many conditions ranging from cancer and pain, to obesity and hypertension (Bao et al., 2014; Elder et al., 2007; Xiong et al., 2015). Although there are some clinical studies suggesting qigong as a potential treatment for CRPS-I (Lee et al., 2007; W. H. Wu et al., 1999), the small number of participants involved precludes any solid conclusion. Further research may be warranted for its use in resolving depression, anxiety and other psychiatric complications resulting from CRPS diagnosis.

One event known to trigger CRPS complications is surgery. Casting and tourniquet use have been suggested as possible initiating noxious events for CRPS. A preventative, rather than therapeutic, approach to CRPS has been studied for

surgical cases by using vitamin C. One-gram daily of vitamin C treatment was found to be effective at preventing CRPS after both upper and lower limb surgery (Besse et al., 2009; Zollinger et al., 1999). More recently, Vitamin C was found to have an antiallodynic effect in rats, when delivered once per day for 3 days before a hindpaw ischemia-reperfusion injury was induced (the chronic post-ischemia pain model (CPIP) (Park et al., 2013). Co-administration with vitamin E had a greater antiallodynic effect in the experiment, and together these vitamins were suggested as modulators of spinal cord neuropathic pain processing.

A relatively radical approach to CRPS is a ketamine coma, where extended use of anesthetic dosages of ketamine is used to place a CRPS patient in a coma. Ketamine has been found to reduce pain significantly in many patients when applied topically or by IV; however, the ketamine coma has been suggested to reset NMDA receptors and block the central sensitization existing in CRPS (Henson & Bruehl, 2010). Studies examining its use are limited and the treatment is still considered controversial and unproven in Canada.

A common theme in the discussion of most therapeutic approaches for CRPS is that further study is required. Many studies lack quality controls or a large sample size to confidently state conclusions. Clinical trials on both CRPS patients and those at risk of developing CRPS (e.g., receiving knee replacement surgery) is to assess the efficacy of various treatments and to better understand the best multi-disciplinary approach to treating CRPS.

1.5 PATHOPHYSIOLOGY

Current knowledge of the pathophysiologic mechanisms driving CRPS symptoms suggests they are multifactorial. A recent comprehensive review by Bruehl (2010) presents a summary of the most widely accepted and documented pathophysiologic mechanisms that may contribute to CRPS; they include peripheral and central nervous system sensitization, inflammation (increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines), altered sympathetic and catecholaminergic function, altered somatosensory representation in the brain, genetic factors and psychophysiologic interactions (Bruehl, 2010). It is now clear that the above multiple mechanisms are involved, and that CRPS presentation depends on the relative contribution of each mechanism.

The majority of CRPS patients have a clinical history of some noxious injury (trauma, ischemia, nerve injury) that initiates their symptoms. Often, as a result, clinicians observe signs of inflammation (e.g. edema, redness and hyperemia). Increased local and systemic levels of pro-inflammatory cytokines (e.g. TNF-alpha, IL-1beta, IL-2 and IL-6) have been observed and correlated with CRPS symptoms in patients (Maihofner et al., 2005; Uceyler et al., 2007). Decreased anti-inflammatory cytokines (e.g. IL-10) are observed in CRPS patients as well (Uceyler et al., 2007). Pro-inflammatory cytokines are released after trauma by classical inflammatory mechanisms, by action of immune cells (lymphocytes, mast cells, etc); the effect of is plasma extravasation, and subsequently edema, which probably explains the edema and swelling often observed in CRPS patients. In addition to pro-inflammatory cytokines, neuropeptides Substance P and calcitonin gene-related

peptide (CGRP) can trigger neurogenic inflammation (Bruehl, 2010). Neuropeptides and pro-inflammatory cytokines have also been found to produce peripheral nerve sensitization, leading to increased nociception (Bruehl, 2010). These molecules have also been found to stimulate osteoclasts, explaining the osteoporosis frequently observed in CRPS patients (Birklein & Schmelz, 2008).

Sensitization of peripheral nerves, which leads to persistent pain, is triggered by the initial tissue trauma and inflammation as explained above and is present early in CRPS symptomatology. After trauma, release of neuropeptides and inflammatory cytokines can increase background firing of nociceptors and decrease the firing threshold for mechanical and thermal stimuli, resulting in allodynia and hyperalgesia observed in CRPS patients (Bruehl, 2010; J. Cheng & Ji, 2008; Couture, 2001). Central sensitization is the increased excitability of nociceptive neurons in the spinal cord, often resulting from persistent noxious input after tissue damage or nerve injury (Bruehl, 2010). Sensitization of the central nociceptors is often controlled through neuropeptides and at N-methyl-D-aspartate (NMDA) receptors, leading to extremely amplified responses to noxious stimuli (hyperalgesia) as well as to non-painful stimuli (allodynia) (Bruehl, 2010; Gracely, 1992; Gracely et al., 1992).

Reduced cutaneous innervation of the affected limb has also been observed in CRPS patients. Although CRPS-I has no clinical signs of nerve injury, Albrecht and colleagues observed a reduced density of nerve fibres (both C- and A-delta fibres) in the affected limbs of CRPS-I patients (Albrecht et al., 2006). No causative role in the development of further CRPS symptoms has been proven; however,

needle stick injury in rodents to distal nerves has resulted in the development of similar symptoms to CRPS-I patients (Albrecht et al., 2006; Siegel et al., 2007b).

Altered sympathetic nervous system (SNS) function has historically been linked to CRPS, as classical symptoms include 'cool' and 'blue' affected limbs, caused by vasoconstriction from SNS outflow. It is believed that pain in some CRPS cases is sympathetically maintained, meaning that there is excessive SNS outflow that results in pain. Sympathetic nerve blocks as a treatment option directly arose from this ideology. Studies in rodents have found that increased adrenergic receptors are expressed on nociceptive nerve fibres after trauma, perhaps explaining why nociceptive signals are triggered (Janig & Baron, 2002). The receptor expression may be linked to sympatho-afferent coupling, which results in sympathetic nerve activity stimulating these receptors on nociceptive fibres. Afferent nociceptive fibres become sensitized to adrenergic excitation, leading to increased firing in the presence of sympathetic outflow or circulating catecholamines. (Arnold & Delbos, 2003; Baron & Maier, 1996; Harden et al., 1994; H. A. Kurvers, 1998). The persistent activation leads to sensitization of the central nervous system as well.

Hypofunction of the SNS directly after injury is believed to upregulate the expression of peripheral catecholaminergic receptors, resulting in supersensitivity to circulating catecholamines, causing vasoconstriction (Birklein et al., 1998; Harden et al., 1994; H. Kurvers et al., 1998). Despite a lower level of catecholamines in the affected limb of CRPS patients, vasoconstriction is observed; this is probably the result of sensitization of peripheral adrenergic receptors during the acute phase of CRPS (Bruehl, 2010). Therefore, vasoconstriction may still occur even with

decreased local sympathetic outflow. The sensitization of adrenergic receptors also explains the exaggerated sweating and vasoconstriction observed after exposure to circulating catecholamines. Catecholamines can increase in the event of regular life stress or pain sensations. An odd vicious cycle may occur where catecholamine release leads to nociceptive input, maintaining an altered central process that generates more pain, and subsequently more catecholamine release (Wilson et al., 2005).

There are limited studies examining CRPS using imaging techniques, however one review of neuroimaging literature has concluded that there is little evidence supporting the concept of a “pain network” in neuropathic pain syndromes (Moisset & Bouhassira, 2007). After acute pain experiments, researchers have been able to correlate activity in specific areas of the brain with various experimental stimuli inducing pain; however, brain activity as a result of clinical pain, more specifically chronic pain, is not well understood and does not correlate well with the “pain network” or “pain matrix” characterized after inducing experimental pain. Also, there is no evidence for a consistent brain activation pattern associated with allodynia. All of this considered, there have been several studies of CRPS using neuroimaging techniques that observe one consistent brain alteration: a reorganization of the somatotopic map. More specifically, the alteration is a reduction in the size of the representation of the affected limb in the somatosensory cortex (Juottonen et al., 2002; Maihofner et al., 2003). The degree of this brain plasticity has been directly correlated to the level of hyperalgesia, as well as CRPS pain intensity (Bruehl, 2010; Maihofner et al., 2003). Somatotopic reorganization in

CRPS patients has also been linked to impaired sensory perception, like the ability to discriminate between two-point tactile stimulation. Although it is not known when in CRPS development the reduced limb representation occurs in the brain, these findings have clinical importance and reflect the multifaceted nature of CRPS.

Genetic factors have been suggested to play a role in CRPS. Small sample studies have correlated CRPS development with a familial link. Onset of CRPS in siblings of CRPS patients occurs 3-times as often as non-familial cases (de Rooij et al., 2009). Genes involved with major histocompatibility complex encoding the human leukocyte antigen (HLA) molecule, and genes involved in inflammation (like a TNF-alpha promoter gene) have been thought to play a role in CRPS development (Vaneker et al., 2005). A polymorphism of the TNF-alpha promoter gene has been suggested to increase TNF-alpha levels, perhaps contributing to the exaggerated inflammatory response observed in CRPS patients. The hypothesis that genetic factors play a role in CRPS development is still being assessed, and no study has provided evidence from a large sample size to link the two.

A psychological cause for the development of CRPS has been hypothesized since the syndrome was formally recognized; today, some still continue to hold this idea (Ochoa & Verdugo, 1995). The literature currently holds very few strong prospective studies examining this hypothesis, although theoretically it is possible that psychophysiological mechanisms may contribute to the development of CRPS (Bruehl, 2010). However, psychogenic factors alone are not sufficient to produce the signs of CRPS. One study by Harden et al (2003) found that higher anxiety levels before total knee arthroplasty surgery were associated with a greater likelihood of

being diagnosed with CRPS by 1-month post-procedure (Harden et al., 2003). This may be related to the increase in catecholamine activity observed during psychological distress (Charney et al., 1990; Harden et al., 2004), potentially contributing to the adrenergic mechanisms involved in CRPS (Bruehl, 2010). Although there are theoretical links and a few prospective studies that suggest psychological factors impact on the development of CRPS, further prospective work is required to provide any empirical evidence.

No empirical studies have been performed evaluating the interactions between many of the pathophysiological mechanisms described above; however, Bruehl (2010) described a speculative model of how these mechanisms may come together in CRPS development and maintenance (Bruehl, 2010). His model is illustrated in Figure 1.1. CRPS is clearly multifaceted in its pathophysiologic mechanisms, and further studies are required to comprehensively evaluate the contribution of each mechanism to CRPS signs and symptoms. Considering that mechanism-based treatment is a goal in CRPS therapy, our lack of understanding the pathophysiology is detrimental not only to patients and the healthcare system, but arguably also to our economy, since many CRPS patients are unable to work. Further research into identifying pathophysiologic mechanisms may eventually permit the development of effective clinical treatment options and, perhaps, a “cure”.

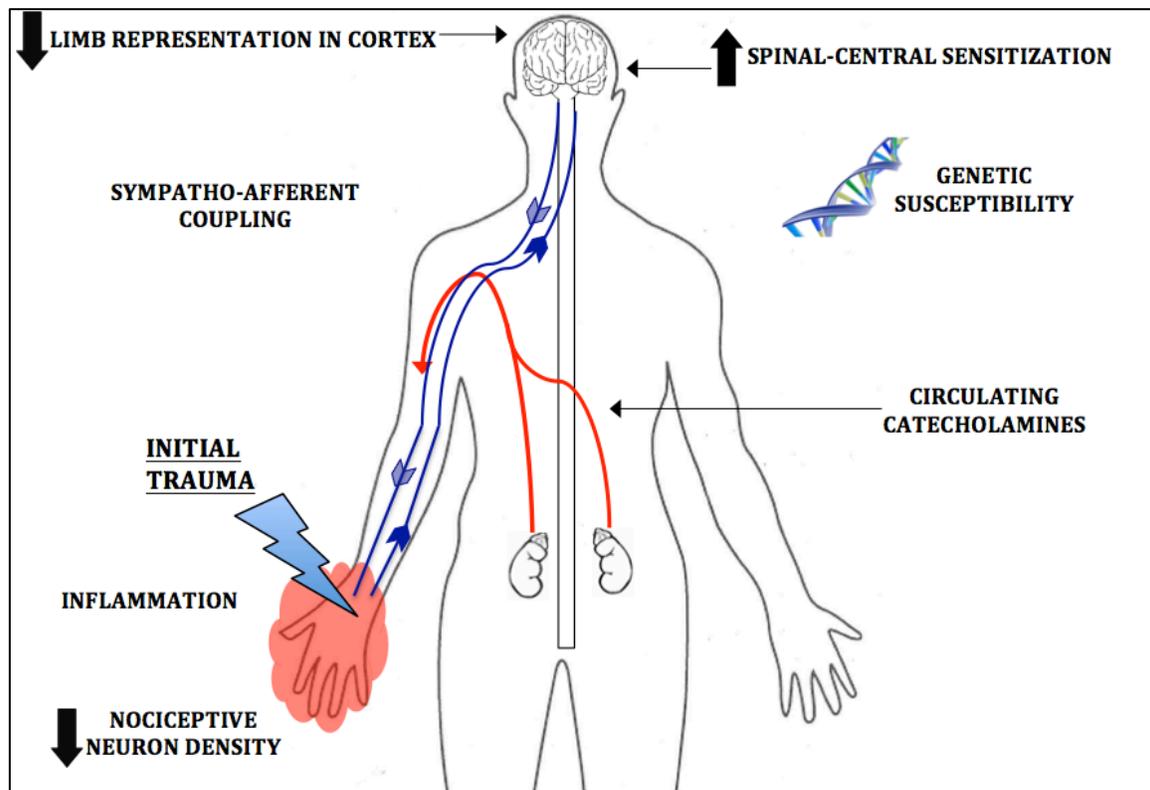


Figure 1.1. Speculative contributions of pathophysiologic mechanisms in CRPS

Adapted from Bruehl 2010.

1.6 ANIMAL MODELS OF CRPS-I

Limited in number, there have been several attempts to model CRPS-I in small animals. Compared with CRPS-II animal models, which have been well characterized through the use of nerve injury, the key to developing a CRPS-I animal model has been the induction of neuropathic-like pain without initiating a major nerve injury.

One of the first animal models describing CRPS-I symptomatology was developed by van der Laan et al (van der Laan et al., 1997). It involved the administration of a free-radical donor called tert-butyl-hyperperoxide into the femoral artery of conscious rats for 24 hours. The result was mechanical allodynia, lasting for at least 4 weeks. The authors hypothesized that free-radical application triggered a sensitization of both peripheral nerves and central processing and subsequently, the hyperalgesia and allodynia (van der Laan et al., 1997). Plasma extravasation and significant inflammatory signs (redness, increased temperature and edema) were also observed. After treatment with a free-radical scavenger before and after injury, the authors found significantly altered effects. While this model presented a very interesting attempt at modelling CRPS-I, no further studies have been done.

Another model, developed in 1998 by Vatine et al, used electrical stimulation of the sciatic nerve to initiate hyperalgesia and allodynia (Vatine et al., 1998). The authors applied a 0.5 Hz and 8mA electrical stimulation to the sciatic nerve supramaximally for 10 minutes and found that animals developed significant thermal hyperalgesia, as well as some cold and mechanical allodynia symptoms. They hypothesized that sensory changes were due to sensitization of the nerve or its

central connections, and not a nerve injury. Symptoms normally observed in CRPS-I patients, like swelling, redness and dystrophy, were not observed, thus no follow-up studies were conducted.

Inducing signs of mechanical allodynia, ischemia and inflammation, Gradl et al (2005) developed a CRPS-I animal model initiated by a controlled-impact soft-tissue injury and intra-arterial infusion of inflammatory mediators (Gradl et al., 2005). The authors found that mechanical allodynia and local inflammatory effects were triggered after intra-arterial infusion of Substance P for 24 hours. This model presents an interesting method for initiating CRPS-I-like symptoms; however, behavioural observations were only followed for four days post-injury and there was no evidence of hyperalgesia or spontaneous pain behaviours.

A study by Oaklander et al (2006) showed evidence for minor nerve injury in CRPS-I patients, suggesting that minor nerve injury may induce CRPS symptomatology (Oaklander et al., 2006). Complementing these findings, Siegel et al (2007) developed a novel model of CRPS-I by initiating a minor nerve injury of the tibial or sural nerve using needle puncture (Siegel et al., 2007a). After nerve injury, 30-50% of rats developed mechanical allodynia that lasted 14-days post-injury, with mechanical hyperalgesia and cold allodynia also evident in some rats. Interestingly, there was no correlation between sensory changes and needle size used to initiate injury, and there was no difference in sensory changes between tibial or sural minor nerve injuries.

