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Therapeutic Application of Carbon Monoxide in Acute Limb Compartment Syndrome

Aurelia Bihari, *The University of Western Ontario*

Supervisor: Dr Gediminas Cepinskas, *The University of Western Ontario*

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ABSTRACT

Acute limb compartment syndrome (CS), a devastating complication of musculoskeletal trauma, develops in response to elevation of the pressure within a closed osseofascial compartment, producing muscle- and limb-threatening ischemia. Full decompression of all involved compartments by fasciotomy is the current gold-standard therapy, but it must be performed within a surgical window of 6-8 hours, before tissue damage becomes permanent.

Carbon monoxide (CO), a byproduct of heme metabolism, has been shown protective in ischemia. While inhalation of CO leads to elevation of carboxyhemoglobin (COHb), recent development of transitional metal carbonyls, CO-releasing molecules (CO-RMs), particularly the water-soluble CORM-3, delivers CO in a controlled manner without COHb formation, making it well suited to clinical applications.

The purpose of this thesis was to examine the effects of CORM-3-derived CO on microvascular dysfunction due to elevated compartment pressure within skeletal muscle, using clinically relevant models of CS. The efficacy of both CO and CORM-3 was tested in the rat, demonstrating that CORM-3, just like inhaled CO, was able to prevent the CS-associated microvascular perfusion deficits, tissue injury and inflammation, all without the CO-generated elevation of COHb.

The effects of CORM-3 were then tested in a preclinical large animal model of CS (pig), demonstrating the abolition of CS-induced systemic leukocyte

activation correlated with the inhibition of systemic TNF-α release, improved tissue microvascular perfusion, diminished tissue injury and apoptosis.

To lay the foundation for translation of animal CS research to humans, CS was modelled *in vitro*, employing tissue culture of human vascular endothelial cells and serum of CS patients. CORM-3 was able to prevent free-radical formation, breakdown of endothelial barrier, apoptosis, leukocyte activation and transendothelial migration in response to CS stimulus.

Thus, CORM-3 appears to have an enormous clinical potential; it might be capable of at least prolonging the surgical window, if not significantly reducing the need for fasciotomy.

Keywords: *compartment syndrome, elevated compartment pressure, fasciotomy, tissue injury, inflammation, leukocyte activation, cytokines, carbon monoxide, CORM-3, oxidative stress.*

THE CO-AUTHORSHIP

While each of the co-authors listed below made important contributions to this work, I am the principal author who designed all the projects, performed all of the experimental data acquisition, collection and analysis. All manuscripts presented in this thesis were prepared by me, with the consultation and critical review by the co-authors.

Abdel-Rahman Lawendy, MD, PhD, FRCSC, in his role as my research partner and the principal investigator of the lab, provided strong leadership on the project, offering direction and guidance on data interpretation, and keeping things in perspective. In addition to his clinical expertise, which was crucial to the project, he also secured all serum samples from patients.

Gediminas Cepinskas, DVM, PhD, in his role as a supervisor, provided direction and guidance on data interpretation, and critical evaluation of all work. In addition, he made sure that everything remained on track.

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Richard Potter, PhD, in his role of a mentor taught me ‘the ropes’. He gave me a solid basis in the realm of the research world, critically reviewed the manuscripts, and provided necessary advice on publishing in scientific journals.

DEDICATION

To my parents, Ivan and Aurelia, who left their country of birth with nothing but clothes on their backs, two children in tow, in pursuit of freedom and better opportunities for our family. They abandoned life of plenty, privilege, high position and convenience for that of hard toils and sacrifice. Since I 'cheated' them out of attending my previous post-secondary graduations, I would now like to fulfill the promise of letting them see me collect my Doctorate diploma.

To Frank, who years ago married a young student, hoping that the schooling would soon come to an end and we would finally lead a peaceful life, only having to put up with more of the same again, in the form of grad studies; my 'lightning rod' and a grounding force throughout these years, a true friend and someone to always rely on. Without him I would have lost my mind long time ago.

To the memory of my beloved grandmother, who, sadly, faded away long before this stage of my education could be attained. She was crucial to the formation of my early years, by instilling in me her love of books and literature that remains with me until today. Our Taffy (may she rest in peace) is now the lucky one to have her as a companion.

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TABLE OF CONTENTS

Page

ABSTRACT ii

CO-AUTHORSHIP iv

DEDICATION v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS vii

LIST OF TABLES xvi

LIST OF FIGURES xvii

LIST OF APPENDICES xx

LIST OF ABBREVIATIONS xxi

CHAPTER 1. INTRODUCTION AND HISTORICAL REVIEW 1

1.1 COMPARTMENT SYNDROME 2

1.2 DIAGNOSIS OF CS 3

1.2.1 Clinical Diagnosis 3

1.2.1.1 Pain 3

1.2.1.2 Paresthesia, Paralysis 4

1.2.1.3 Pallor, Pulselessness 5

1.2.2 Compartment Pressure Monitoring 6

1.2.2.1 Devices for ICP Measurement 7

1.2.2.2 ICP Measurement Threshold 9

1.2.3 Consequences of Missed Diagnosis 10

1.3 THERAPEUTIC APPROACH TO CS 12
1.3.1 Limb Anatomy 12
1.3.1.1 Leg 12
1.3.1.2 Forearm 15
1.3.2 Fasciotomy 17
1.3.2.1 Surgical Techniques for Fasciotomy 18
1.3.3 Complications of Fasciotomy 18
1.3.4 Non-Operative Treatment of CS 20
1.3.4.1 Mannitol 22
1.3.4.2 L-Ascorbic Acid 22
1.3.4.3 N-Acetyl Cysteine 23
1.3.4.4 Non-Steroidal Anti-Inflammatory Drugs 23
1.3.4.5 Tissue Ultrafiltration 24
1.3.4.6 Hyperbaric Oxygen Therapy 24
1.3.5 Outcomes 25
1.4 PATHOPHYSIOLOGY OF CS 26
1.4.1 Historical Progression of Understanding CS Pathophysiology 27
1.4.2 Current Understanding. 30
1.5 MICROCIRCULATION IN CS 31
1.5.1 Tissue Ischemia 33
1.5.1.1 Critical Closing Pressure Theory 33
1.5.1.2 Microvascular Occlusion Theory 35

1.5.1.3 Arterio-Venous Gradient Theory 36
1.5.2 Reperfusion and Inflammation 37
1.5.2.1 Endothelial Activation 38
1.5.2.2 Increased Vascular Permeability 39
1.5.2.3 Vasomotor Response 40
1.5.2.4 Cytokine Release 40
1.5.2.5 Leukocyte Activation 42
1.5.2.6 Reactive Oxygen Species 45
1.6 HEME METABOLISM AND OXIDATIVE STRESS 47
1.6.1 Heme Oxygenase 47
1.6.2 Carbon Monoxide 50
1.6.2.1 CO Toxicity 50
1.6.2.2 Endogenous Sources of CO 51
1.6.3 Biological Effects of CO 52
1.6.3.1 Cellular Signalling 53
1.6.3.2 Vasodilation 55
1.6.3.3 Anti-Inflammatory Effects 56
1.6.3.4 Anti-Apoptotic Effects 57
1.6.3.5 Anti-Proliferative Effects 58
1.6.4 Carbon Monoxide Releasing Molecules (CO-RMs) 58
1.6.5 CORM-3 62
1.7 AIM OF THIS THESIS 64
1.8 REFERENCES 66

CHAPTER 2. THE EFFECT OF EXOGENOUS APPLICATION OF OF CARBON MONOXIDE IN THE RAT MODEL OF ACUTE LIMB COMPARTMENT SYNDROME 86
2.1 INTRODUCTION 87
2.2 MATERIALS AND METHODS 89
2.2.1 Animal Preparation 89
2.2.2 Exogenous Application of CO 91
2.2.3 Experimental Groups 91
2.2.4 Intravital Video Microscopy (IVVM) 91
2.2.5 Offline Video Analysis 92
2.2.6 Statistical Analysis 93
2.3 RESULTS 93
2.3.1 Microvascular Perfusion 93
2.3.2 Tissue Injury 93
2.3.3 Inflammation 96
2.3.4 Systemic COHb 96
2.4 DISCUSSION 99
2.5 REFERENCES 103
CHAPTER 3. THE SEVERITY OF MICROVASCULAR DYSFUNCTION DUE TO COMPARTMENT SYNDROME IS DIMINISHED BY THE SYSTEMIC APPLICATION OF CO-RELEASING MOLECULE-3 (CORM-3) 108
3.1 INTRODUCTION 109

3.2 MATERIALS AND METHODS 111
3.2.1 Animal Preparation 111
3.2.2 CORM-3 Synthesis 112
3.2.3 Experimental Groups 112
3.2.4 Intravital Video Microscopy (IVVM) 113
3.2.5 Offline Video Analysis 114
3.2.6 Serum TNF-α Measurements 114
3.2.7 Statistical Analysis 115
3.3 RESULTS 115
3.3.1 Systemic Leukocyte Counts and Carboxyhemoglobin 115
3.3.2 Microvascular Perfusion 115
3.3.3 Tissue Injury 118
3.3.4 Serum TNF-α 118
3.3.5 Inflammation 118
3.4 DISCUSSION 122
3.5 REFERENCES 127
CHAPTER 4. SYSTEMIC ADMINISTRATION OF CARBON MONOXIDE RELEASING MOLECULE-3 (CORM-3) PROTECTS THE SKELETAL MUSCLE IN PORCINE MODEL OF COMPARTMENT SYNDROME 131
4.1 INTRODUCTION 132
4.2 MATERIALS AND METHODS 134
4.2.1 Animal Preparation 134

4.2.2 CORM-3 136
4.2.3 Experimental Design 136
4.2.4 OPS Imaging 137
4.2.4.1 Offline Video Analysis 137
4.2.5 Tissue Leukocyte Infiltration and Necrosis 138
4.2.6 Tissue Injury and Apoptosis 138
4.2.7 Systemic Leukocyte Isolation and Activation Assay 140
4.2.8 Serum TNF-α Measurements 140
4.2.9 Statistical Analysis 141
4.3 RESULTS 141
4.3.1 Systemic Leukocyte Count and COHb 141
4.3.2 Organ Function 141
4.3.2.1 Liver Enzymes 141
4.3.2.2 Kidney 143
4.3.2.3 Muscle – Lactate 143
4.3.3 Systemic Leukocyte Activation and TNF-α Levels 143
4.3.4 Microvascular Perfusion 146
4.3.5 Tissue Injury and Apoptosis 146
4.3.6 Tissue Leukocyte Infiltration and Necrosis 150
4.4 DISCUSSION 150
4.5 REFERENCES 159