Perhaps a more clinically relevant model than those listed above, Guo et al (2004) induced a number of CRPS-I signs and symptoms after initiating a rat tibial

fracture and casting for four weeks (Guo et al., 2004). Around 31% of CRPS cases may indeed be the result of distal tibial fractures (Sarangi et al., 1993). After fracture and casting, animals developed mechanical allodynia that lasted 16 weeks, as well as edema and hyperthermia. Increased cytokines in the hind paw skin and decreased mineral bone density was also observed. After glucocorticoid treatment, edema and hyperthermia were attenuated; however, mechanical allodynia wasn't affected. After application of a neurokinin-1 (NK-1) antagonist and soluble TNF-alpha receptor, mechanical allodynia was reversed, suggesting that Substance P and TNF-alpha may play important roles in CRPS-I symptomatology.

The above-mentioned rat models each possess some features similar to CRPS symptoms. As mentioned by Wilson and colleagues (2005), there is a need for validation of existing standards and the generation of new models that can recapitulate CRPS's unique features (Wilson et al., 2005). Each animal model briefly explained above lacks in representing most symptoms of CRPS. Thus, a chronic post-ischemia pain (CPIP) model developed by Coderre et al (2004) through ischemia-reperfusion injury may represent the literature's best option for the study of CRPS-I (Coderre et al., 2004).

1.6.1 CPIP Model

Coderre et al (2004) was able to produce a neuropathic pain-like syndrome in rats after initiating a reperfusion injury due to prolonged hindpaw ischemia. In this model, anesthetised rats were subjected to complete ischemia of one hindpaw, through use of a tight fitting ring, for 3 hours. Hyperalgesia to noxious mechanical stimuli and cold, as well as mechanical allodynia were evident and lasted for at least

four weeks post injury in most animals. Spontaneous pain behaviours, such as licking, shaking and favouring of the hindpaw were also observed. Spread of hyperalgesia and allodynia to the contralateral limb were also observed, similar to the spread of symptoms in CRPS-I patients. In addition to sensory changes, rats exhibited hyperemia and edema for several hours post-injury, all without ischemia-induced damage to the tibial nerve (Coderre et al., 2004).

CPIP presents a good tool for CRPS study as it displays several symptoms that are observed in CRPS patients (Janig & Baron, 2002; van der Laan et al., 1998). Considering CRPS is most common after fracture, sprain, crush injury and surgery (i.e physical injury), this model is clinically relevant as it induces symptoms through physical injury: ischemia-reperfusion. This model has been used several times in the literature in order to assess the effect of different analgesic/anti-allodynic treatments (Laferriere et al., 2014; Nahm et al., 2014; Kwak et al., 2011; de Mos et al., 2009).

Coderre and colleagues propose that many cases of CRPS-I are the result of microcirculatory abnormalities following IR and persistent inflammation from injury. Ischemia-reperfusion injury and inflammation can lead to a persistent state of reduced perfusion and/or reduced oxygenation of tissue, which is likely involved in the sensitization and activation of afferent innervation of the affected tissue (Coderre et al., 2004). This activation and sensitization of muscle nociceptors would result in deep, persistent pain, and can lead to the central sensitization that contributes to mechanical allodynia and hyperalgesia. This means that chronic sensory

disturbances present in CRPS-I patients may be strongly linked to the inflammatory changes and microvascular deficits occurring after ischemia-reperfusion injury.

While CPIP provides evidence for the development of inflammatory and pain symptoms after ischemia-reperfusion injury similar to CRPS-I, it would benefit from functional analyses before and after injury, considering CRPS-I results in significant functional deficits and behavioural changes in patients. Additionally, it may also provide a proper means for the testing of other potential therapeutics.

1.7 HEME OXYGENASE

It may surprise many, but carbon monoxide (CO) – the so-called “silent killer” gas – is endogenously produced in the body via the heme oxygenase (HO) system during heme metabolism (Bauer et al., 2008). As displayed in Figure 1.2, heme from hemoglobin is converted to biliverdin by HO enzyme, resulting in the formation of CO and ferrous iron (Fe^{2+}) byproducts (Kikuchi et al., 2005). Biliverdin is then quickly converted to bilirubin through the biliverdin reductase.

Heme oxygenase is present in three isoforms: HO-1, HO-2 and HO-3. HO-1, which is in high abundance in the liver, spleen, vascular endothelial cells and smooth muscle, is inducible in response to oxidative stress, hypoxia, heavy metals and cytokines (Ryter & Otterbein, 2004; Ryter et al., 2002). HO-2 is constitutively found and expressed under homeostatic conditions; it is found primarily in neuronal cells, liver, heart, vascular endothelial cells and smooth muscle tissue (Maines, 1997). The function of HO-3 is not well understood.

The HO system has often been associated with cytoprotective functions. The anti-oxidant properties are evident in all byproducts of this system (Motterlini & Foresti, 2014; Rytter et al., 2002). Both biliverdin and bilirubin have been found to be potent scavengers of peroxy radicals, as well as inhibiting the effect of other mutagens (T. W. Wu et al., 1991). Ferrous iron, a byproduct of heme breakdown, has also been found to reduce the formation of iron free radicals, contributing to the cytoprotective effect of HO. Last, but definitely not least, CO is often regarded as the most important player in the action of HO (Maines, 1997; Motterlini et al., 1998; Otterbein et al., 1999). CO has been shown to be effective in diminishing the severity of microvascular dysfunction after ischemia, as well as effective in reducing inflammation and cell injury.

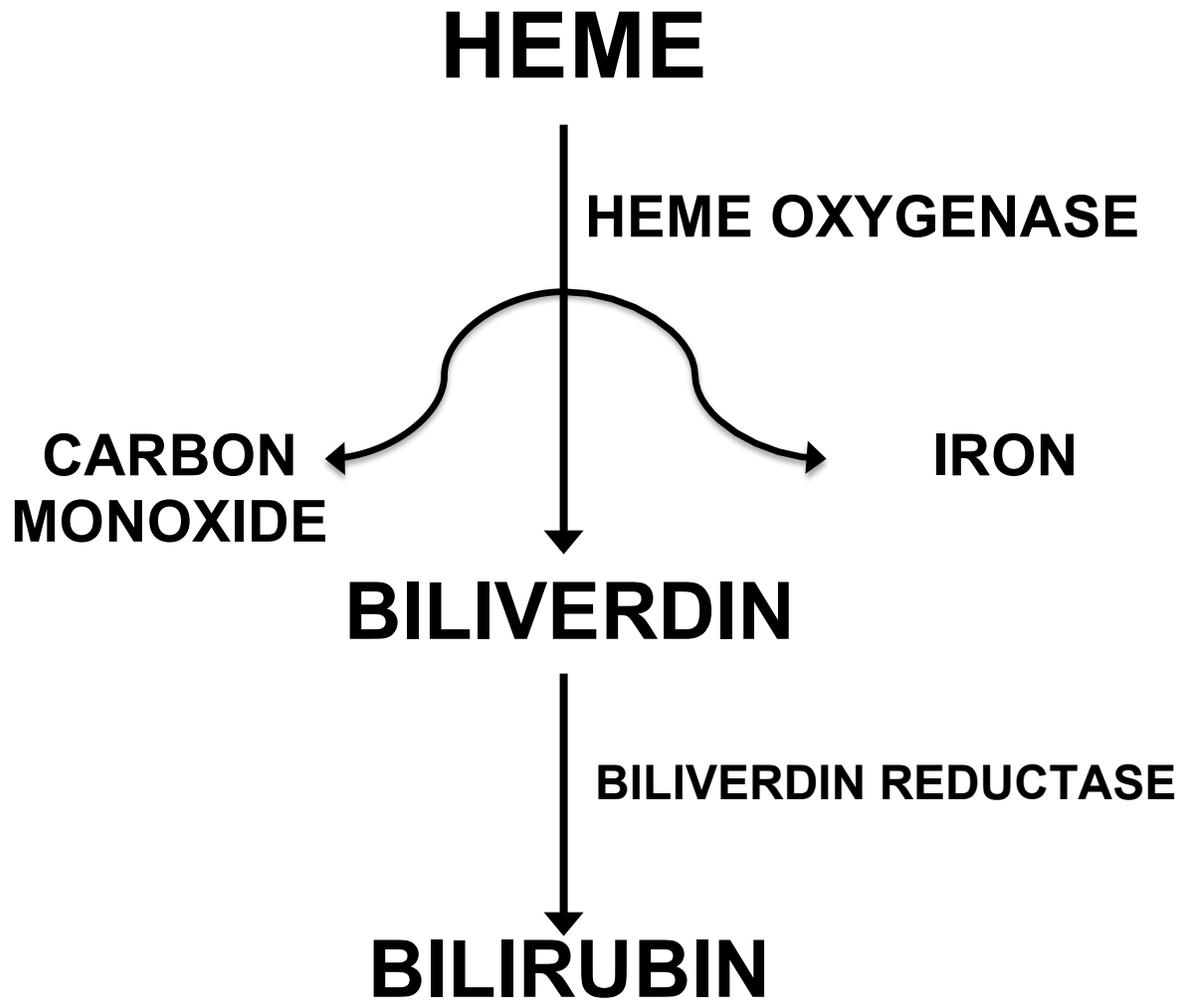


Figure 1.2. Heme catabolism: conversion of heme to bilirubin through heme oxygenase and biliverdin reductase, with carbon monoxide and iron as byproducts.

1.7.1 Carbon Monoxide

At normal conditions, CO exists as a colourless, tasteless and odourless gas that is often the product of incomplete combustion (Varon et al., 1999). Currently, the leading cause of death by poisoning in the United States is due to CO intoxication (Meredith & Vale, 1988; Varon et al., 1999). Upon CO exposure, many detrimental side effects may begin to appear, including headache, nausea, vomiting, impaired memory, confusion and dizziness. Death is said to occur when carboxyhemoglobin levels reach 50-80% (Burg, Ryter and Otterbein 2004).

Although there seems to be a number of reasons why carbon monoxide should never be considered for use as a therapy, investigations into its uses in many unique settings are currently underway and are showing promise for its potential as a treatment in several conditions.

In 2001, Fujita and colleagues showed the paradoxical rescue of ischemic lung tissue by CO in a HO-1 deficient murine model (Fujita et al., 2001). They found that inhaled CO was able to potentiate fibrinolysis by suppressing the induction of the gene encoding plasminogen activator inhibitor-1 in phagocytes. They concluded that suppression of this hypoxia-induced protein was the result of action through soluble guanylate cyclase (Fujita et al., 2001). From this work, and several other from the late 1990's and early 2000's, the proposition of using CO in a clinical setting in the future has been garnering attention.

In 2008, a review by Foresti and colleagues outlined the promises and challenges associated with the use of CO in therapy. In examining inhaled CO, they found that there were indeed several studies showing remarkable results in the

treatment of inflammatory processes and cardiovascular disorders, in pre-clinical models; however, they could not ignore the inherent toxic effects CO can produce if uncontrolled amounts were delivered (Foresti et al., 2008). They concluded that administration of gaseous compounds in a clinical setting would come with many difficulties and potential complications; they suggested the use of carbon monoxide-releasing molecules (CO-RMs) would provide a much more clinically applicable therapeutic option.

1.7.1.1 Inhaled CO

Exogenous application of CO through inhalation has been investigated in several studies and has been shown to have beneficial effects (Fujita et al., 2001; Mishra et al., 2006; Ott et al., 2005); however, CO toxicity is still a very serious and potential risk. CO binds haemoglobin at approximately 220 times the strength oxygen does (Motterlini, 2007). This significant difference in affinity poses potential risks of reducing the oxygen-carrying capacity, resulting in hypoxia, or the formation of carboxyhemoglobin (HbCO) molecules, resulting in CO-poisoning. Although the level of CO delivered through inhalation could be minimized so as to reduce the levels of HbCO, CO poisoning is poorly understood and cannot rely solely on HbCO levels as indication of toxicity (Foresti et al., 2008). HbCO levels, however, are still good markers to predict the amount of CO present in the body; literature states that a proportion of 15-20% HbCO is not detrimental and is the biological threshold for CO tolerance (Foresti et al., 2008). Beyond, or around, this biological threshold and co-mediated injury is likely to occur in most.

In examining the effect of inhaled CO on lung disease or injury, several studies have showed positive effect. Otterbein and colleagues (1999) found inhaled CO provided protection against hyperoxic lung injury; they were the first to suggest anti-inflammatory and anti-apoptotic actions of CO (Otterbein et al., 1999). Beneficial effects of inhaled CO were confirmed in models of allergen-induced asthma (Chapman et al., 2001), lung transplantation (Song et al., 2003), oxidative lung injury (Otterbein et al., 2003), and lung hypertension (Zuckerbraun et al., 2006). Interestingly, Clayton and colleagues (2001) challenged this positive view of CO effects in their study that showed no benefits after CO treatment (Clayton et al., 2001).

Inhaled CO has provided benefit in systemic inflammation and the cardiovascular system as well. In several *in vitro* and *in vivo* models, CO was able to reduce the production of inflammatory cytokines TNF-alpha and interleukins. In a lung transplantation model, inhaled CO was able to prevent ischemia-reperfusion injury (Kohmoto et al., 2006). It was also found that administration of CO provided full protection even in the absence of HO-1 (Otterbein, 2002).

Although inhaled CO provides remarkable benefits in several pre-clinical models, its administration in a clinical setting may be very limited.

1.7.1.2 Carbon Monoxide-Releasing Molecules (CO-RMs)

As a result of the potential risks associated with inhaled CO therapy, a novel class of transition metal carbonyl compounds have been developed, in order to deliver exogenous CO through an oral or injectable route (Foresti et al., 2008;

Motterlini et al., 2002; Motterlini, Mann, et al., 2005). Carbon monoxide-releasing molecules (CO-RMs), have the general chemical formula $M(\text{CO})_x\text{L}_y$, whereby “M” is the transition metal, “x” the number of CO ligands and “y” the number of additional ligands (Santos-Silva et al., 2011).

The first CO-RMs developed, CORM-1 and CORM-2, were lipid soluble, fast-releasing compounds (Figure 1.3). CORM-1 and CORM-2 both have half-lives equal to or less than one minute (Motterlini, Mann, et al., 2005). As a result of the need for solubility in solutions like DMSO or ethanol, these CO-RMs proved difficult to administer, as they are relatively inapplicable to biological solutions. Eventually, a water-soluble ruthenium-based CO-RM, CORM-3, was developed.

CORM-3 (molecular formula $\text{Ru}(\text{CO})_3\text{Cl}(\text{glycinate})$), also a short half-life compound, is stable at physiological conditions (pH 7.4, 37°C, aqueous solution). With a half-life of approximately one minute, CORM-3 rapidly liberates CO through a ligand substitution reaction (Motterlini, Mann, et al., 2005). Both CORM-3 and a newer CO-RM called CORM-A1, are promising compounds for the use of CO-RMs in therapy (Motterlini, Sawle, et al., 2005).

Newer CO-RMs have been developed as of late, such as CORM-368, CORM-401, CORM-371, CORM-409, and CORM-313; however, limited investigations have been completed in terms of their therapeutic properties. These newly synthesized CO-RMs are manganese-containing compounds (rather than ruthenium) and therefore may provide less risk as a potential drug.

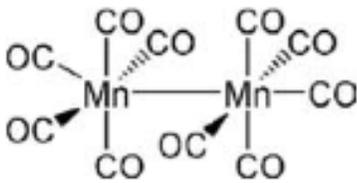
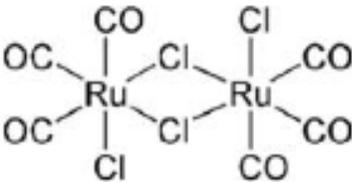
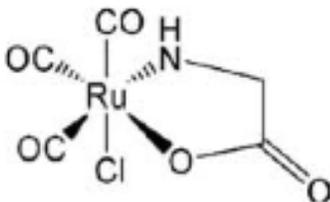
Molecule	Chemical structure
CORM-1	
CORM-2	
CORM-3	

Figure 1.3. Chemical structures of three carbon monoxide-releasing molecules (CO-RMs). Adapted from (Gullotta et al., 2012).

1.7.1.3 CORM-3

One of the key limitations of the first CO-RM molecules developed was its solubility. Lipid soluble molecules tend to be more difficult to administer clinically and are therefore less favourable. In order to confer solubility in aqueous solutions, biochemists were able to add a glycine amino acid molecule to the metallic carbonyl compound (Motterlini, Mann, et al., 2005). With this amino acid addition, the half-life of the compound remained similar to CORM-1 and -2 at about 1 minute, assuming in physiological solutions. Motterlini and colleagues were able to show positive results for CORM-3's ability to release CO molecules upon administration in a rat model and provide beneficial vasodilation. By also showing that CORM-3 administration was safe up to a concentration of 500 μ M, Motterlini was able to meet all of the criteria required for a potential clinical carrier for pharmaceutical carbon monoxide (Motterlini, Mann, et al., 2005).

CORM-3 has been used in both *in vivo* and *in vitro* modelling of several disorders: downregulation of inflammation, cardio-protective effects, anti-thrombotic effects, effects on hypertension, bactericidal effects, effects on intraocular pressure, nephrotoxicity, and pain. Evidence for beneficial effects of CORM-3 has been described by many authors (Bani-Hani et al., 2006; Desmard et al., 2009; Failli et al., 2012; Hervera et al., 2012; Lawendy et al., 2014; Mizuguchi et al., 2009; Sato et al., 2001; Sawle et al., 2005; Stagni et al., 2009; Tayem et al., 2006; Urquhart et al., 2007; Varadi et al., 2007).

The mechanism(s) by which CORM-3 acts are still relatively unknown. Some scholars state that CORM-3 (and CO-RMs in general), may act differently than their

inhalational counterpart (Gullotta et al., 2012); however, based on the evidence that CO possesses beneficial functions in controlling vessel tone, apoptosis, cell proliferation, platelet aggregation, inflammation, neurotransmission and ion channel activation, most researchers agree that CO (including CORM-3-generated CO) act through several different signalling pathways (Alberto & Motterlini, 2007; Motterlini et al., 2003). Specifically, the effect of CO appears to be mediated by cyclic GMP (cGMP), which is part of a soluble guanylate cyclase signalling cascade (Failli et al., 2012; Foresti et al., 2004; Fujita et al., 2001). Another pathway by which CORM-3 has been stated to act is the mitogen-activated protein kinase (MAPK) (Chlopicki et al., 2006; Mishra et al., 2006; Zhang et al., 2003). While both the soluble guanylate cyclase and MAPK pathways are suggested to mediate CO action, other possible targets may include calcium-activated potassium channels, cytochrome P450 or the mitochondrial respiratory chain (Chlopicki et al., 2006).

1.7.2 CO and Pain

Studies investigating CO and CO-RMs have been strongly suggesting their beneficial effects in multiple disorders; however, a question arises whether CO could have an effect on pain and/or sensory disorders. In 2012, Hervera and colleagues discovered that CORM-3-derived CO was able to reduce mechanical allodynia, as well as thermal hyperalgesia and allodynia in sciatic injury in mice. They suggested that CORM-3 might produce these effects through inhibition of nitric oxide (NO) pathways and synthesis, as well as through inhibition of microglial activation,

implicated in the initiation and maintenance of neuropathic pain (Hervera et al., 2012; Watkins et al., 2001).

Several other studies have investigated the effects of CO in pain (Bijjem et al., 2013; Hervera, Gou, et al., 2013; Hervera, Leanez, et al., 2013; Negrete et al., 2014); however, none have examined the effect of CO on functional behaviour.

1.8 FUNCTIONAL TESTING AND CRPS

The clinical presentation of CRPS is dominated by a combination of sensory and autonomic symptoms. However, mounting evidence indicates that many patients with CRPS suffer from some forms of motor dysfunction and movement disorders (Birklein et al., 2000; Schwartzman & Kerrigan, 1990; Veldman et al., 1993). With the growing consensus being that movement disorders should be included in the diagnostic criteria of CRPS, functional analysis is an area in need of exploration.

Functional analysis can be performed through several methods. In rodent models, however, there are two chief methods used to assess functional changes: (1) electrophysiological testing and (2) locomotor analysis. In electrophysiological testing, by using electrodes and isolated muscle, contraction amplitude and frequencies can be measured and analyzed in comparison with the afferent stimulation provided. These, however, do not reveal the true function of the limb.

Locomotion, as a consequence of neurological stimulation, muscle contraction and coordination between limbs, is a better measure of function in all models. Several methods of locomotor analysis exist.

Open field locomotion, was initially developed to examine behaviour of rodents (Hall, 1934). It utilizes an open field apparatus to acquire locomotor information through the capture of images, or through the use of infrared photocells. Recently, open field locomotion analysis has begun to examine the use of infrared illumination for capturing data, rather than visible light, which could interfere with an animal's behaviour (Aragao Rda et al., 2011). Although useful for examining some behavioural and locomotor changes in the rat, these open field locomotion methods are time-consuming and cannot measure some very important parameters of gait.

Other methods of locomotor analysis include the paper paw print method, the electric grid method, as well as Cheng's glass plate method (Afelt et al., 1983; Basso et al., 1995; H. Cheng et al., 1997; de Medinaceli et al., 1982). Each of these, however, has its own set of drawbacks and cannot provide a rapid and objective means to examining a large set of gait parameters.

1.8.1 CatWalk™ Automated Gait Analysis

The CatWalk™ automated gait analysis system is a computerized functional assessment tool for rats and mice, rapidly and objectively quantifying many parameters of gait. The system has been validated in numerous animal models, including spinal cord injuries (Koopmans et al., 2005; Miyagi et al., 2013), allodynia (Gabriel et al., 2007; Vrinten & Hamers, 2003), sciatic nerve injury (Bozkurt et al., 2008; Chiang et al., 2014) and arthritis (Angeby-Moller et al., 2008; Ferland et al., 2011).

This system uses a 1-metre long glass-floored walkway with a green light source attached, such that the light is completely internally reflected in the glass. Once a paw makes contact with the glass, light escapes and scatters; this can be detected by a digital video camera fixed below. After videos are recorded, and each paw print has been classified, the CatWalk™ software calculates static and dynamic parameters of gait (Appendix A).

To ensure precision in acquiring data, rodents must be thoroughly trained before the commencement of baseline tests. Ideally, rodents should be able to run from one end of the walkway to the other in an un-interrupted fashion. Some factors, however, may significantly alter gait parameters. Gabriel et al (2007) demonstrated that a 40% body mass increase affects many gait parameters. It is therefore crucial to the experiment to ensure rodents stay within a bracket of body mass.

In comparison with von Frey mechanical stimulation testing – the gold standard test for measuring allodynia – Vrinten and Hamers (2003) demonstrated that the CatWalk™ provided similar results, suggesting gait analysis may be a more rapid, reproducible and objective tool for measuring allodynia.

1.8.2 Functional Testing in CRPS Patients

In examining and publicizing CRPS, pain is usually the main focus, and not the disabilities that come with it. The pain is often so debilitating that joints become locked, bones become osteoporotic and muscles become spastic and atrophy. Disuse of the affected limb is a common feature of CRPS and is often the result of patients trying to avoid potential painful stimuli. This can lead to skin changes (colour

and temperature) as well as hyperalgesia (Butler, 2001). By avoiding the use of the limb, patients may operant condition themselves, reinforced by the avoidance of actual pain and even the reduced anxiety of anticipated pain. This would result in the prevention of de-sensitization as well as the elimination of any tactile or proprioceptive input that may help restore central signal processing of the limb (M. Stanton-Hicks et al., 1998; H. K. Watson & Carlson, 1987). Disuse may also result in a lack of natural blood pumping from limb musculature, perhaps resulting in an accumulation of catecholamines or tachykinin that could further exacerbate CRPS symptoms (Drummond et al., 2001; Weber et al., 2001).

As a result, Wilfrid Janig proposed several research directions towards understanding the functional deficits associated with CRPS (Wilson et al., 2005). He suggested the generation of new models that recapitulate the syndrome's unique features. The CPIP model mentioned earlier does indeed present several features observed in CRPS patients; however, no study has been found to examine the deficits accumulated through functional testing modalities like gait analysis.

1.9 AIM OF THIS THESIS

Despite the breadth of work dedicated to understanding CRPS-I, there are still several gaps in knowledge, and no cemented conclusion as to specific cause(s). With recent studies observing microvascular deficits in CRPS-I patients (Bellingham et al., 2014), the development of the CPIP model by Coderre et al has provided a suitable avenue for the investigation of both pathophysiology and treatment options. However, as suggested by Wilson et al (Wilson et al., 2005), future directions in the study of this syndrome must be geared towards validation of existing models of CRPS and generation of new models that recapitulate the unique features of this syndrome; changes in gait and motor abnormalities are certainly unique features that need to be assessed.

As a modulator of several signalling pathways that affect vascular properties, CORM-3 may be a good potential treatment for vascular deficit disorders. With the understanding that microvascular dysfunction may play an important role in the development and maintenance of CRPS-I, the investigation into the use of CORM-3 as treatment of CRPS-I is warranted.

Therefore, the aim of this thesis was twofold: to examine the use of the CatWalk™ automated gait analysis system in quantifying functional changes following initiation of CRPS-like symptoms in the CPIP rodent model, and to examine the potential therapeutic use of CORM-3 in CPIP.

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CHAPTER 2

FUNCTIONAL ASSESSMENT OF ALLODYNIA IN A RAT MODEL OF COMPLEX REGIONAL PAIN SYNDROME TYPE-1 USING AUTOMATED GAIT ANALYSIS

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1 USING AUTOMATED GAIT ANALYSIS

2.1 INTRODUCTION

Complex regional pain syndrome type-1 (CRPS-I) is a debilitating chronic pain condition, characterized by hyperalgesia and allodynia. Many studies suggest a major factor in the development and maintenance of CRPS-I may be inflammation and/or microcirculatory dysfunction (Albrecht et al., 2006; Bellingham et al., 2014; Coderre & Bennett, 2010), while others suggest it to be a disease of the central nervous system (Janig & Baron, 2002). Most clinicians however, prefer to believe the syndrome to be multifactorial and is not driven by a single mechanism of injury (Janig & Baron, 2006). Both of types I and II of CRPS develop after trauma and present similar symptoms; however, CRPS-I occurs without nerve injury and the severity of its symptoms are disproportionate to the severity of the trauma (Janig & Baron, 2006).

A rodent model of CRPS-I, called chronic post-ischemia pain (CPIP), developed by Coderre et al, induces microcirculatory dysfunction through ischemia-reperfusion injury (Coderre et al., 2004). Consequent allodynia (mechanical and cold), edema, hyperemia and hyperalgesia were observed to mimic those in patients suffering from CRPS (Coderre et al., 2004; Harden et al., 2013; Janig & Baron, 2002; van der Laan et al., 1998; van der Laan et al., 1999). However, no objective assessment of function was included in this model.

Automated gait analysis has been proposed to be a quality indicator of behaviour and mechanical allodynia in many rodent pain and/or injury models (CatWalk™). This computer-assisted technique allows for rapid quantification of both static paw and dynamic inter-limb gait parameters (Angeby-Moller et al., 2008; Bozkurt et al., 2008; Ferland et al., 2011; Gabriel et al., 2007; Gabriel et al., 2009; Hamers et al., 2001; Huehnchen et al., 2013; Koopmans et al., 2005; Miyagi et al., 2013; Sakuma et al., 2013; Vogelaar et al., 2004; Vrinten & Hamers, 2003). Depending upon the model used, automated gait analysis has varying results as a measure of allodynia. In some chronic pain models (Gabriel et al., 2009), the CatWalk™ system detected allodynia at different time points compared to standard assessments of mechanical allodynia (the von Frey test, (Chaplan et al., 1994)), and may also have understated measures of allodynia (Gabriel et al., 2009). Alternatively, a study using carrageenan injections to induce neuropathic pain provided significant correlation between CatWalk™ parameters and von Frey data (Gabriel et al., 2007). Analysis of gait changes using the CatWalk™ system as a measure of mechanical allodynia may be dependent on the model of chronic pain being used (Gabriel et al., 2009). At this time, to our knowledge, there have been no reports that examine the usefulness of the gait analysis to assess allodynia in the CPIP model of CRPS-I. In addition, in keeping with the more stringent diagnostic criteria for research purposes, functional tests should be required in many models.

Therefore, the purpose of this study was to investigate whether an automated gait analysis system, the Catwalk™, would provide a more thorough and complete assessment of allodynia in the CPIP model of CRPS-I.

2.2 METHODS

2.2.1 Animal Description and Care

The experimental protocol was approved by the Canadian Council on Animal Care at the University of Western Ontario. Animals were cared for in accordance to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP). All animals were housed in pairs with access to food and water *ad libitum*.

Male Wistar rats (185 – 260 g) were anesthetized with inhalational isoflurane (5% induction, 2% maintenance) in 1:1 oxygen/nitrogen gas mixture. Body temperature was measured using a rectal probe thermometer and maintained at 37 degrees Celsius using a heating lamp. CPIP was generated by ischemia-reperfusion (IR) injury of the right hind limb, as per Coderre et al (Coderre et al., 2004). Briefly, ischemia was induced by application of tourniquet (#4 silk, Johnson and Johnson) around the distal portion of the right hind limb, completely occluding the blood flow (i.e. no-flow ischemia), and maintained for 3 hours. Reperfusion was initiated by a tourniquet release. Tourniquet placement was at a standardized distance distal to the tibial tuberosity of the distal portion of the hind limb, differing slightly from tourniquet placement by Coderre and colleagues.

In a separate group of animals, the tourniquet was applied just proximal to the knee joint (proximal IR group), so as to induce ischemia over the entire lower aspect of the limb. The position of each tourniquet was standardized to minimize variation in injury.

2.2.2 Experimental Groups

Rats were randomized into three groups: CPIP (n=9), proximal IR (n=5) and sham (n=8). Compared with the CPIP group, the proximal IR group differed in the location of where the ischemia-reperfusion injury was induced. Sham animals underwent all procedures as the CPIP and proximal IR groups, but the tourniquet was not tightened.

2.2.3 Mechanical Allodynia Testing

To assess mechanical allodynia, hindpaw withdrawal thresholds to von Frey filament stimulation were measured. Animals were placed on a raised, mesh-floored platform and covered with a transparent plastic box. Animals were left for a minimum of 5-10 minutes to allow for familiarization to the new environment before measurements were taken. The plantar surfaces of both right and left hind paws were stimulated by von Frey filaments with calibrated bending forces (grams) (Stoelting Co., Wood Dale, IL) in order to determine a 50% withdrawal response threshold, similar to the method developed by Chaplan et al (Chaplan et al., 1994). Filaments were applied 10 times each, in ascending strength (1.0 g to 15.0 g). Withdrawal thresholds were determined by a positive response observed a minimum of 5 times at a specific filament strength. Positive responses were recorded as a lift or lick. Withdrawal thresholds were measured pre-injury, as well as 5, 7, 8, 9, 10 and 14 days post-injury.

2.2.4 Functional Analysis of Gait

The CatWalk™ (Noldus Information System, Wageningen) is an automated gait analysis system that delivers a large collection of gait parameters, including paw print area, duty cycle, weight load, swing phase and step regularity index. Static gait parameters, including paw prints and weight load, as well as dynamic parameters like duty cycle, are automatically measured and calculated by the system software. Definitions of each parameter are listed in Table 2.1.

The system consists of a glass plate platform illuminated with fluorescent tube; when contact is made with the glass, light is reflected downwards toward a high-definition camera, connected to a computer, where it is then interpreted by the accompanying software.

In order to assess gait, animals were trained to walk the length of the glass plate platform end-to-end, prior to the induction of ischemia. A minimum of five days of training was conducted for each animal until each animal was competent at walking the length of the platform without interruption. General, dynamic and paw-specific gait parameters were collected pre-injury, as well as 1, 5, 7, 8, 9, 10 and 14 days post-injury. At least 3 runs, that were uninterrupted and 1-3 seconds in duration, were recorded for each trial.

2.2.5 Statistical Analysis

Using GraphPad Prism® software, repeated measures two-way analysis of variance (ANOVA) was performed to assess differences in gait parameters, as well as paw withdrawal thresholds, compared to baseline and control values.

Table 2.1. CatWalk™ automated gait analysis tool parameters and definitions. Static and dynamic gait parameters were collected and assessed using the assisting software package. The parameters discussed in the results section were those showing the largest changes from baseline measurements.