CHAPTER 5. CARBON MONOXIDE RELEASING MOLECULE-3 (CORM-3) OFFERS PROTECTION IN AN *IN VITRO* MODEL OF COMPARTMENT SYNDROME 165
5.1 INTRODUCTION 166
5.2 MATERIALS AND METHODS 168
5.2.1 Reagents 168
5.2.2 Cells 169
5.2.3 *In vitro* Model of CS 169
5.2.4 Reactive Oxygen Species Production 170
5.2.5 Measurement of the Endothelial Monolayer Integrity 171
5.2.6 Quantification of Apoptosis 171
5.2.7 PMN Adhesion/Rolling Assay 172
5.2.8 Transendothelial PMN Migration Assay 172
5.2.9 Statistical Analysis 173
5.3 RESULTS 173
5.3.1 ROS Production 173
5.3.2 Transendothelial Electrical Resistance (TEER) 175
5.3.3 Apoptosis 178
5.3.4 PMN Rolling/Adhesion 178
5.3.5 PMN Migration 181
5.4 DISCUSSION 183
5.5 REFERENCES 192
CHAPTER 6. GENERAL DISCUSSION 198

6.1 OVERVIEW OF RESULTS 199
6.1.1 Pathophysiology of CS and Therapeutic Considerations 199
6.1.2 Carbon Monoxide and CS 200
6.1.2.1 CORM-3 as a Source of CO 201
6.1.3 Preclinical Testing of CORM-3 in Porcine CS 202
6.1.4 Human *in vitro* Model of CS 203
6.2 LIMITATIONS AND FUTURE DIRECTIONS 204
6.3 CONCLUSIONS 206
6.4 REFERENCES 207
APPENDICES 210
APPENDIX I. SURGICAL APPROCHES TO LIMB COMPARTMENT SYNDROME 211
I.1 FASCIOTOMY IN THE LEG 212
I.1.1 Surgical Technique: Single-Incision Fasciotomy 214
I.1.2 Surgical Technique: Two-Incision Fasciotomy 216
I.2 FASCIOTOMY IN THE FOREARM 218
I.2.1 Surgical Technique: Volar Approach 219
I.2.2 Surgical Technique: Dorsal Approach 222
I.3 REFERENCES 222
APPENDIX II. PERMISSIONS TO USE COPYRIGHTED MATERIAL ... 224
II.1 Journal of Orthopaedic Trauma 2014; 28(11): e263-8 225
II.2 Operative Techniques: Orthopaedic Trauma Surgery 2010 .. 226
APPENDIX III. ANIMAL PROTOCOL APPROVAL 228

APPENDIX IV. HUMAN RESEARCH ETHICS BOARD APPROVAL..... 230

VITA..... 232

LIST OF TABLES

Table		Page
1.1.	Most common carbon monoxide-releasing molecules (CO-RMs)	60
3.1	The effects of CORM-3 on systemic leukocyte count and COHb levels	116
4.1	Histopathology grading scale for skeletal muscle tissue leukocyte infiltration and necrosis in porcine model of CS	139
4.2	The effect of CORM-3 on hematological and biochemical parameters in porcine model of CS	142

LIST OF FIGURES

Figure	Description	Page
1.1	Compartments of the leg	13
1.2	Compartments of the forearm	16
1.3	Schematics of normal microcirculation	32
1.4	Microcirculatory dysfunction in CS	34
1.5	Leukocyte activation sequence in inflammation	43
1.6	Heme degradation pathway	48
2.1	Schematics of the experimental setup of a rat model of compartment syndrome	90
2.2	The effect of CO inhalation on skeletal muscle microvascular perfusion following CS	94
2.3	The effect of CO inhalation on skeletal muscle tissue injury following CS	95
2.4	The effect of CO inhalation on leukocyte adhesive interactions with endothelium of skeletal muscle microvasculature in CS-challenged rat	97
2.5	The effect of inhaled CO on systemic COHb levels	98
3.1	The effect of CORM-3 on skeletal muscle microvascular perfusion following CS	117
3.2	The effect of CORM-3 on skeletal muscle tissue injury following CS	119
3.3	The effect of CORM-3 on serum TNF-α levels in CS	120

3.4 The effect of CORM-3 on modulation of leukocyte recruitment to the skeletal muscle vasculature following CS 121
4.1 The effect of CORM-3 on systemic leukocyte activation in porcine model of CS 145
4.2 The effect of CORM-3 on serum TNF- α levels in porcine model of CS 147
4.3 The effect of CORM-3 on skeletal muscle perfusion in porcine model of CS 148
4.4 The effect of CORM-3 on skeletal muscle tissue injury and apoptosis in porcine model of CS 149
4.5 The effect of CORM-3 on skeletal muscle tissue necrosis and leukocyte infiltration in porcine model of CS 151
5.1 The effect of CORM-3 on the oxidative stress response in HUVECs elicited by stimulation with human CS serum 174
5.2 The effect of CORM-3 on leukocyte activation (quantified by the production of superoxide by PMNs) in response to stimulation with human CS serum 176
5.3 The effect of CORM-3 on the integrity of HUVEC monolayer following stimulation with CS serum 177
5.4 The effect of CORM-3 on the level of apoptosis in HUVEC, elicited by stimulation with human CS serum 179
5.5 The effect of CORM-3 on leukocyte (A) rolling and (B) adhesion in response to stimulation of HUVEC by CS serum 180