Static Gait Parameter	Definition	Dynamic Gait Parameter	Definition
Paw Print Area (mm ²)	Total area of glass plate in contact with paw during stance phase	Duty Cycle (%)	Stance as a percentage of step cycle (=stance/step cycle)
Paw Print Width (mm)	Total width of paw print	Step Cycle (s)	Duration in seconds between two consecutive initial contacts of the same paw (stance + swing phases)
Paw Print Length (mm)	Total length of paw print	Stance Phase Duration (s)	Duration in seconds of contact of paw with glass plate
Weight Load (a.u/pixel)	Paw pressure is indicated by light intensity. Weight load is mean intensity (arbitrary units, a.u) per pixel	Swing Phase Duration (s)	Duration in seconds of no contact of paw with glass plate
		Step Regularity Index (%)	Number of normal step sequence patterns (NSSP) relative to the total number of paw placements (PP) (=NSSPx4)/PP x 100%
		Swing Speed (distance units/s)	Speed of the paw during swing phase

Bonferroni post-tests were also performed. To determine if CatWalk™ parameters correlated with the gold standard paw withdrawal thresholds, Pearson correlation coefficients were calculated. A $p < 0.05$ was considered significant.

2.3 RESULTS

2.3.1 CPIP And Proximal IR

For almost the entire duration of ischemia, the right hind paw (ipsilateral) was observed as cold and cyanotic in all animals. Directly after the removal of the tourniquet, evidence of severe edema and hyperemia were observed. Rats displayed spontaneous pain behaviours, such as shaking, licking and lifting of the injured limb. In the CPIP group, by 14 days post-reperfusion injury, most animals displayed a slightly atrophied and less robust right hind limb when compared to the contralateral limb. Animals in the proximal IR group showed less substantial changes in spontaneous pain behaviours over the duration of the experiment. Sham animals displayed no spontaneous pain behaviours or changes in the appearance of the right hind limb.

2.3.2 Mechanical Allodynia

Ischemia-reperfusion injury led to the development of mechanical allodynia in both the CPIP and proximal IR groups, as demonstrated by decreased paw withdrawal thresholds of the ipsilateral limbs (Figure 2.1). In the CPIP group, by 7 days post-reperfusion injury, paw withdrawal thresholds of the injured right hind limb decreased to 2.444 ± 0.689 g, from 13.889 ± 0.735 g at baseline ($p < 0.001$).

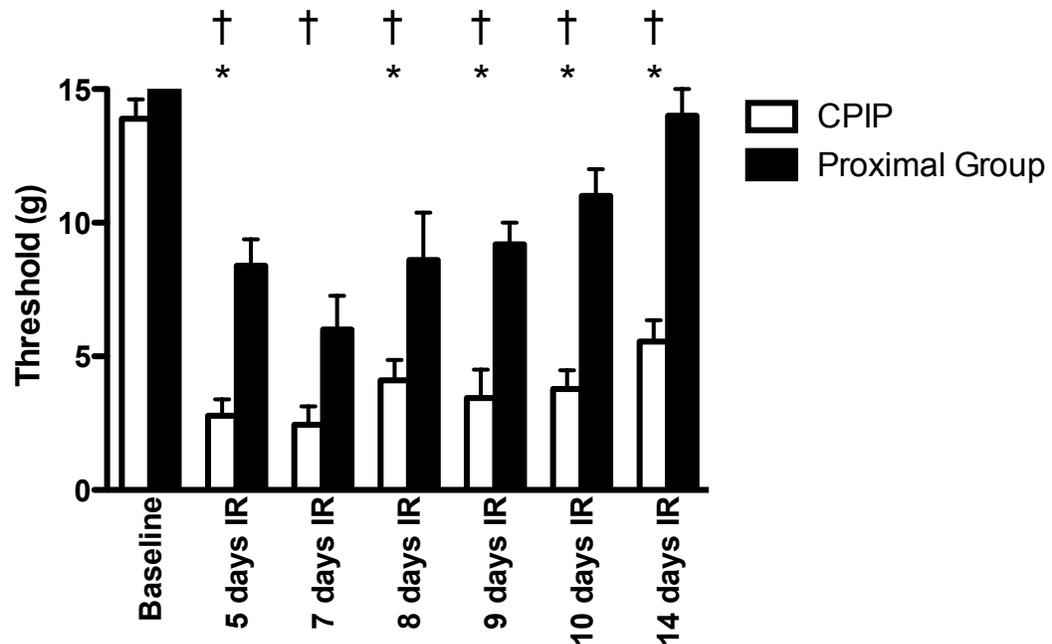


Figure 2.1. Paw withdrawal thresholds (PWT) of the ipsilateral/right hindpaw.

PWTs were assessed using mechanical stimulation via von Frey filaments and analyzed via two-way repeated measures ANOVA. PWTs of the ipsilateral/right hindpaw showed significant differences in both CPIP and proximal IR groups; however, only the CPIP group displayed sustained differences over the entire time course. (* $p < 0.05$ from proximal IR group, † $p < 0.05$ from baseline) Error bars shown represent the standard error of the mean (SEM).

The right hind limb displayed reduced paw withdrawal thresholds throughout the duration of examination. At 14 days post-reperfusion injury, paw withdrawal thresholds were still significantly lower than baseline values (5.556 ± 0.801 g, compared to 13.889 ± 0.735 g at baseline ($p < 0.05$)). At 7 days post-reperfusion injury the contralateral limb also displayed a mild, though not significant, reduction in the paw withdrawal thresholds (12.444 ± 1.334 g in CPIP versus 13.889 ± 0.735 g at baseline, n.s.) (Figure 2.2).

In the proximal IR group, ipsilateral paw withdrawal thresholds decreased from 15.00 ± 0.00 g at baseline to 6.00 ± 1.265 g after ischemia-reperfusion injury ($p < 0.05$). Paw withdrawal thresholds of the contralateral limb did not significantly change from baseline, similar to the CPIP group. Withdrawal thresholds of the injured (ipsilateral) hind limb were restored, without intervention, to baseline levels by 14 days post-injury (Figure 2.2).

Sham animals displayed no significant changes in paw withdrawal thresholds for the duration of the experiment (data not shown).

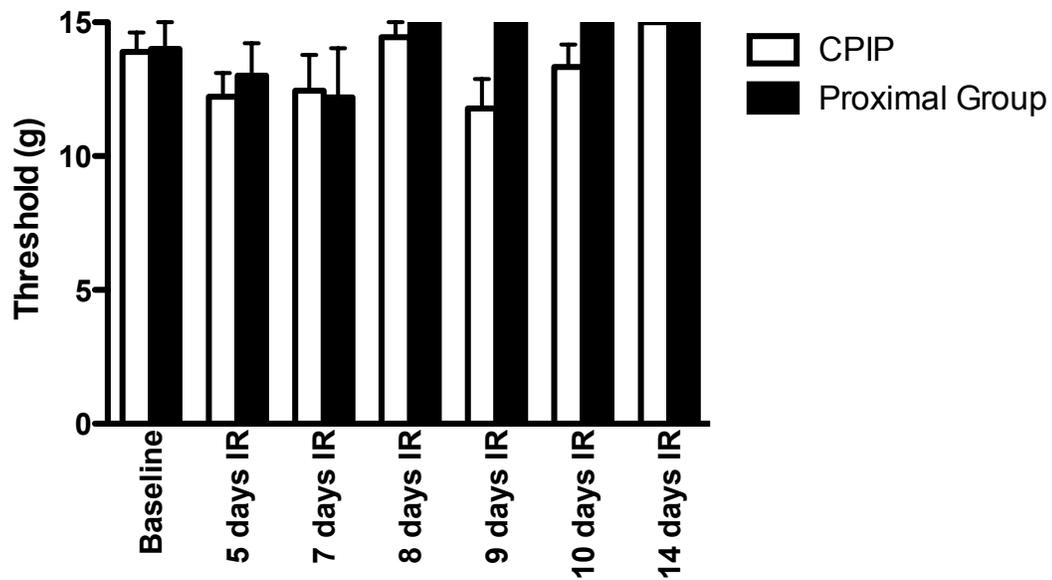


Figure 2.2. Paw withdrawal thresholds (PWT) of the contralateral/left hindpaw. PWTs were assessed using mechanical stimulation via von Frey filaments and analyzed via two-way repeated measures ANOVA. No significant differences in paw withdrawal thresholds were observed in the contralateral/left hindpaw. Error bars shown represent the standard error of the mean (SEM).

2.3.3 Functional Analysis of Gait

Automated gait analysis detected several alterations of gait parameters post-reperfusion. Both static (paw print area, paw print width, paw print length and weight load) and dynamic (duty cycle, stance phase and swing cycle) parameters markedly changed by 24 hours post-reperfusion injury (Figures 2.3 to 2.7). Paw print area decreased from the baseline of $56.18 \pm 8.38 \text{ mm}^2$ in CPIP and $76.27 \pm 18.65 \text{ mm}^2$ in proximal IR group to $1.113 \pm 0.77 \text{ mm}^2$ and $4.834 \pm 2.97 \text{ mm}^2$, respectively, after 1 day reperfusion injury ($p < 0.05$) (Figure 2.3). By 5 days post-injury, print area in the proximal IR group was restored back to baseline values. At 14 days post-injury, print area of the CPIP group was still significantly decreased, at $25.39 \pm 5.305 \text{ mm}^2$ ($p < 0.05$).

Print length and width demonstrated very similar significant trends (Figures 2.4 and 2.5). Using the Pearson correlation coefficient, all paw print parameters in the CPIP group displayed correlation with paw withdrawal threshold data ($p < 0.05$). Correlation data is shown in Table 2.2.

Duty cycle in the CPIP group decreased after injury from the baseline of $53.2 \pm 2.1\%$ to $6.8 \pm 4.5\%$ at 1 day post-injury ($p < 0.05$) (Figure 2.6). The change in duty cycle was sustained throughout the course of the experiment ($32.6 \pm 4.2\%$ at 14 days post-injury, $p < 0.05$). Correlation with paw withdrawal thresholds, using the Pearson correlation coefficient, was confirmed in the CPIP group ($p < 0.05$).

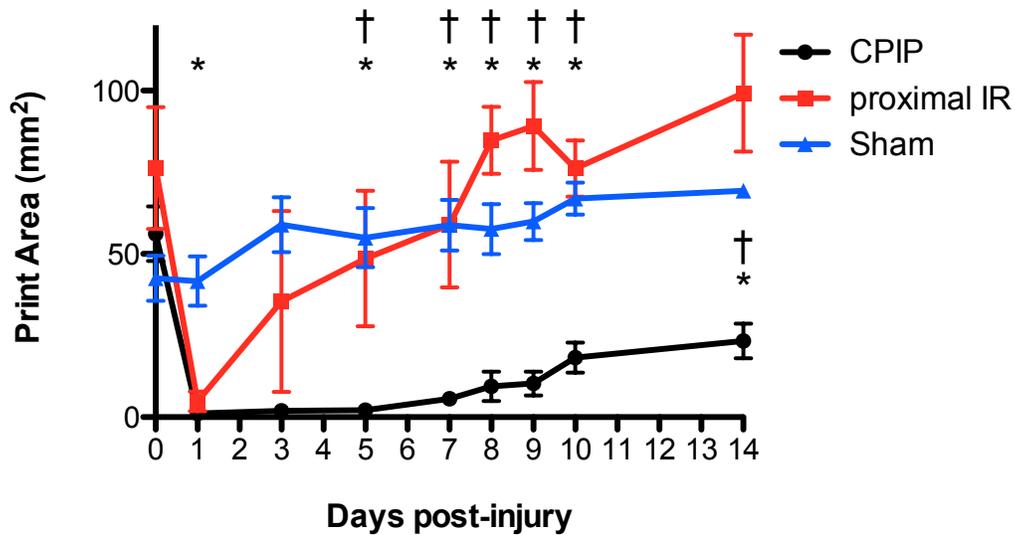


Figure 2.3. Paw print area of the right hindpaw (mm²) in CPIP, proximal IR and sham groups. Print area was calculated using the CatWalk system software and analyzed via two-way repeated measures ANOVA. CPIP animals show significant difference from sham group after ischemia-reperfusion injury for the duration of the experiment. Print area of proximal IR animals restore to baseline quickly after injury. († $p < 0.05$ from proximal IR group, * $p < 0.05$ from baseline) Error bars shown represent the standard error of the mean (SEM).

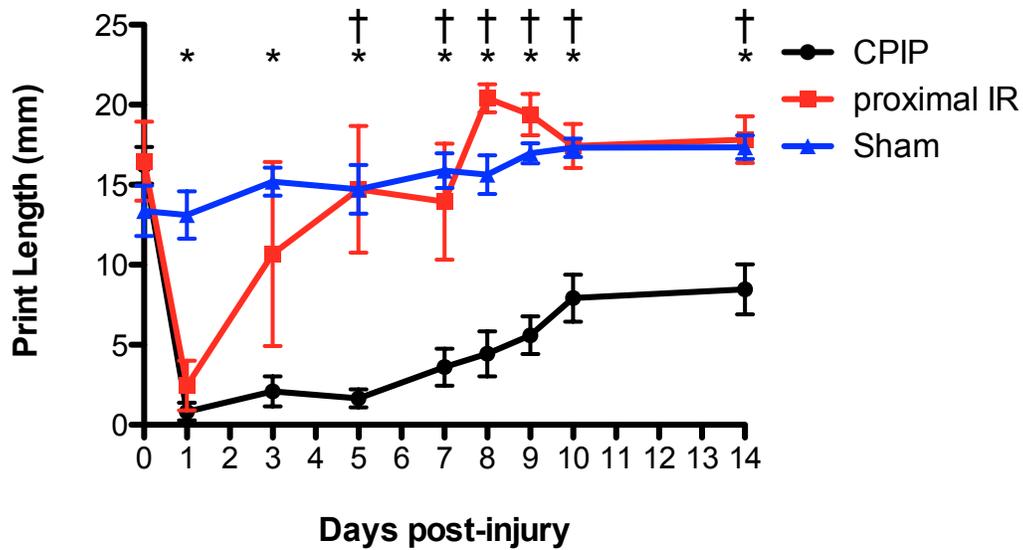


Figure 2.4. Paw print length of the right hindpaw (mm). After ischemia-reperfusion injury, CPIP animals demonstrated substantially lower print length than sham and proximal IR groups through the duration of testing. († $p < 0.05$ from proximal IR group, * $p < 0.05$ from baseline) Print area was calculated using the CatWalk system software and analyzed via two-way repeated measures ANOVA. Error bars shown represent the standard error of the mean (SEM).

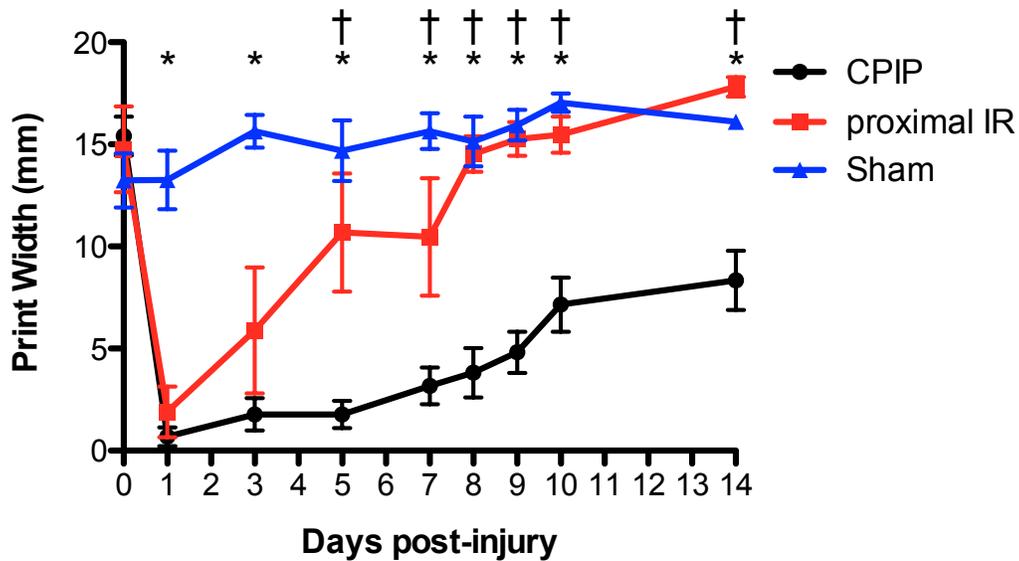


Figure 2.5. Paw print width of the right hindpaw (mm). Print width was markedly lower in CPIP animals after ischemia-reperfusion injury compared to proximal IR and sham animals. († $p < 0.05$ from proximal IR group, * $p < 0.05$ from baseline) Print width was calculated using the CatWalk system software and analyzed via two-way repeated measures ANOVA. Error bars shown represent the standard error of the mean (SEM).