5.6 The effect of CORM-3 on transendothelial leukocyte migration in response to stimulation of HUVEC with CS serum 182

I.1 Single incision fasciotomy 215

I.2 Two-incision fasciotomy 217

I.3 Fasciotomy of the forearm 215

LIST OF APPENDICES

Appendix **Page**

Appendix I. Surgical Approaches to Compartment Syndrome 211

Appendix II. Permissions to Use Copyrighted Materials..... 224

Appendix III. Copy of Animal Protocol Approval..... 228

Appendix IV. Copy of Research Ethics Board Approval..... 230

LIST OF ABBREVIATIONS

AdHO-1, adenovirus containing HO-1 gene construct

AP-1, activator protein-1

BB, bisbenzimide

BR, bilirubin

BV, biliverdin

BVR, biliverdin reductase

CCL2, chemokine (C-C motif) ligand 2

CCL4, chemokine (C-C motif) ligand 4

cGMP, cyclic guanosine monophosphate

CO, carbon monoxide

COHb, carboxyhemoglobin

CO-RMs, carbon monoxide-releasing molecules

CORM-3, carbon monoxide-releasing molecule-3

COX, cyclooxygenase

COX-1, cyclooxygenase-1

COX-2, cyclooxygenase-2

CPC, continuously-perfused capillaries

CS, compartment syndrome

CXCL1, chemokine (C-X-C motif) ligand 1

CXCL8, chemokine (C-X-C motif) ligand 2

CXCL10, chemokine (C-X-C motif) ligand 10

DMSO, dimethyl sulfoxide

EB, ethidium bromide

EDL, extensor digitorum longus

ELISA, enzyme-linked immunosorbent assay

FLIVO, fluorescence *in vivo* labelling of apoptosis

FLICA, fluorescence cellular labelling of apoptosis

GRO, growth-regulated oncogene

HO, heme oxygenase

HO-1, heme oxygenase-1

HO-2, heme oxygenase-2

HO-3, heme oxygenase-3

HSP32, heat shock protein 32

HUVEC, human vascular endothelial cells

ICP, intra-compartmental pressure

ICAM-1, intracellular adhesion molecule-1

Ig, immunoglobulin

IL-1β, interleukin-1 beta

IL-6, interleukin-6

IL-8, interleukin-8

IM, intra-muscular

IP-10, interferon gamma-induced protein 10

IPC, intermittently-perfused capillaries

IR, ischemia-reperfusion

IVVM, intravital video microscopy

KC, keratinocyte chemoattractant

LFA-1, lymphocyte function-associated antigen-1

LPS, lipopolysaccharide

Mac-1, macrophage-associated protein-1

MAPK, mitogen-activated protein kinases

MCP-1, monocyte chemotactive protein 1

MIP-1β, macrophage inflammatory protein 1β

NAC, N-acetyl cysteine

NADPH, nicotinamide adenine dinucleotide phosphate

NIR, near infra-red spectroscopy

NFκB, nuclear factor kappa B

NO, nitric oxide

NOS, nitric oxide synthase

NPC, non-perfused capillaries

NSAIDs, non-steroidal anti-inflammatory drugs

OPS, orthogonal polarized spectroscopy

PAF, platelet activating factor

PECAM-1, platelet-associated cell adhesion molecule-1

PI, propidium iodide

PI3K, phosphatidylinositol 3-kinase

PL, peroneus longus

PMN, polymorphonuclear leukocytes

PPS, pain on passive muscle stretch

PSGL-1, P-selectin glycoprotein ligand-1

PT, peroneus tertius

RFU, relative fluorescence units

RLU, relative luminescence units

ROS, reactive oxygen species

sGC, soluble guanylate cyclase

TA, tibialis anterior

TEER, trans-endothelial electrical resistance

TNF-α, tumor necrosis factor alpha

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling

VCAM-1, vascular cell adhesion molecule-1

VLA-4, very late antigen-4

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

CHAPTER 1: INTRODUCTION AND HISTORICAL REVIEW

1.1 COMPARTMENT SYNDROME

Compartment syndrome (CS) is a devastating limb- and life-threatening condition caused by elevated pressure within a closed osseofascial compartment, resulting in microvascular compromise, cellular anoxia and cell death (Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen, Winquist et al. 1980, Hartsock, O'Farrell et al. 1998). If left untreated, it produces significant tissue ischemia, which usually leads to severe functional impairment of the affected limb. CS can develop in response to a multitude of traumatic injuries and medical co-morbities: fractures, burns, exercise, crush injuries, and ischemia-reperfusion injury (McQueen, Gaston et al. 2000); less common causes may include bleeding disorders (Hope and McQueen 2004), diabetes, administration of statins (Chautems, Irmay et al. 1997, Jose, Viswanathan et al. 2004), infection (Schnall, Holtom et al. 1994), hypothyroidism (Hsu, Thadhani et al. 1995), lithotomy position (Mathews, Perry et al. 2001), snake bites (Vigasio, Battiston et al. 1991), arterial rupture (Brumback 1990) and blast injuries (Born 2005).

Case reports of extremity contracture after trauma have been reported as early as 1840; most were, at that time, believed to be a result of direct neurologic injury. CS was first described in depth by Richard von Volkmann in 1881. Volkmann, a German surgeon, noted deformities of the hand and wrist following

supracondylar fractures of the distal humerus; he believed that these contractures were related to (and always preceded by) the application of tight bandages to the injured limb, producing an “inflammatory myositis”. Eventually, Volkmann suggested that resulting limb paralysis and ischemic contracture were due to the interruption in arterial blood supply, leading to tissue ischemia; however, the precise cause of CS remained open for debate for decades to come (von Volkmann 1881).

1.2 DIAGNOSIS OF CS

1.2.1 Clinical Diagnosis

Diagnosis of acute CS is challenging, as there is no ‘true’ diagnostic or imaging test; instead, the surgeon must rely on the clinical examination and observation of certain signs and symptoms. The first description of clinical criteria for the diagnosis of CS was provided by Griffiths in 1940. Griffiths established the original “four Ps”: pain out of proportion and pain on passive stretch, paraesthesia, paralysis and ‘puffiness’ (Griffiths 1940). Eventually, pallor and pulselessness were added to the signs of CS (Cascio, Wilckens et al. 2005). Today, those six criteria represent the basis of CS diagnostics.