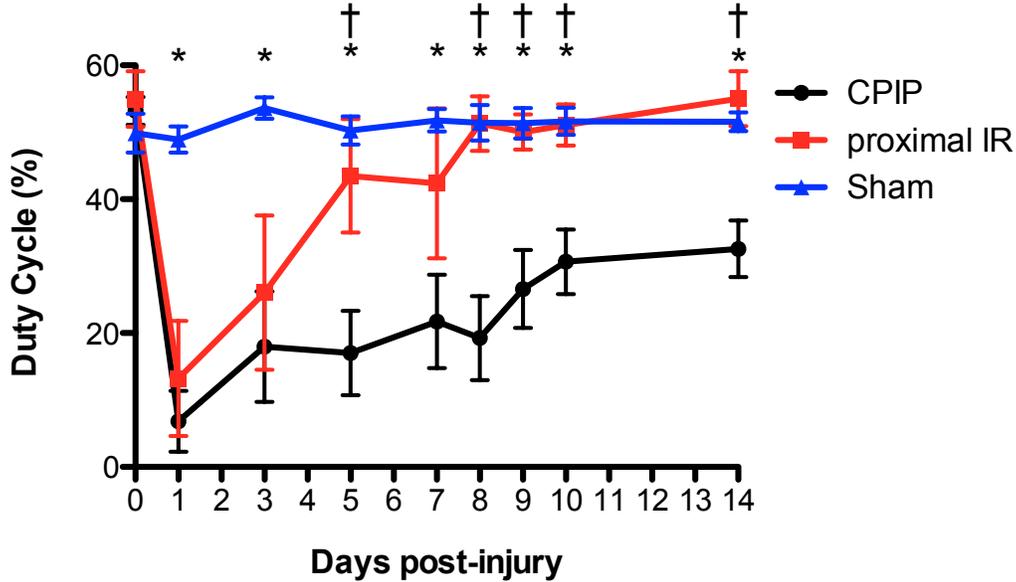


Figure 2.6. Changes in Duty Cycle (stance phase/swing phase + stance phase). Duty cycle was calculated using the CatWalk system software and analyzed via two-way repeated measures ANOVA. After injury, duty cycle in CPIP animals was significantly different from both proximal IR and sham animals, for the duration of the experiment. († $p < 0.05$ from proximal IR group, * $p < 0.05$ from baseline) Error bars shown represent the standard error of the mean (SEM).

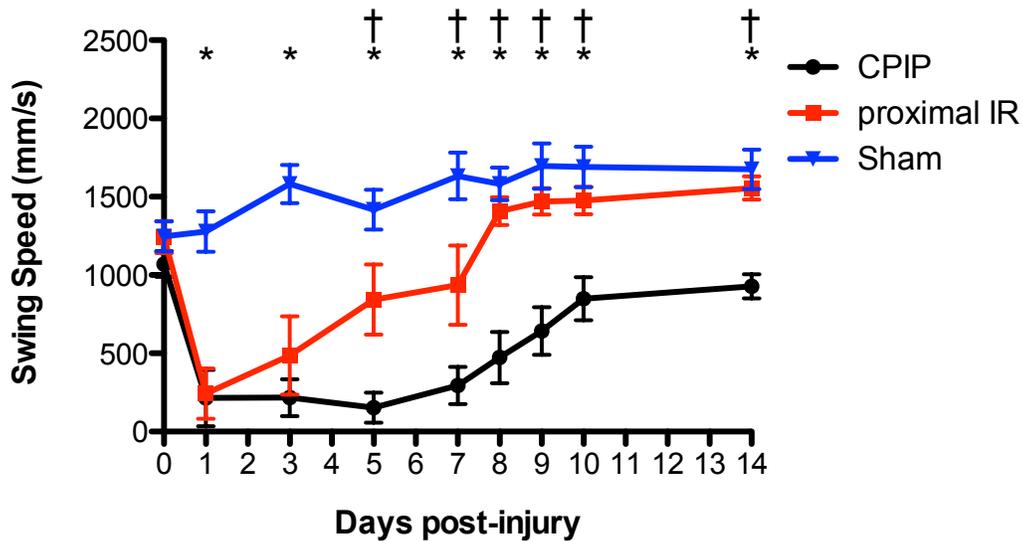


Figure 2.7. Swing speed of the right hindpaw. Swing speed was calculated using the CatWalk system software and analyzed via two-way repeated measures ANOVA. CPIP animals demonstrated a smaller swing speed than proximal IR and sham groups throughout experiment; however, swing speed at 14 days post-injury was not different from baseline speed in CPIP animals. († $p < 0.05$ from proximal IR group, * $p < 0.05$ from baseline) Error bars shown represent the standard error of the mean (SEM).

Table 2.2. Correlation coefficients comparing gait parameters and mechanical stimulation. Using the Pearson Correlation statistical test in our GraphPad Prism software, we compared gait parameters with the gold standard mechanical stimulation measurements for mechanic allodynia.

Gait Parameters	p-value	Correlation Coefficient
Duty Cycle	0.0019	0.8760
Paw Print Area	0.0001	0.9609
Paw Print Length	0.0015	0.8817
Paw Print Width	0.0011	0.9002
Step Regularity Index	0.2320	0.2700
Swing Speed	0.0654	0.5253

Step regularity index decreased from $89.8 \pm 4.3\%$ at baseline to $11.7 \pm 7.8\%$ ($p < 0.05$) at 1 day after injury, however it was restored to baseline without intervention by 9 days post-injury. At 14 days post-injury, regularity index returned to $89.3 \pm 4.5\%$. No correlation was observed using the Pearson correlation coefficient.

Swing speed decreased from the pre-injury value of 1071.0 ± 82.7 mm/s to 215.1 ± 180.3 mm/s at 1 day after injury, ($p < 0.05$), and increased back to 928.6 mm/s ± 77.39 by 14 days post-injury in the CPIP group. In the proximal IR group, the swing speed decreased from the pre-injury value of 1242.6 ± 101.3 mm/s to 242.5 ± 159.6 mm/s at 1 day after injury and returned to 1556.2 ± 73.8 mm/s at 14 days post-injury. Although significantly different from proximal IR and sham groups at 14 days post-injury, swing speed in CPIP animals was not markedly different from its baseline value. Swing speed showed no correlation with von Frey data, as well. (Figure 2.7)

Sham animals showed no significant changes in gait parameters throughout the entire length of the experiment.

2.4 DISCUSSION

In the present study, we demonstrated the use of automated gait analysis (CatWalk™) to rapidly and objectively quantify allodynia in the CPIP model of CRPS-I. Four gait parameters proved to display results correlating with the gold standard von Frey mechanical stimulation tests, which were paw length, width, and area as well as duty cycle. Through successful validation with gait analysis, we have

provided a more objective, practical and perhaps more clinically relevant model of CRPS-I.

Sensitivity was substantially heightened and spontaneous pain behaviours were observed after injury, resulting in symptoms similar to those presented in patients with CRPS-I (Coderre et al., 2004). After comparing the distal tourniquet placement to one placed proximal to the knee joint, paw withdrawal thresholds confirmed that tourniquet placement in the CPIP animals produced chronic mechanical allodynia symptoms, whereas proximal tourniquet placement resulted in paw withdrawal thresholds returning to baseline levels by 14 days post-injury. Although sensitivity did indeed increase with IR injury in the proximal IR group, these symptoms were not long lasting. This suggests the CPIP model is more representative of chronic pain symptoms of CRPS-I compared to the proximal IR group. The proximal IR group was added in order to both examine chronic pain symptoms using a different tourniquet placement, as well as to assess muscular tissue injury in the rodent after ischemia-reperfusion injury; however, our results indicate the proximal IR group does not display a chronic pain response.

Mechanical stimulation using von Frey filaments demonstrated a persistent increase in sensitivity and symptoms of allodynia in the CPIP group. Correlating with results from the development of the CPIP model (Coderre et al., 2004), paw withdrawal threshold was significantly lower than that of the pre-injury. Although the O-ring band used to initiate ischemia in the original model was not used, our use of a ligature (#4 silk, Johnson & Johnson) showed similar results, suggesting that the same tension was applied.

Confirmation of the CPIP model through mechanical stimulation testing (Coderre et al., 2004) allowed for comparison with and examination of gait analysis, through the automated CatWalk™ method. After sensitivity and gait analysis, some correlation in mechanical allodynia measurements was observed. The decrease and sustainment of paw withdrawal thresholds observed after ischemia-reperfusion injury confirm the CPIP model produces mechanical allodynia; changes in gait parameters, correlating well with changes in paw withdrawal thresholds, also suggest that animals experienced allodynia. Meanwhile, the proximal IR animals did not display persistent or correlating gait parameter changes, which again suggests that the CPIP model best displays CRPS-I symptoms. This is important as it demonstrates that the automated gait system is indeed assessing changes as a result of allodynia rather than muscle injury, which would likely be greater in those animals undergoing IR injury with a proximally placed tourniquet. In the proximal IR group though, standard error is greater in both mechanical stimulation and gait analyses, perhaps suggesting more variable occlusion of blood flow and less standardization of injury.

Gait parameters showed significant changes through the 14-day testing period. At one day post-injury, all gait parameters were significantly different than those at baseline (i.e. pre-injury); however, only duty cycle and paw print parameters (area, length and width) maintained the significant changes throughout the duration of the experiment. The time of contact of one paw in one single stepcycle, defined as the stance phase duration, can be directly related to pain perception in the rat; therefore, duty cycle, which is the ratio of stance phase duration to stepcycle, has been suggested to be the gait parameter most indicative of mechanical allodynia

(Gabriel et al., 2009). Static paw print parameters such as area, width, and length, also provide indication of potential pain symptoms, as it would be reasonable to expect a reduction in paw use when said paw is experiencing pain symptoms. Weight load did not show chronic changes in contrast to other gait parameters. This suggests that even with similar loads placed on the limb, the limb use is functionally different in the CPIP animals.

Slight differences in gait parameter values between the control and sham group were observed, especially in static paw print parameters. Sham animals were slightly lesser in mass (and as such, smaller in size) upon commencement of testing which may explain these findings. No significant differences were observed in dynamic gait parameters. This was expected, even with the slight differences in mass between the groups, as it has been shown that a 40% variation in mass is required for significant difference in paw print intensity to be observed (Gabriel et al., 2009).

Although gait parameter changes and von Frey tests differed in their assessment of mechanical allodynia, these may be explained through the mechanisms underlying the responses observed. Reaction to von Frey filament stimulation involves minimal central processing (Gabriel et al., 2009), whereas gait is a centrally controlled daily activity that can be affected by several factors, including pain (Jordan et al., 2008; MacKay-Lyons, 2002; Pearson, 2000). Since human chronic pain conditions are also centrally processed, gait is relevant to human experiences and therefore its analysis may be more clinically applicable. Pain and allodynia are obvious factors that impact gait, but behaviours changes and

neurological and/or muscular tissue damage definitely have a role as well. In relevant literature, reversible nerve damage is usually not evident until at least 3-4 hours of ischemia, and in the CPIP model publication by Coderre and colleagues, no nerve injury was observed through light microscopy (Coderre et al., 2004) Although muscle tissue damage may impact our gait measurements, our correlation data suggests that the gait changes were significantly correlated with changes in mechanical allodynia, according to our gold standard mechanical stimulation tests. These markedly correlated changes are shown in Figure 2.2.

Validation of the CPIP model provides additional support for the notion of microcirculatory dysfunction and inflammation may be part of the pathophysiology driving CRPS-I signs and symptoms. A recent clinical study by Bellingham et al observed a decrease in deep tissue oxygen saturation in CRPS-I patients using near infrared spectroscopy, supporting the theory that deep tissue hypoxia is part of the pathogenesis behind CRPS-I, providing more validity to an inflammation/microvasculature dysfunction-based CRPS-I model (Bellingham et al., 2014).

As stated by Gabriel et al, the use of the CatWalk™ method for assessment of mechanical allodynia needs to be carefully restricted to specific injury models (Gabriel et al., 2009). This study, through comparison of von Frey test and CatWalk™ analysis, suggests that functional examination of gait provides a powerful additional tool for the study of mechanical allodynia in the CPIP model of CRPS-I. Supplementation with the CatWalk™ system provides an additional significant measure of function and can provide a more robust account of the pain experience

in the rodent. Ultimately, comparing therapeutic interventions in the management of CRPS-I is currently very difficult; the addition of objective, practical and functional testing adds substantially to our ability to develop more effective therapies.

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CHAPTER 3

CARBON MONOXIDE REDUCES THE LEVEL OF ALLODYNIA IN A RODENT MODEL OF COMPLEX REGIONAL PAIN SYNDROME TYPE-1

CHAPTER 3: Carbon Monoxide Reduces the Level of Allodynia in a Rodent Model of Complex Regional Pain Syndrome Type-1

3.1 INTRODUCTION

Complex regional pain syndrome type-1 (CRPS-I) is a clinical condition where disproportional symptoms of pain and mechanical allodynia are experienced after distal extremity trauma (Bellingham et al., 2014; Harden, 2010; Sandroni et al., 1998; P. Wilson et al., 2005; P. R. Wilson, 2010). Sprains, crush injuries, surgery and fractures have all been known to lead to a CRPS-I diagnosis (Bean et al., 2014; Harden, 2010; Sandroni et al., 1998).

Recently, studies by Coderre and colleagues have led to the development of a rodent model of CRPS-I, through initiation of the “slow-flow/no-reflow” phenomenon by ischemia-reperfusion (IR) injury, triggering chronic post-ischemia pain and allodynia, similar to those observed in patients suffering from CRPS-I. Studies suggest that the pain perceived by some CRPS-I patients may indeed be the result of I-R injury and the subsequent inflammation developed as a result of microcirculatory changes from the IR injury (Bellingham et al., 2014; Coderre & Bennett, 2010; Coderre et al., 2004). Another recent study has demonstrated impaired tissue oxygen saturation in the hands of patients with CRPS-I, demonstrating that deep tissue hypoxia and microvascular dysfunction are involved in the pathogenesis (or at least maintenance) of CRPS-I (Bellingham et al., 2014).

Lately, attention has been directed to the potential use of carbon monoxide (CO) in a clinical setting, despite the detrimental and deadly effects commonly associated with it. As a byproduct of the heme oxygenase (HO) system, CO is

endogenously produced at low doses and, along with HO-1, has been correlated to either the degree of injury/disease or increased survival in a variety of syndromes (Foresti et al., 2008).

Although the mechanisms of CO action are poorly understood, exogenous application through inhalation has been shown to elicit protection against multiple organ injury (MOI), inflammation, apoptosis, cell proliferation, vasoconstriction and hypertension (Chapman et al., 2001; Foresti et al., 2008; Fujita et al., 2001; Mishra et al., 2006; Otterbein et al., 1999; Song et al., 2003). However, CO application through inhalation is a clinically challenging therapy as carboxyhemoglobin (HbCO) levels increase quickly, possibly leading to hypoxia and further co-mediated injury (Clayton et al., 2001). Recent developments have led to the production of CO-releasing molecule-3 (CORM-3), which is a water soluble, quick-releasing transition metal carbonyl capable of efficiently releasing CO in the bloodstream (Foresti et al., 2008; Foresti et al., 2004; Yabluchanskiy et al., 2012). Compared with CO inhalation, CORM-3 application does not significantly alter HbCO levels (Foresti et al., 2008; Foresti et al., 2004; Yabluchanskiy et al., 2012).

The anti-inflammatory and vasodilatory actions of CO are perhaps the most intriguing and potentially useful. Previous studies have shown that the inflammatory reaction after IR injury is reduced after application of CORM-3 (Lawendy et al., 2014). Inflammation and oxidative stress resulting from ischemic conditions appear to be the underlying factors for several chronic pathologies. Recent work suggests that microvascular dysfunction and inflammation may be factors contributing to the development of one such pathology, CRPS-I (Bruehl, 2010).

Previously, in order to investigate symptoms of CRPS-I, mechanical allodynia has been assessed using mechanical stimulation via the von Frey method. This, however, does not test the function of the affected limb. The 'CatWalkTM' automated gait analysis system rapidly and objectively quantifies several parameters of gait and has been shown to be a quality supplement to the von Frey method in some injury models (Angeby-Moller et al., 2008; Bozkurt et al., 2008; Chiang et al., 2014; Ferland et al., 2011; Gabriel et al., 2007; Sakuma et al., 2013; Vrinten & Hamers, 2003).