1.2.1.1 Pain

The first symptoms of acute CS appear to be the disproportionate pain relative to the injury and pain on passive muscle stretch (PPS) (Whitesides and

Heckman 1996). Thus, a progressive increase in analgesia requirements (or total unresponsiveness to narcotics) may be an indication of impeding CS (Bae, Kadiyala et al. 2001).

Both pain out of proportion to what is expected of the injury (based on the physical examination) and PPS are the most sensitive clinical findings (19%) and are often *the only* observation that precede ischemic dysfunction in the nerves and muscles of the affected compartment (Whitesides and Heckman 1996, Ulmer 2002). While the specificity of both pain measures is high (97%), the sensitivity is very poor (19%); thus, pain as a diagnostic criterion fails to identify a high percentage of individuals with acute CS (Ulmer 2002). Rather, the absence of pain is a more useful measure in ruling out acute CS, given the low false positive rate. However, an adequate level of suspicion must be maintained by the attending surgeon, as the absence of pain may indicate individual variation, altered states of pain perception, compartment syndrome of the deep posterior compartment, or missed acute compartment syndrome that has resulted in altered sensation (Whitesides and Heckman 1996).

1.2.1.2 Paraesthesia, Paralysis

Approximately 1 hour after the onset of ischemia, the patient may experience the first sensory changes (Whitesides and Heckman 1996). The first neurological signs of acute CS are usually hypoesthesia and paraesthesia in the dermatomal distribution of the nerve(s) of the involved compartment (Hargens, Akeson et al. 1978, Mubarak, Owen et al. 1978, Matsen, Winquist et al. 1980).

For example, altered sensation in the first web space of the foot is indicative of the involvement of the deep peroneal nerve and anterior compartment syndrome, while numbness of the dorsum of the foot may indicate lateral compartment syndrome with compression of the superficial peroneal nerve. Yet, these signs may also be caused by direct trauma to the nerve (Mubarak, Owen et al. 1978).

As a clinical measure of acute CS, paraesthesia has a sensitivity of 13% and a specificity of 98% (Ulmer 2002). Unfortunately, paresis and/or paralysis of the muscles of the involved compartment are considered to be signs of a late acute CS; at this stage, the patient is less likely to respond to fasciotomy (Matsen and Clawson 1975, Ulmer 2002).

1.2.1.3 Pallor, 'Puffiness', Pulselessness

A pale, tense, swollen compartment resulting from increased intra-compartmental pressure, sometimes with bruising of the skin, is recognized as an early physical sign of acute CS (Mubarak, Owen et al. 1978), although the measure may not be evident with isolated involvement of a deep compartment. Dressings and casts should be immediately removed to accurately assess swelling.

The lack of a pulse rarely occurs in CS patients, as pressure that causes CS is often well below arterial pressure, and thus is not a feature of acute CS; additionally, the relevant artery may not be contained within the affected compartment (Shears and Porter 2006). Alternatively, the presence of a pulse does not exclude CS.

While congestion of the digits and prolonged capillary refill time may also indicate acute CS, these measures should not be relied upon, as they may be affected by many different external factors (e.g. shock, dehydration, decreased peripheral perfusion (Shears and Porter 2006).

In addition to the observation and monitoring of the above-mentioned clinical signs, it has been suggested that intra-muscular (IM) pH monitoring may also be of benefit, as IM pH of less than 6.38 was found in 80% of CS cases (Elliot 2014).

1.2.2 Compartment Pressure Monitoring

Once a surgeon suspects a development of acute limb CS, diagnosis requires confirmation by the actual measurement of intra-compartmental pressure (ICP) (Hargens and Ballard 1995). Thus, measurement of ICP is a valuable tool for providing objective criteria for the diagnosis of acute CS, provided the proper technique is used. In order to capture the peak ICP value, measurements should be taken at the level of the fracture, as well as at sites up to 5 cm proximal and distal to injury (Heckman, Whitesides et al. 1994). In addition, pressures should also be measured in the other compartments of the affected limb, to ensure that a compartment syndrome is not missed.

The indications for ICP measurement include unconscious patients (Gelberman, Garfin et al. 1981, Hargens, Akeson et al. 1989, Schwartz, Brumback et al. 1989); difficult-to-assess patients (e.g. young children, patients with psychiatric problems, or those under the influence of narcotics) (Whitesides,

Haney et al. 1975); patients with equivocal signs and symptoms (Gelberman, Garfin et al. 1981), especially when accompanied by nerve injury (Whitesides, Haney et al. 1975, Wright, Bogoch et al. 1989); and patients with multiple injuries (Schwartz, Brumback et al. 1989). Additionally, all tibial diaphyseal fractures (especially those in young men), high energy distal radial and forearm diaphyseal fractures in young patients, high energy fractures of the tibial metaphysis, and soft tissue injury or bleeding diathesis should also be monitored (McQueen, Gaston et al. 2000).

1.2.2.1 Devices for ICP Measurement

Current routine clinical compartment pressure monitoring uses one of the three main types of invasive devices: needle manometer, wick catheter (an adaptation of needle manometer), slit catheter (a modification of needle manometer), or electronic transducer-tipped catheter (Hargens and Ballard 1995). Non-invasive ICP measurements, using near-infrared spectroscopy (NIR) to measure tissue oxygenation (Arbabi, Brundage et al. 1999), have also been proposed as an additional diagnostic tool, but require further research prior to their clinical use (Arbabi, Brundage et al. 1999, Gentilello, Sanzone et al. 2001, Katz, Nauriyal et al. 2008). Additionally, it has been demonstrated that temperature differences between the thigh and the foot show a unique pattern in individuals with acute CS (Katz, Nauriyal et al. 2008).

The needle manometer consists of a 20cc syringe full of air, attached to a column that contains both air and saline. The ICP is the pressure required to

flatten the meniscus between the saline and the air (Whitesides, Haney et al. 1975). The technique had been modified by Matsen et al (Matsen, Winquist et al. 1980) to measure ICP as the amount of pressure required to overcome the pressure in the circuit and infuse a small amount of saline into the compartment. While this method is simple and low-cost, it is the least reliable, as the needle can easily be occluded (Moed and Thorderson 1993).

The wick catheter is an adaptation of the needle manometer in which fibers project from the end of the catheter (Hargens and Ballard 1995). The fibers prevent tissue plugging, thus maintaining patency of the catheter to improve accuracy (Hargens and Ballard 1995). Disadvantages of this device include possible occlusion of the catheter tip by a blood clot and air in the fluid column, yielding falsely low measures.

The slit catheter, described by Rorabeck et al (Rorabeck, Castle et al. 1981) is another modification of the needle manometer technique that relies on the principle of increased surface area and increased patency (Hargens and Ballard 1995). The tip of the catheter is cut longitudinally, forming plastic petals. A fluid column, connected to a transducer, measures pressure.

Transducer-tipped catheters designed with the transducer housed in the catheter tip have improved the accuracy of compartment measurements (Hargens and Ballard 1995). An early variant of this was the solid-state transducer intra-compartmental catheter (STIC), which offered increased accuracy compared to the slit and wick catheters; the disadvantage is that it still relies on an infusion for pressure measurement (McDermott, Marble et al. 1984).

This is not the case with the newer electronic transducer-tipped systems: electronic devices are independent of limb position and the height of the pressure transducer and do not require calibration (Mubarak, Hargens et al. 1976, Mubarak, Owen et al. 1978, McDermott, Marble et al. 1984, Moed and Thorderson 1993, Willy, Gerngross et al. 1999). Disadvantages of these devices are their high cost and difficulty with re-sterilization.

1.2.2.2 ICP Measurement Threshold

Although controversial, the role of ICP measurement in acute CS remains valuable. While the comparative benefit of ICP measurements relative to clinical assessment and the definition of an ICP measurement determining the need for fasciotomy are unclear, appropriately utilized ICP monitoring is a valuable diagnostic tool. Continuous compartment pressure monitoring decreases the delay to fasciotomy and may, therefore, decrease the long-term complications of the disorder (McQueen, Christie et al. 1996). In addition, ICP monitoring confirms clinical findings in difficult cases.

While ICP monitoring is utilized in the diagnosis of acute CS, there is no clear protocol for a specific pressure threshold at which fasciotomy should be carried out. The threshold ICP for decompression has been listed as 30mmHg (Mubarak, Owen et al. 1978), 40mmHg (Schwartz, Brumback et al. 1989) and 45mmHg (Matsen, Winquist et al. 1980). Whitesides et al (Whitesides, Haney et al. 1975) proposed the idea that the differential pressure (ΔP) is indicative of tissue ischemia. They suggested that tissue ischemia began when the difference

between ICP and diastolic pressure was 20mmHg (Whitesides and Heckman 1996). McQueen et al (McQueen and Court-Brown 1996) recommended that the threshold ΔP be 30mmHg, based on the retrospective observation that this value lead to no apparent missed cases of acute compartment syndrome. As of this day, many trauma surgeons prefer ΔP to the use of an absolute ICP threshold. The advantages of a differential pressure threshold include better utility in hypotensive trauma patients and a lower overall fasciotomy rate, compared to an absolute pressure threshold (Matsen, Winquist et al. 1980, McQueen and Court-Brown 1996).

1.2.3 Consequences of Missed Diagnosis of CS

Early diagnosis of acute CS is absolutely critical to its successful management and subsequent clinical outcome. The failure of timely diagnosis appears to be the single most important cause of adverse outcomes (Matsen and Clawson 1975, Rorabeck 1984, McQueen, Christie et al. 1996, Mars and Hadley 1998). Early diagnosis of CS is facilitated by the recognition of patient risk factors, understanding of the early clinical symptoms of CS, and the judicious use of compartment pressure monitoring (Matsen, Winquist et al. 1980, McQueen, Gaston et al. 2000). Risk factors for the development of acute compartment syndrome include gender (males are more prone to CS than females), young age group (below 30 years of age), tibial fracture, high-energy forearm fracture, high-energy femoral diaphyseal fracture and bleeding diathesis or anticoagulation (McQueen, Gaston et al. 2000).

Missed or late diagnosis of acute CS can result in serious complications: muscle infarction, muscle contracture, secondary deformity, weakness, and neurologic dysfunction (Whitesides and Heckman 1996). Less common sequelae include infection, gram-negative sepsis, amputation and end-organ involvement (Whitesides and Heckman 1996). Time from onset to necrosis is variable, with an accepted upper limit of 6 hours (Elliott and Johnstone 2003). Determination of the exact time of onset of acute compartment syndrome is often difficult, as it may not parallel the onset of injury.

Once irreversible myoneural ischemia occurs, some degree of permanent neurological deficit and muscle dysfunction will always be present. Depending on the proportion of tissue involvement, the severity can range from mild weakness to ischemic contractures. If there is a sufficient amount of muscle tissue involved, CS can lead to crush syndrome: rhabdomyolysis, renal failure (secondary to myoglobinuria) and shock (Sanghavi, Aneman et al. 2006, West 2007). Limb amputation will not be life saving at this point.