Through the use of a previously validated model of CRPS-I, the purpose of this study was to examine the effects of CORM-3 on the symptoms of CRPS-I, specifically on symptoms of allodynia.

3.2 METHODS

3.2.1 Animal Description and Care

The experimental protocol was approved by the Canadian Council on Animal Care at the University of Western Ontario. Animals were cared for in accordance to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP). All animals were housed in pairs with access to food and water *ad libitum*.

Male Wistar rats (185-260g) were anesthetized with inhalational isoflurane (5% induction, 2% maintenance) in a 1:1 oxygen/nitrogen gas mixture.

Chronic post-ischemia pain (CPIP) was generated by ischemia-reperfusion (IR) injury of the right hindpaw, as per Coderre et al (Coderre et al., 2004). A tourniquet (#4 silk, Johnson and Johnson) was applied to the distal portion of the

right hind limb in each animal, completely occluding the blood flow. Tourniquet application was maintained for 3 hours. The position of each tourniquet was standardized so as to minimize variation in injury, as stated above in Chapter 2. Reperfusion was then initiated by release of the tourniquet.

3.2.2 CORM-3

CORM-3 has been synthesized by us, in accordance with previously published methods (Lawendy et al., 2014; Motterlini & Otterbein, 2010). CORM-3, or its inactive counterpart, iCORM-3, were administered to animals at a dose of 10mg/kg, given IP.

3.2.3 Experimental Groups

Twenty male Wistar rats were randomized into two groups: control group, treated with iCORM-3 (n=9), and an experimental group, treated with CORM-3 (n=11). Both CORM-3 and iCORM-3 groups were administered their respective injections at 7 days post-injury via intra-peritoneal injection.

3.2.4 Mechanical Allodynia Testing

To assess allodynia, hind paw withdrawal thresholds (PWT) to von Frey filament stimulation were measured. Animals were placed on a raised, mesh-floored platform and covered with a transparent plastic box. To allow for familiarization to the new surroundings, animals were left for a minimum of 10 minutes before measurements were taken. The plantar surfaces of both right and left hind paws were stimulated with von Frey filaments (Stoelting Co., Wood Dale, IL) in order to

determine a 50% withdrawal response threshold in accordance with similar techniques found in literature (Bennett, 2010). Filaments were applied 10 times each in ascending strength (1.0 g to 15.0 g). Withdrawal thresholds were determined by a positive response, observed a minimum of 5 times, at a specific filament strength. Positive responses were recorded as a lift or lick. Withdrawal thresholds were measured pre-injury, as well as 5, 7 (pre- and post-injection), 8, 9, 10, and 14 days post-reperfusion.

3.2.5 Functional Analysis of Gait

The CatWalk™ (Noldus Information System, Wageningen) is an automated gait analysis system that delivers a large collection of gait parameters, including paw print area, duty cycle, weight load, swing phase and step regularity index. Static gait parameters, including paw prints and weight load, as well as dynamic parameters like duty cycle, are automatically measured and calculated by the system software.

The system consists of a glass platform illuminated by a fluorescent tube; when contact is made with the glass, light is reflected downwards toward a high-definition camera, connected to a computer, where it is then interpreted by the accompanying software.

In order to assess gait, animals were trained to walk the length of the glass plate platform end-to-end, prior to the induction of ischemia. A minimum of five days of training was conducted until each animal was competent at walking the length of the platform without interruption. During training, animals were motivated to traverse the walkway by the use of sweet treats. General, dynamic and paw-specific gait parameters were collected pre-injury, as well as 1, 5, 7 (pre- and post-injection), 8,

9, 10 and 14 days post-injury. Each trial had a minimum of 3 runs, and only runs 1-3 seconds in duration were accepted.

3.2.6 Intravital Video Microscopy (IVVM)

In some iCORM-3 (n=5) and CORM-3 (n=5) animals, microscopic evaluation of skeletal muscle microcirculation was undertaken using intravital video microscopy (IVVM).

The extensor digitorum longus (EDL) muscle was isolated and prepared, as described previously (Lawendy et al., 2011; Potter et al., 1993). Briefly, the tibialis anterior and lateral gastrocnemius muscles were divided through blunt dissection to expose the EDL. A suture ligature was applied around the distal tendon of the EDL and the tendon was then cut from its bony attachment in order to reflect the muscle onto the microscope stage, with its arterial and venous blood flow intact. Once prepared, animals were carefully placed on the stage of an inverted microscope (Nikon Diaphot 300) and the EDL reflected onto a slide with saline bath containing 5µg/ml each of the fluorescent vital dyes bisbenzimidazole (BB; Ex. 343nm, Em. 483nm) and ethidium bromide (EB; Ex. 482nm, Em. 616nm). As BB stains the nuclei of all cells while EB stains the nuclei of only those cells with damaged cell membrane, EB/BB ratio provided an index of tissue injury. A cover slip was then placed atop the EDL. The temperature of the exposed muscle and the animal itself was maintained at 37 degrees Celsius by the use of a heat lamp. Care was taken to minimize time between EDL exposure and the first microscope recording.

The inverted microscope was connected to a charge-coupled device camera (Dage-MTI VE1000), a time-date generator (WJ-810, Panasonic), as well as a

computer. To provide appropriate white light illumination, flexible fibre-optic guides were positioned above the EDL.

Microvascular perfusion and leukocytes within the post-capillary venules were recorded by transillumination with 20x and 40x objectives, respectively, in five randomly chosen fields of view. Fluorescence microscopy was used to visualize the BB and EB from the same fields of view that had been selected for the measurement of capillary perfusion. At the conclusion of the experiment, rats were euthanized by an overdose of isoflurane anesthetic agent.

3.2.7 Offline Video Analysis

3.2.7.1 Perfusion Analysis

Capillary perfusion was measured in each 60-second clip at 20x objective (final magnification of 700x), as per previously validated methodology (Lawendy et al., 2014). Perfusion was quantified by counting the number of continuously-perfused (CPC), non-perfused (NPC) and intermittently perfused (IPC) capillaries crossing 3 equidistant parallel lines drawn on the computer monitor, perpendicular to the capillary axis. Capillaries with continuous flow were deemed CPC, those with flow interrupted at any point during the 60-second clip were deemed IPC, and those with no flow throughout the 60-second clip were deemed NPC. These values were expressed as the percent of total capillaries per field of view.

3.2.7.2 Analysis of Leukocytes

Leukocyte activation, i.e. rolling and adherence, was measured in post-capillary venules at 40x objective (final magnification of 1400x). Thirty second clips

from each 45 second recording were randomly chosen; the total number of rolling and adherent leukocytes were measured during this time and expressed per 1000 μm^2 . Venular area was measured using ImageJ (NIH, Bethesda, MD). Adherent leukocytes were defined as those cells remaining stationary over the entire duration of the 30-second clip.

3.2.7.3 Injury Analysis

BB and EB are nuclear dyes; BB labels nuclei of all cells, as it is membrane-permeable, while EB is membrane impermeable and therefore only stains cells with injured (permeable) membranes. EB labeling cannot distinguish between cell injury and cell death, as a wide range of injury may cause increased membrane permeability. Tissue injury in the randomly chosen fields of view was assessed by counting the number of EB- and BB-labelled nuclei, and expressed as EB/BB ratio.

3.2.8 Statistical Analysis

Repeated measures two-way analysis of variance (ANOVA), using GraphPad Prism® software, was performed to assess differences in gait parameters, paw withdrawal thresholds, tissue perfusion, muscle injury, leukocyte rolling and leukocyte adherence between iCORM-3 and CORM-3-treated animals. Statistical difference was defined at $p < 0.05$; Bonferroni post-tests were also performed.

3.3 RESULTS

3.3.1 CRPS-I Model

For the duration of ischemia, the right hind paw (ipsilateral) was observed as cold and cyanotic in all animals. Directly after the removal of the tourniquet, evidence of severe edema and hyperemia were observed. Upon recovery, rats displayed spontaneous pain behaviours, such as shaking, licking, and lifting of the injured limb. By 14 days post-reperfusion injury, most animals displayed an atrophied and less robust right hind limb when compared to the contralateral limb. Two animals did not develop CRPS-I like symptoms or display pain behaviours, and hence were excluded from further analysis.

3.3.2 Mechanical Allodynia

Ischemia-reperfusion injury led to increased tactile sensitivity, as demonstrated by decreased paw withdrawal thresholds of both the ipsilateral and contralateral limbs. By 7 days post-reperfusion injury, paw withdrawal thresholds of the injured right hind limb decreased from 14.25 ± 0.41 g at baseline to 3.00 ± 0.59 g ($p < 0.001$) (Figure 3.1). At 7 days post-reperfusion injury, the left hind limb displayed paw withdrawal thresholds at an average of 11.25 ± 0.85 g, compared to 14.25 ± 0.41 g at baseline ($p < 0.05$). (Figure 3.1)

In the right hind limb, paw withdrawal thresholds were significantly less than baseline levels in the iCORM-3 group throughout the duration of the experiment. However, in the experimental group, upon administration of CORM-3 at 7 days post-reperfusion injury, accelerated restoration of paw withdrawal thresholds was observed.

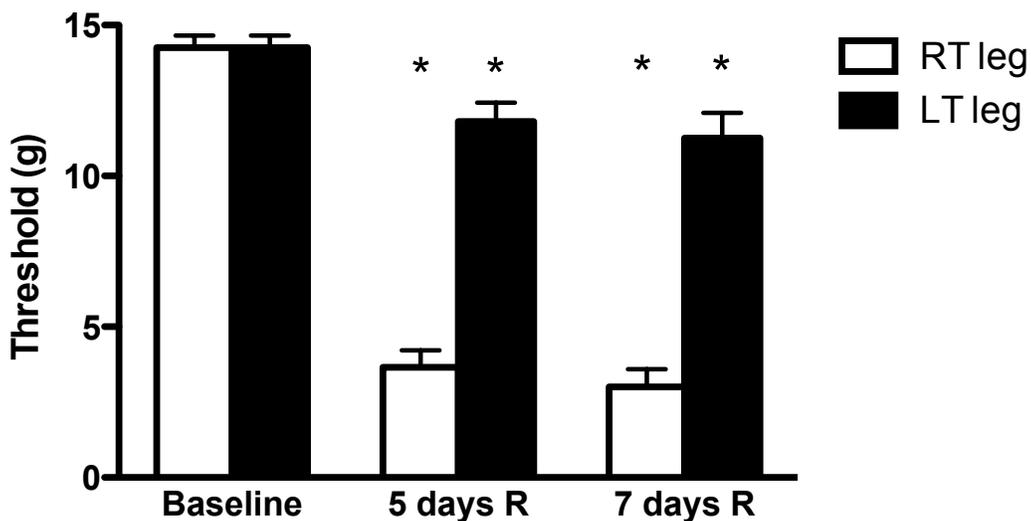


Figure 3.1. The effect of ischemia-Reperfusion on paw withdrawal threshold (PWT) of the ipsilateral/right and contralateral/left hindpaws. PWTs were assessed using mechanical stimulation via von Frey filaments. PWTs of both the ipsilateral/right and contralateral/left hindpaw showed significant differences after reperfusion injury. (* $p < 0.05$ from iCORM-3, † $p < 0.05$ from baseline). Error bars shown represent the standard error of the mean (SEM).

Compared to application of iCORM-3, application of CORM-3 significantly increased paw withdrawal thresholds ($6.636 \pm 0.96\text{g}$ versus $2.222\text{g} \pm 0.49\text{g}$, $p < 0.05$) at 1 hour post-injection. By 7 days post-injection (14 days post-reperfusion injury), right hind limb paw withdrawal thresholds of CORM-3-administered animals were restored to baseline levels. (Figure 3.2).

In the left hind limb, restoration of paw withdrawal thresholds back to baseline values was observed quickly without intervention. CORM-3 had no effect on the contralateral limb.

3.3.3 Functional Analysis of Gait

Gait analysis using the CatWalk™ method detected several alterations of gait parameters post-reperfusion injury. Both static and dynamic parameters of gait dramatically changed by 24 hours post-reperfusion injury, including paw print parameters, weight load, duty cycle, stance phase and swing cycle, among several others. However, by 7 days post-injury, most gait parameters were restored to baseline without intervention, with the exception of paw print parameters (area, length and width) and duty cycle. Application of CORM-3 did not affect duty cycle, resulting in no significant difference from baseline. Similarly, paw print length and width showed no differences between CORM-3 and iCORM-3 injections. Paw print area demonstrated a trend of an increased rate of restoration of print area after application of CORM-3; however, no significance was achieved (Figure 3.3).

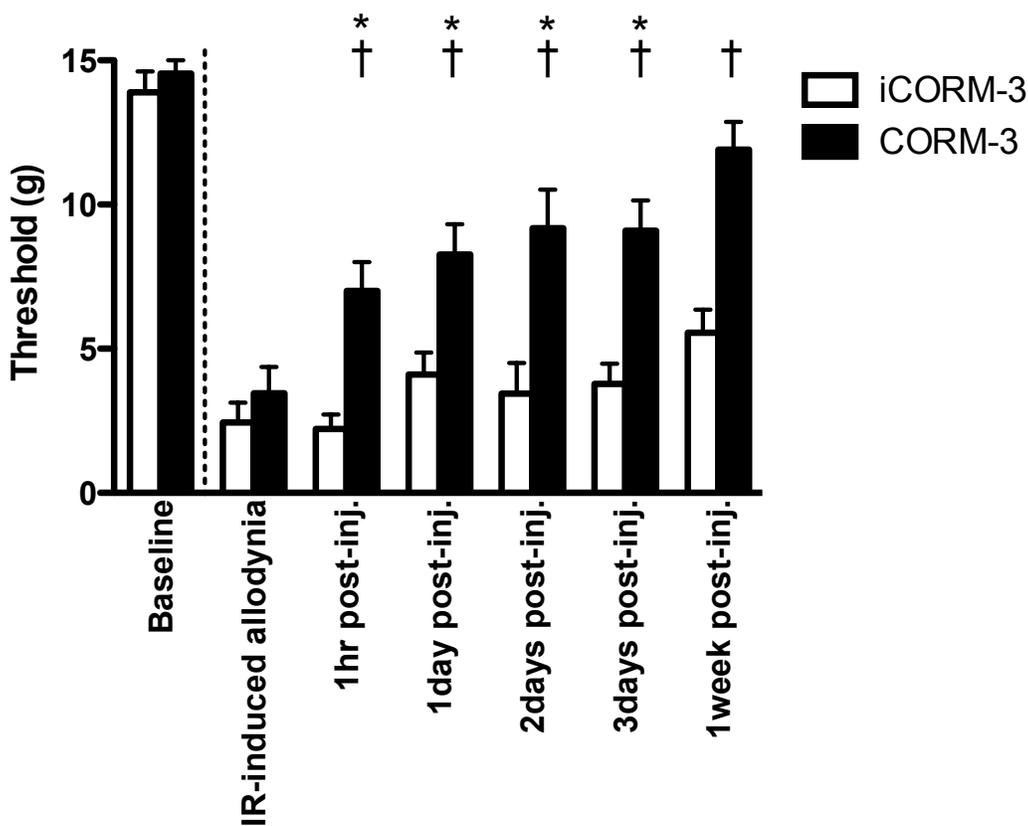


Figure 3.2. The effect of CORM-3 on paw withdrawal threshold (PWT). CORM-3 was administered at 7 days post-injury and PWTs were assessed via mechanical stimulation testing at 1 hour post-injection, and at 1, 2, 3 and 7 days post-injection. PWTs of the ipsilateral/right hindpaw displayed significant changes after CORM-3 injection. No changes in PWTs were observed in the contralateral hindpaw of iCORM-3 and CORM-3 animals. (* $p < 0.05$ from iCORM-3, † $p < 0.05$ from baseline). Error bars shown represent the standard error of the mean (SEM).