Missed or late diagnosis is often a result of clinical inexperience, lack of suspicion, or a confusing clinical presentation (McQueen, Christie et al. 1996). Altered pain perception, as seen with changes in level of consciousness, regional anesthesia, patient-controlled analgesia and nerve injury are risk factors for late diagnosis (Mubarak and Wilton 1997, Harrington, Bunola et al. 2000). Maintaining an appropriate index of suspicion is important in preventing the negative sequelae of late-diagnosed acute compartment syndrome, as well as malpractice litigation (Bhattacharyya and Vrahas 2004).

1.3 THERAPEUTIC APPROACH TO CS

The management goals of acute CS are to minimize permanent injury of the affected limb by restoring microcirculation to the muscle and nerve, and therefore avoiding the sequelae of ischemic contracture. While non-operative techniques may be employed initially in *impending* CS, surgical decompression of all involved compartments through fasciotomy remains the only gold standard therapy for acute *established* CS to this day, provided the diagnosis of CS is made within the recommended surgical window of 6-8 hours (Matsen, Winquist et al. 1980, McQueen and Court-Brown 1996, Lawendy and Sanders 2010). Given the fact that the consequences of delaying the fasciotomy are severe, non-operative measures have been shown to have a very limited role; rather, medical management is restricted to an adjunctive role supplemental to fasciotomy.

Although leg is most frequently involved (80% of all cases), followed by the forearm, CS has been reported in every muscle compartment of the upper extremity, lower extremity and trunk (McQueen, Gaston et al. 2000).

1.3.1 Limb Anatomy

1.3.1.1 Leg

The leg consists of tibia and fibula, and is divided into four compartments: anterior, lateral, superficial posterior and deep posterior (Figure 1.1) (Gray 2000) Muscles of the anterior compartment, innervated by deep peroneal nerve and vascularized through anterior tibial artery, are responsible for the dorsiflexion of the foot and ankle. The compartment is comprised of tibialis anterior, extensor

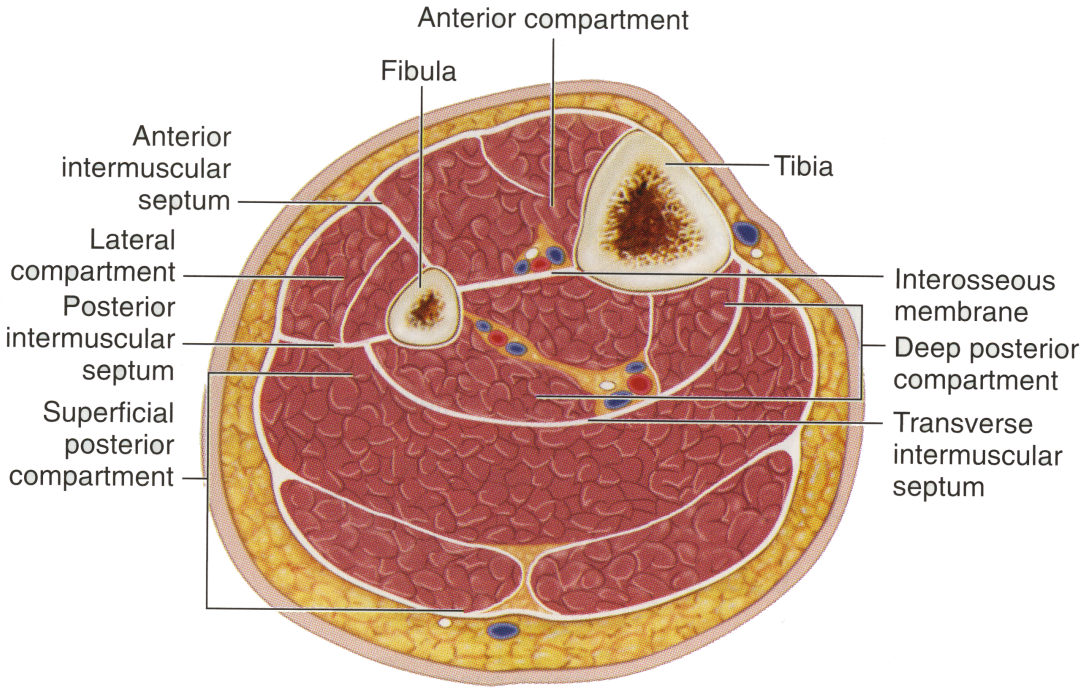


Figure 1.1. Compartments of the leg. Leg (shown in cross-section) consists of two bones, tibia and fibula, and several muscles that are separated into four different osseofascial compartments: anterior, lateral, superficial posterior and deep posterior.

Reproduced with permission from Lawendy and Sanders (2010).

digitorum longus, extensor hallucis longus and peroneus tertius muscles (Gray 2000). The compartment boundary is delineated medially by the extensor surface of the tibia, laterally by the intermuscular septum, and posteriorly by the extensor surface of the fibula and the interosseous membrane. The anterior compartment is completely enclosed by the deep fascia of the leg.

Muscles of the lateral compartment, innervated by superficial peroneal nerve and vascularized by anterior tibial and fibular arteries, are responsible for plantarflexion and eversion of the foot. The compartment is comprised of peroneus longus and peroneus brevis muscles. The compartment boundary is delineated medially by the fibula, while the intermuscular septum surrounds it both anteriorly and posteriorly (Gray 2000).

Muscles of the posterior compartments, innervated by the tibial nerve and vascularized through posterior tibial, fibular and popliteal arteries, are responsible for plantarflexion of the foot and ankle. The deep posterior compartment consists of popliteus, flexor hallucis longus, flexor digitorum longus and tibialis posterior muscles. The anterior boundary is delineated by the fibula, interosseous membrane, posterior intermuscular septum and the posterior surface of the tibia, while the posterior boundary is delineated by the transverse intermuscular septum (Gray 2000).