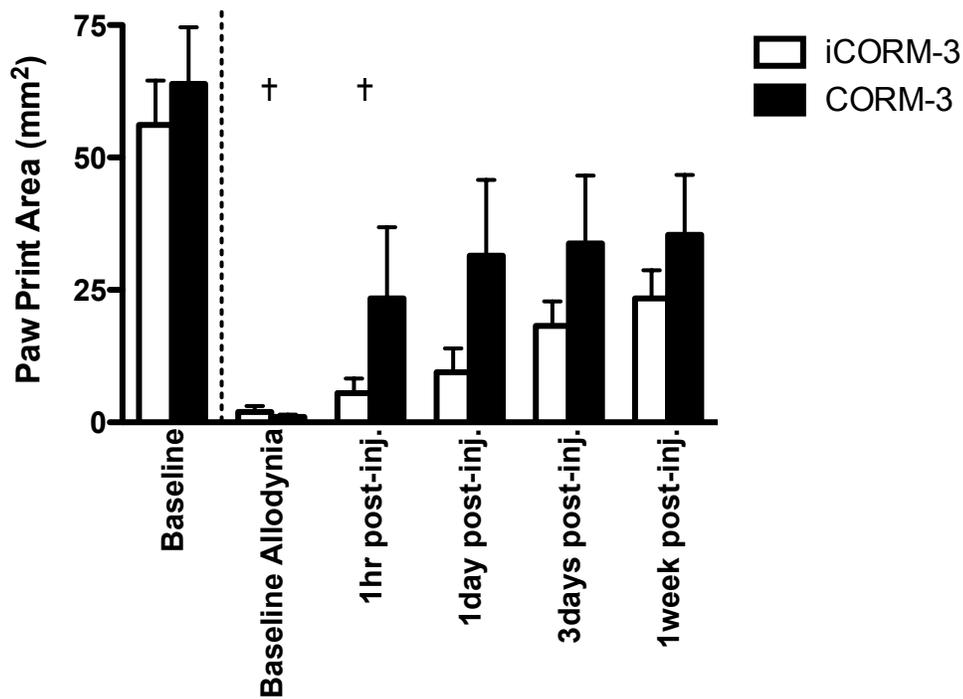


Figure 3.3. Paw print area of the right hindpaw (mm²). Paw print was calculated using the automated gait analysis software tool. († p<0.05 from baseline) Error bars shown represent the standard error of the mean (SEM).

3.3.4 Microcirculation

3.3.4.1 Muscle Perfusion

When compared to CORM-3 animals at 14 days, CPC of the EDL in CORM-3 animals were significantly increased when compared to iCORM-3 animals ($87\pm 1\%$ versus $77\pm 3\%$, $p < 0.05$) (Figure 3.4). No significant difference between IPC in the CORM-3 and iCORM-3 animals was observed ($3\pm 1\%$ versus $6\pm 2\%$, n.s.).

There was no significant difference in NPC in CORM-3 animals compared to iCORM-3 animals at 14 days post-reperfusion injury ($10\pm 1\%$ versus $17\pm 3\%$, n.s.), although a trend for increased perfusion with CORM-3 was observed.

3.3.4.2 Activated Leukocytes

Decreased adherence of leukocytes was observed in CORM-3 animals when compared to iCORM-3 animals (0.7836 ± 0.116 leukocytes/ $1000\mu\text{m}^2$ in CORM-3 group versus 2.482 ± 0.556 leukocytes/ $1000\mu\text{m}^2$ in iCORM-3 group, $p < 0.05$). No significant difference in rolling leukocytes within the EDL was observed (8.320 ± 1.807 leukocytes/ $30\text{s}/1000\mu\text{m}^2$ in CORM-3 group versus 9.12 ± 2.992 leukocytes/ $30\text{s}/1000\mu\text{m}^2$ in iCORM-3 group, n.s.) (Figure 3.5).

3.3.4.3 Injury

The level of cell injury was not significantly different between iCORM-3 and CORM-3 animals after intervention. Animals administered with CORM-3 demonstrated a $0.3202\pm 0.1229\%$ level of injury, compared with $0.4588\pm 0.1369\%$ (n.s.) in iCORM-3-treated animals (Figure 3.6).

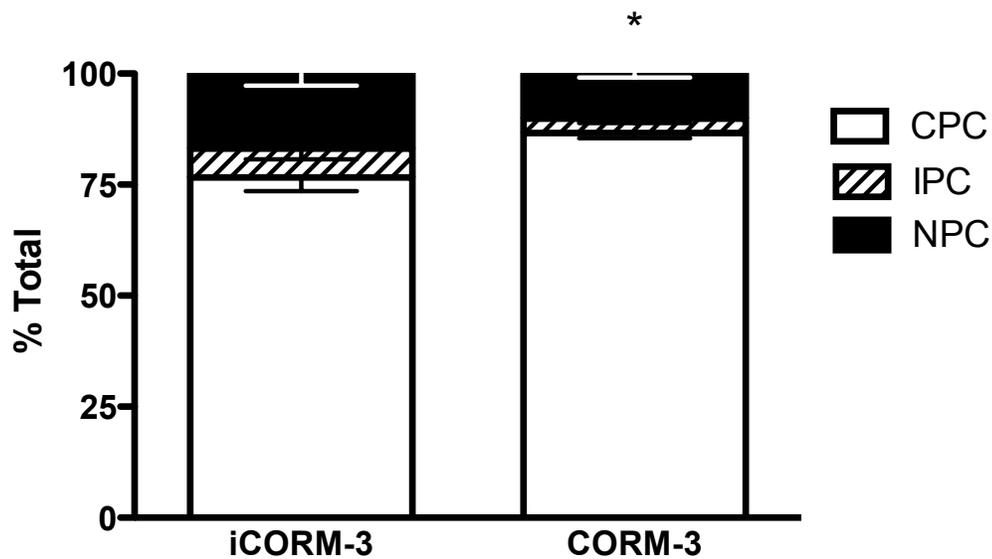


Figure 3.4. Skeletal muscle capillary perfusion. Values are expressed as a percentage of the total number of capillaries per field of view within EDL muscle. Significant difference in CPC was observed between CORM-3 and iCORM-3 animals. (* $p < 0.05$ from iCORM-3). No statistical differences were observed in IPC or NPC between CORM-3 and iCORM-3 groups. Error bars shown represent the standard error of the mean (SEM).

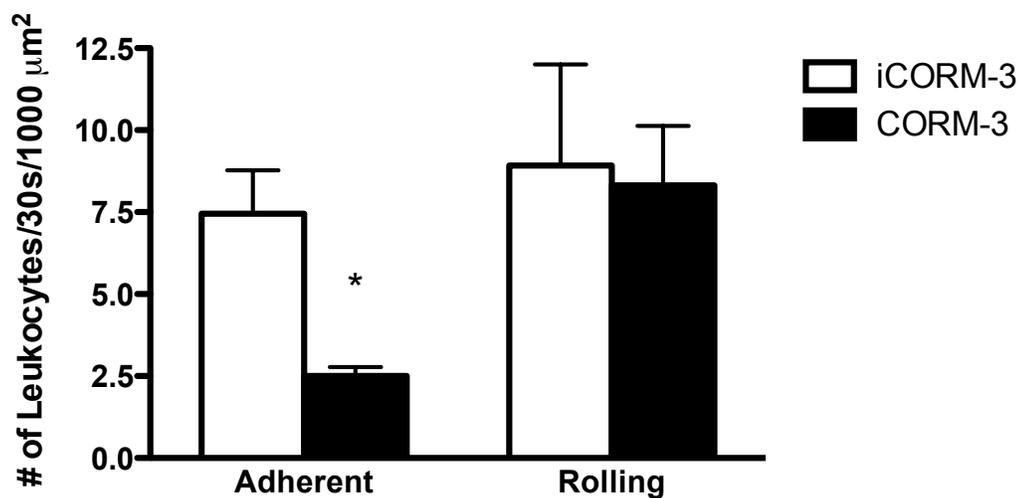


Figure 3.5. Leukocyte activation (adherence and rolling) in post-capillary venules of the extensor digitorum longus after IVVM. Significant difference in the number of adherent leukocytes was observed between iCORM-3 and CORM-3 animals. (* $p < 0.05$ from iCORM-3). Error bars shown represent the standard error of the mean (SEM).

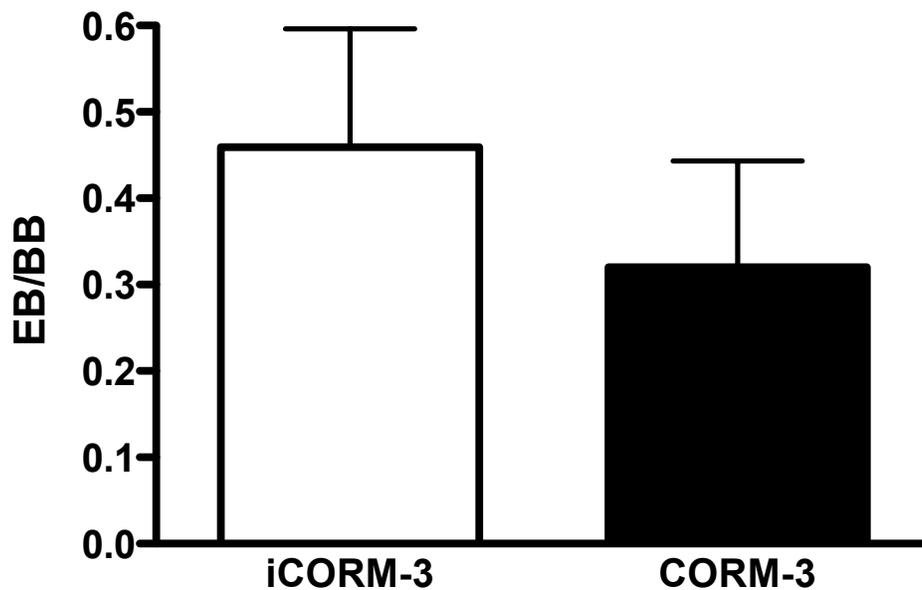


Figure 3.6. The effect of CORM-3 on cellular injury within the skeletal muscle.

The index of injury was assessed as the ratio of EB-labeled nuclei to BB-labeled nuclei (EB/BB). No statistical difference was observed, although there was a trend towards a decrease in CORM-3-treated animals. Error bars shown represent the standard error of the mean (SEM).

3.4 DISCUSSION

The chronic post-ischemia pain model, established by Coderre et al (Coderre et al., 2004) and validated with gait analysis, appeared to successfully model symptoms of CRPS-I in the rat, allowing for a comprehensive examination of possible therapeutic interventions. In the present study, we demonstrated the effect of CORM-3 on the reduction of mechanical allodynia symptoms, as well as its anti-inflammatory and vasodilatory action through direct observation of microcirculation.

Prolonged ischemia-reperfusion injury led to production of mechanical allodynia in the injured, as well as the uninjured, rat hind limbs. Consistent with previous results (Coderre et al., 2004), animals displayed reduced paw withdrawal thresholds after injury. Although this CRPS-like symptom was observed in both hind limbs, allodynia was much more pronounced in the ischemia-injured limb. In animals treated with inactive CORM-3, the reduced paw withdrawal threshold was maintained throughout the duration of the experiment; CORM-3 injection restored paw withdrawal thresholds to baseline levels, demonstrating a reduction in the pain sensation and tactile sensitivity. Previously, other CO-RMs, (i.e. CORM-2), have shown to have similar anti-nociceptive effects, as demonstrated in nerve constriction models and inflammation-based models (Hervera, Gou, et al., 2013; Negrete et al., 2014). Unlike CORM-2, CORM-3 is water-soluble, making it more clinically relevant therapeutic, if found to be effective.

While the mechanisms of CORM-3 actions are not fully understood, studies suggest that some may involve the suppression of oxidizing compound production and up-regulation of free radical scavengers (Mizuguchi et al., 2010; Patterson et al., 2014). This is consistent with the idea that CRPS-I may be induced or maintained

through free radical generation and inflammatory processes, as treatment with other free radical scavengers also appeared to reduce mechanical allodynia symptoms (Coderre et al., 2004). Thus, our data may also provide support for inflammatory and microvascular dysfunction as important factors in the development and maintenance of CRPS-I.

As suggested previously (Gabriel et al., 2007; Gabriel et al., 2009), the functional limb assessment by the use of the CatWalk™ automated gait analysis system may be limited to certain injury models. In the case of the CPIP as a model of CRPS-I, we have previously identified certain gait parameters as being important in the correlation between von Frey filament testing for allodynia and gait analysis. These included several paw print parameters (length, width, area) and duty cycle. In response to ischemia-reperfusion injury, some gait parameters changed significantly. Application of CORM-3 had no effect; however, there was a trend for increased paw print measurements (Figure 3.3). Given that changes in gait are a more complex measure of allodynia as compared to mechanical stimulation, it is reasonable to assume that pain sensation might not have as dramatic an effect. Previously, changes in CatWalk™ gait parameters have been observed after certain treatments, however, these were delivered multiple times over the course of the injury period (Koopmans et al., 2009), as opposed to a single injection of CORM-3 in our study. Thus, potential changes in gait parameters in response to CPIP should be explored in the future, with multiple dosing regimen.

It has been suggested that CRPS-I may be maintained by microcirculatory abnormalities (Bellingham et al., 2014; Coderre et al., 2004; Millecamps & Coderre,

2008). Consistent with this, our IVVM data in the CPIP model shows that the muscle tissue in the allodynic limb appears to have clear microvascular deficiencies. In response to CPIP, cell injury, leukocyte activation and capillary perfusion all show significant changes from the control parameters of sham animals previously published in ischemia studies (Potter et al., 1993). At 7 days post-injury, CORM-3 altered leukocyte adhesion within the post-capillary venules of the EDL (Figure 3.5). Additionally, CORM-3 also appeared to alter the degree of capillary perfusion (Figure 3.4). These results confirm the anti-inflammatory and vasodilatory actions of CO (McCarter et al., 2004; Mizuguchi et al., 2009), perhaps providing reason for the reduced pain sensation and the trend for restored gait. While not significant, there was a trend towards a reduced tissue injury in CORM-3-treated animals (Figure 3.6). Perfusion, which may be affected by leukocyte activity (Granger & Senchenkova, 2010), is an important factor in functional use of a tissue. Thus, increased tissue perfusion may help to rationalize the observed trend towards restoration of gait parameters. Delivery of CORM-3 at seven days post-reperfusion injury may give it little opportunity to act to reduce cell injury, since circulating endothelial progenitor cells may have repaired damaged endothelium within seven days.

Considering the effects of CORM-3-released CO on the observed changes of IVVM parameters in CPIP, restoration of microvascular perfusion may underlie the mechanism by which CORM-3-derived CO is able to alleviate allodynia symptoms. Thus, given the notion of microvascular dysfunction as one of the important factors in the development and maintenance of CRPS-I, improving capillary perfusion and

reducing leukocyte activation could explain the significant difference in the withdrawal thresholds of CORM-3-administered animals.

Previously, magnetic resonance spectroscopy has demonstrated that CRPS patients have hypoxic muscles in the affected limbs, which causes difficulty in regulating normal limb functions (Heerschap et al., 1993). Transcription factor NF κ B, which has been shown to be involved in ischemia, inflammation and sensitization pathologies, appears to be potently inhibited by CO preconditioning (Sun et al., 2008). Some studies have shown a direct link of NF κ B to the development of allodynia in the CPIP model; thus it has been suggested that NF κ B may play a role in the pathogenesis of CRPS-I (de Mos et al., 2009). Application of NF κ B inhibitor was able to relieve both mechanical and cold allodynia symptoms. Therefore, it could be hypothesized that application of CORM-3 may interfere with this potential pathogenic mechanism of CRPS-I.

Another molecule recently linked to CRPS-I pathogenesis is the inflammatory cytokine TNF-alpha (Maihofner et al., 2005; Munnikes et al., 2005; Sabsovich et al., 2008; Wesseldijk et al., 2008a, 2008b). Increasing evidence suggests that TNF-alpha plays a critical role in the pathogenesis of altered pain sensation. Although it has been previously shown that TNF-alpha returns to baseline levels quickly after a reperfusion injury (Bihari et al., 2014), Kramer et al found elevated TNF-alpha levels in the skin of affected limbs of CRPS-I compared to "normal" fracture and osteoarthritis patients (Kramer et al., 2011). This local, but not systemic, increase in TNF-alpha may be linked to the well-documented peripheral effects of TNF-alpha on nociceptor sensitization (Julius & Basbaum, 2001). Considering the ability of

CORM-3 to block the release of TNF-alpha levels during reperfusion (Lawendy et al., 2014), perhaps the substance acts to reduce allodynia sensation in CPIP model through a reduction in TNF-alpha levels. Further molecular examination of harvested tissue from these animals is required to explore this.

Inflammation of the vasa nervorum around peripheral nerves (neuritis) may be involved in CPIP (Coderre & Bennett, 2008); this suggests that relief of the inflammation may reduce pain symptoms associated with CRPS-I. Several other studies have demonstrated CO-RMs to be effective in reducing allodynia in a chronic sciatic nerve constriction model of neuropathic pain (Hervera, Gou, et al., 2013; Hervera, Leanez, et al., 2013). Given the range of pain models where CO was able to provide relief of symptoms (including both nerve constriction and inflammatory pain), CORM-3-derived CO may play an active role in several pathways of pain sensation, either directly or indirectly. However, further comprehensive study into both CORM-3 mechanisms and pain sensation would be required.

Treatment options currently available for CRPS-I patients are relatively extensive. One of the common treatments is a regional nerve block to the affected limb; however, recent studies have shown that regional anesthesia does not consistently block pain, especially in ischemic limbs (Kucera & Boezaart, 2014). Considering the fact that many patients do not respond to the available treatment, the development of additional therapeutic options for CRPS-I is needed. A holistic interpretation of the results from mechanical stimulation testing, gait analysis and IVVM suggests that CORM-3 reduces allodynia symptoms in the CPIP rodent model of CRPS-I. Considering that pain is the most important factor in patients' quality of

life, the reduction in sensitivity, even without significant changes in gait, after administration of CORM-3 is still an important finding. Considering the anti-inflammatory and vasodilatory actions of CO, the reduction in allodynia symptoms after CORM-3 administration again suggests that CRPS-I may have some microvascular abnormalities associated with the maintenance of its symptoms. Thus, our data suggests that CO, along with its current potential application in several other pathologies, may have clinical relevance as a therapeutic agent in CRPS-I.

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CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 OVERVIEW OF RESULTS

4.1.1 CPIP Model

This thesis focuses on the assessment and potential treatment of CRPS-I, using the chronic post-ischemia pain (CPIP) model. The model has proved to be a reliable representation of many CRPS-I-like symptoms, acquired through an insult known to often result in CRPS. In keeping with the proposed hypotheses for the pathophysiology of CRPS-I, the CPIP model appears to present what is perhaps the best animal model for examination of CRPS-I mechanisms and assessment of potential treatments. Compared with others, CPIP provides the hallmark symptoms of CRPS-I, including chronic hyperalgesia, allodynia (both cold and mechanical), inflammatory signs and spontaneous pain behaviours. The results of this thesis also show that the CPIP model produces behavioural and functional deficits similar to those observed in CRPS-I.

4.1.2 Gait Analysis

Chapter 2 describes the use of an automated gait analysis system (the CatWalk™) to assess functional changes in the CPIP model.

The data showed that gait analysis using the CatWalk™ system was able to detect an injury after ischemia-reperfusion injury. Four gait parameters (duty cycle and the three paw print parameters) statistically correlated with the chronic allodynia measurements taken via the gold standard of von Frey filament stimulation. While

other gait parameters showed significant changes immediately after injury, most normalized quickly to baseline by the end of our 14-day testing cycle.

In a separate group of animals, we showed that the CPIP model was an effective model for the development of long-lasting allodynia symptoms. CPIP animals maintained lower threshold of von Frey stimulation compared to the proximal IR group, which received a similar IR insult, only proximal to where the tourniquet had been placed in the CPIP group. The results validated the CPIP model in its ability to develop chronic allodynia. Interestingly, the CPIP group showed that the injury paradigm was not the major factor in affecting gait alterations; rather the gait alterations were probably secondary to allodynia. However, the physiology of tourniquet position just below the knee (both in this model and in general) is not well defined, and this may affect a range of kinematic parameters.

Chapter 2 provides us with an objective method for assessing functional changes in the CPIP model of CRPS-I. As stated by the new Budapest diagnostic criteria for CRPS (Harden et al., 2007), functional assessment is beneficial in examining symptomatology of CRPS. By providing an additional measure for the total pain experience in the rat, we may be able to better test the effects of potential therapeutics in the treatment of CRPS-I.

4.1.3 The Effect of CORM-3

CORM-3 has been found to have some anti-inflammatory, anti-apoptotic and vasodilatory properties (Lawendy et al., 2014). Based on this, we aimed to examine its use as a potential therapeutic agent in reduction of allodynia symptoms, as well

as any gait changes observed in the CPIP model of CRPS-I. In Chapter 3, we showed that CORM-3-derived CO carried anti-allodynic effects after an injection at seven days post-reperfusion. We demonstrated that CORM-3 was able to increase von Frey mechanical stimulation withdrawal threshold values back to baseline within seven days post-injection. Consistent with the previous anti-nociceptive vasodilatory treatments (Coderre et al., 2004), increased perfusion of EDL capillaries was observed after CORM-3 injection. In addition, a decrease in leukocyte adherence was found, although no changes in rolling leukocytes or in cell injury were observed in CORM-3 treated animals. These results suggest that an inflammatory response, albeit a diminished one is still occurring. Considering the length of time, elapsed after CORM-3 treatment (i.e. 7 days) that microcirculation was directly visualised, it is not unreasonable to see no changes in cell injury between CORM-3 treated and CPIP animals, even with the knowledge that CORM-3 has anti-apoptotic properties.

Similar to recent work by Hamam et al (Hamam et al., 2014) on compartment syndrome (CS), no significant changes in gait parameters were observed after CORM-3 application. A trend toward improved function was observed after CORM-3 injection in both studies; however, dynamic gait parameters were most affected by CORM-3 in CS, compared to a trend in static gait parameters in this study. The complex nature of gait movements, in terms of central processing, may have negated some of the positive effects CORM-3 had on the microvasculature, perhaps resulting in a lack of gait improvement.

4.2 STUDY LIMITATIONS

4.2.1 Pain Measurements

As the CPIP model in rats is a representation of the painful human condition, CRPS-I, we are presented with an inherent limitation in regards to pain and its measurement. Non-verbal representations of pain and hypersensitivity, using paw withdrawal from both noxious and non-noxious stimuli, are measured in the CPIP model. One limitation of pain and sensitivity tests is the animal handling required. Often, pain tests require that animals be restrained for measurements. In Chapters 2 and 3, animals were taken from their resting cages and placed in small chambers for allodynia testing with von Frey filaments. Studies have found that simple handling of rats can cause recruitment of endogenous opioids that can result in delayed responses to stimuli, including the stimuli used in our experiments, i.e. von Frey filaments (Jorum, 1988; Jorum & Shyu, 1988). In an effort to remove this potential limitation, we allowed rats to acclimatize to the new environment for a period of at least 10-15 minutes. Even with these additional efforts, some rats tried to escape the testing chamber after the 10-15 minute acclimatization period. Escape behaviour may suggest that these rats had altered responses to stimuli, perhaps as a result of endogenous opioid release.

4.2.2 Animal Model and Age of Rats

In both Chapters 2 and 3, young rats were used to produce CPIP. Animals were ordered at a weight of 150-175 g, although a minimum weight of 200 g was ensured before inducing the model through I-R injury. What was noticed in both

chapters was a resiliency of the rats, as it pertains to their recovery from injury. In some rats, no allodynia symptoms developed; these animals were subsequently excluded from further testing. Considering that CRPS is more common in adult patients, it may be prudent to use older rats in future experiments, in order to account for their resilient nature with respect to recovery from any injury and/or the development of allodynia symptoms similar to CRPS.

Other authors have cited a weight gain of 40% as being significant in the measurement of several gait parameters (Gabriel et al., 2009). Although no significant weight changes were observed in our experiments, the use of older rats may help negate any chance of weight gain affecting gait parameters.

Coderre and colleagues, in the original publication of the CPIP model, used pentobarbital injection to anesthetize rats before surgery. In our experiments, we used inhalational isoflurane. Although isoflurane has been found to have hypotensive and respiratory depression effects, it is an ideal anesthetic to use for extended surgical procedures like ours. Also, if temperature is properly maintained at normal body temperature, these side effects may be avoided. We ensured rats were always approximately 37 degrees Celsius through the use of a heating lamp.

4.2.3 Automated Gait Analysis

To assess functional changes after induction of CPIP to model CRPS-I, we used an automated gait analysis system. The CatWalk™ uses the refraction of light from an animal's paw on the glass walkway to capture images. Light is internally reflected within the glass and only released once a paw (or any other object) touches the

walkway. As a result, error is created if moisture or debris is present on the walkway. Rats often urinate small amount at the ends of the walkway and drag that moisture towards the centre of the walkway. Efforts were taken to ensure the walkway was dry and free from debris at all times, but it was impossible to have all of the recorded runs free of any moisture.

Several times throughout the experimental time-course rats would drag their underside on the glass walkway. The excess contact is normally recorded by the system as noise on the CatWalk™ software, creating a difficult situation for the software to classify paw prints automatically. As a result, many paw prints had to be manually classified, inherently introducing a source of error.

Noldus, the CatWalk™ system manufacturer, has just released an improved and more advanced analyzing software. Currently, our lab does not have this most recent edition, which might prove to be useful in measuring functional changes in our model, as well as in several other models of injury.

4.3 FUTURE DIRECTIONS

Considering the short half-life of CORM-3 in physiological solution, injecting CORM-3 multiple times after initiation of the CPIP model may yield different results. It may be interesting to see the effects this would have on how quickly paw withdrawal thresholds are returned to baseline, or if they would be restored at all.

Building on the above direction, and in order to better observe the effects of CORM-3, it would be beneficial to perform IVVM on some animals directly after injury, as well as at 1, 2, and 3 days post-injection. This approach may provide a

better insight into the effect of CORM-3 on the microvasculature, as it may be possible to correlate the gradual restoration of paw withdrawal thresholds with the changes directly observed within the microvasculature. It may also allow us to examine the effects of tourniquet placement and time on microvascular changes of the distal portion of the limb.

In examining how gait changes are caused by pain-related problems in the CPIP model, including a known analgesic pharmacological agent may be useful. This approach may help to ascertain the exact link between gait changes and pain while monitoring the normalization of gait, similar to the work of Angeby-Moller and colleagues in arthritis studies (Angeby-Moller et al., 2008).

Tissues from the skeletal muscle could be harvested from all animals at the time of euthanasia; although beyond the scope of this study, these could be used to examine the potential mechanisms of action of CORM-3 at a molecular level.

4.4 GENERAL CONCLUSIONS

The work presented in this thesis is the first piece of scientific literature to evaluate gait in the CPIP model of CRPS-I. It was hypothesized, based on the literature describing other inflammatory conditions, that automated gait analysis may be an objective and rapid tool for the assessment of gait changes secondary to allodynia. The results presented here suggest that automated gait analysis in the CPIP model is indeed a beneficial tool. We found several gait parameters correlating with von Frey measurements of allodynia assessment. As CRPS-I patients often lose range of motion and general function, as well as develop dystrophy/atrophy of the affected limb, using the methodology described in this work we may be able to better assess efficacy of potential therapeutics.

In the literature, anti-sympathetic and vasodilatory drugs have often appeared to reduce mechanical allodynia in CPIP model of CRPS-I. Through the use of the known vasodilatory, anti-inflammatory and anti-oxidant properties of CORM-3, this work has shown that CO therapy is capable of reducing mechanical allodynia symptoms in the CPIP, as indicated by an increase in paw withdrawal thresholds. The actual effect of CORM-3 is still unclear; however, with further analysis, mechanisms of CORM-3 action may be delineated and assist with improving our understanding of the complex pain mechanisms involved in CRPS-I.

4.5 REFERENCES

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APPENDICES

APPENDIX A – DEFINITIONS OF GAIT ANALYSIS PARAMETERS

Paw Statistics

The Paw Statistics sheet displays parameters that are based on individual paw prints.



Which paws/parameters are displayed in the Paw Statistics sheet depends on the selection in the currently active Parameter Profile (displayed in blue and bold in the Experiment Explorer).

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 phase) (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:

$$\text{Stand Index} = \frac{a}{x0} \times \text{frame rate}$$

where a is derived from $y = ax + b$, that describes a line that best fits through the data points at $t(\text{Max area})$ and the 90% percentile; $x0$ 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.

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 (in the Distance Unit) 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 pressure. Therefore, Intensity is a measure of pressure exerted on the glass plate.

Chapter 8

The **Intensity** parameter is used to assess the effects of neuropathic pain, including mechanical allodynia (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 **Mac Contact Max Intensity**.

X position

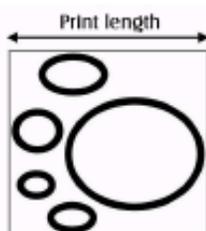
X is the position of the mass-midpoint (center of the print) at **Max Contact** in the horizontal (walking) direction. **X** is the distance in the Distance Unit from the left side of the walkway to the center of the print.

Y position

Y is the position of the mass-midpoint (center of the 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.

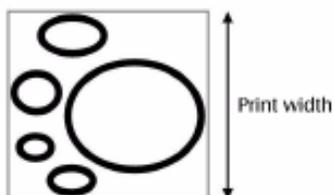
Print Length

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 70).



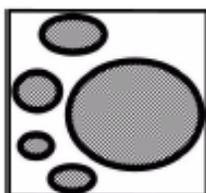
Print Width

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 70).



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:

$$\text{Max Intensity at} = \frac{\text{Max Intensity At (s)} - \text{Initial Contact}}{\text{Stand}} \times 100 \%$$

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.

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 mass-midpoint of two consecutive placements of the same paw during **Max contact**:

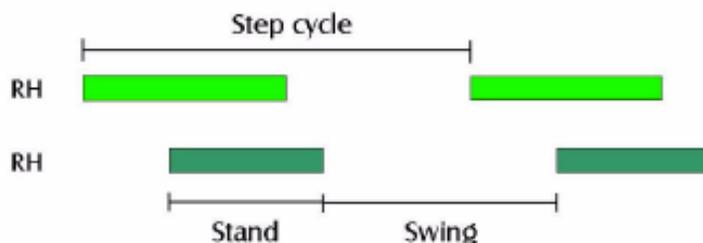
$$\text{Stride Length} = |X_n - X_{n-1}|$$

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:

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



In the **Paw Statistics** sheet, when you click one of the paw rows:

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

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.

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 Table 8.1.). 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 Table 8.1. on page 120).

Accounted Steps

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



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**.

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 8.5.).



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

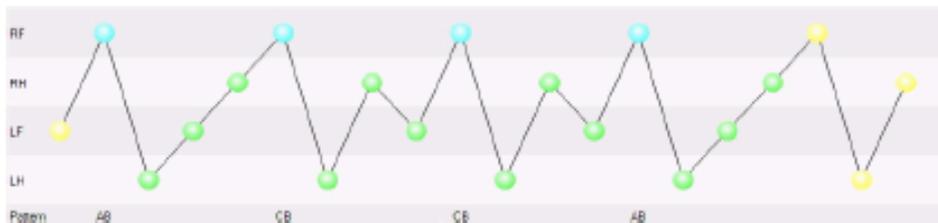


Figure 8.5. A Footfall Patterns diagram. In this example, the animal had 4 normal footfall patterns. All footfall patterns start with the right front paw (RF: blue dot). The first paw print and the last three are not taken into account. The number of accounted steps is '16'. Regularity Index is '100%'

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 8.5.).

APPENDIX B - PERMISSIONS

 Yvonne H. [redacted]
to me ▾

Hi,

I just got approval for "If it is parts of the manual with a reference". Would this work for you?

[redacted]

[redacted]

*Human Resource Administrator
Conference Coordinator*

Naldus Information Technology Inc.

[redacted]

USA

[redacted]

From: Hussein Abdo [redacted]
Sent: Wednesday, April 01, 2015 11:43 AM

[redacted]

 Hussein Abdo [redacted]
to Yvonne ▾

That works great. Thanks Yvonne for your help.

Regards,
Hussein Abdo

APPENDIX C – ANIMAL PROTOCOL APPROVAL

11.01.13

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

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 years
Rat	Wistar	150-350 g	C	680
Pig	Yorkshire-Landrace	50-60 kg	B	30

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 Bihari, T Carter, K Bothwell, P Coakwell

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Publications:

Abdo H, Bihari A, Bellingham GA, Sanders DW and Lawendy AR. Carbon Monoxide Reduces Allodynia in a Rodent Model of Complex Regional Pain Syndrome type-1 (CRPS-I). *American Orthopaedic Association/Canadian Orthopaedic Association Combined Meeting*, Montreal, Quebec, June 19, 2014.

Chadi SA, **Abdo H**, Bihari A, Parry N, Lawendy AR. Hepatic Microvascular Changes in Rat Abdominal Compartment Syndrome. *Journal of Surgical Research*. (in Press)

Desai SJ, Patel J, **Abdo H**, Lawendy AR and Sanders DW. (2014) A Comparison of Surgical Delays in Directly Admitted vs. Transferred Patients with Hip Fractures: Opportunities for Improvement? *Can J Surg*. 57(1): 40-43.