The superficial posterior compartment consists of gastrocnemius, plantaris and soleus muscles, and its boundary is delineated anteriorly by the posterior intermuscular septum and posteriorly by the deep fascia of the leg (Gray 2000).

1.3.1.2 Forearm

Forearm is made up of the antebrachium and hand. The antebrachium consists of the radius and ulna, and is divided into three interconnected compartments: “mobile wad”, anterior (volar) and posterior (dorsal) (Gray 2000) (Figure 1.2). The compartments are separated by antebrachial interosseous membrane between the radius and ulna; fascial extensions from the antebrachial fascia anatomically sequester the mobile wad into its own compartment.

Muscles of the “mobile wad” (sometimes referred to as the lateral compartment) are innervated by the radial nerve and the posterior interosseous nerve; the blood supply comes primarily from the radial artery and the profunda brachii. The muscles of this compartment act collectively as elbow flexors (Gray 2000).

Muscles of the volar compartment, innervated primarily by the median nerve and vascularized by the ulnar and radial arteries, are divided into superficial, intermediate and deep groups; these are primarily involved with flexion and pronation (Gray 2000).

Muscles of the dorsal compartment, innervated by the posterior interosseous nerve and supplied by the branches of ulnar and radial arteries, are further subdivided into superficial and deep groups; they are primarily involved with the extension of the wrist and digits, as well as supination of the forearm (Gray 2000).

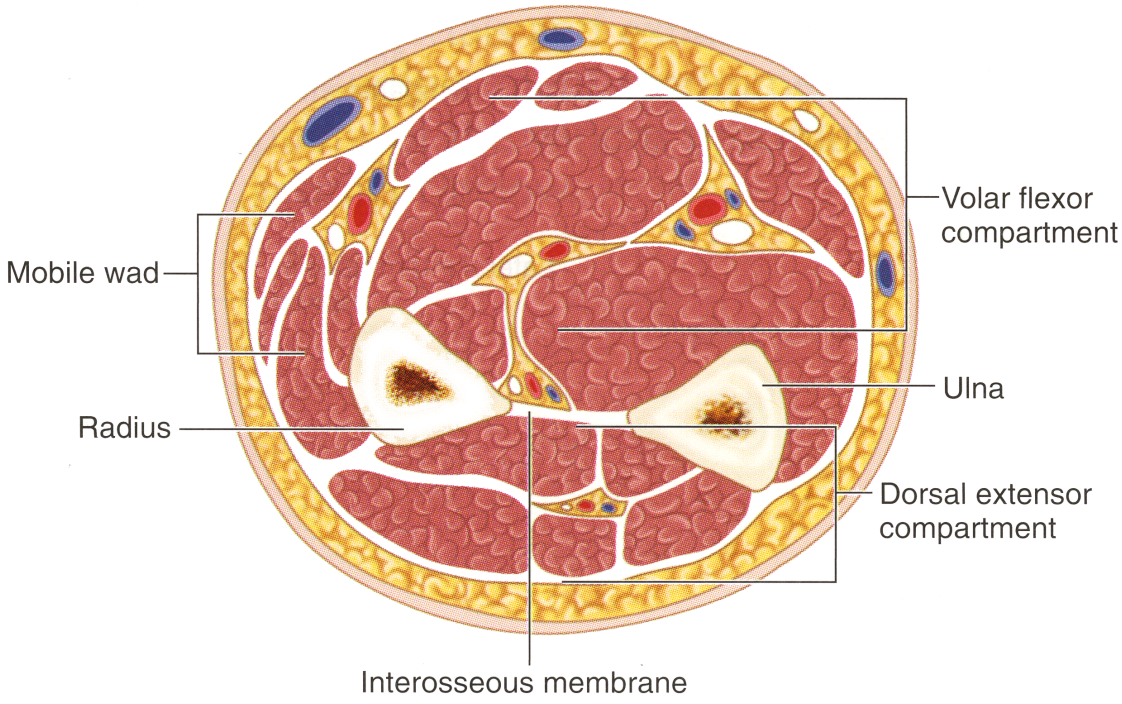


Figure 1.2. Compartments of the forearm. Forearm (shown in cross-section) consists of two bones, radius and ulna, and several muscles that are separated into three different osseofascial compartments: dorsal, volar and mobile wad.

Reproduced with permission from Lawendy and Sanders (2010).

1.3.2 Fasciotomy

Fasciotomy is a surgical procedure where the fascia is cut to relieve the elevated ICP within the affected compartments (Schmidt 2007, Lawendy and Sanders 2010). Wounds are usually left open for 48-72 hours prior to skin closure, with possible skin grafting necessary (Lawendy and Sanders 2010).

Fasciotomy, as a therapeutic option in patients with impeding Volkmann's contracture, was first described in 1911 by Bardenheuer (Bardenheuer 1911); he termed it an 'aponeurectomy'. Eventually, Murphy (Murphy 1914) suggested early fasciotomy as a means of preventing paralysis and contracture when the pressure within fascial-enclosed space was increased due to hemorrhage and edema, stressing urgency in terms of preservation of function and patient outcomes. Murphy's treatment is the mainstay of surgical practice today.

In 1926, Jepson was able to demonstrate that surgical exploration and drainage of affected limb could avoid myonecrosis, and a relatively normal limb function could be resumed (Jepson 1926). Other authors (Jorge 1925, Moulonquet and Seneque 1928, Massart 1935) also stressed the need for fasciotomy in selected patients.

The first detailed record of the actual operative technique for fasciotomy was provided by Benjamin (Benjamin 1957), who described the surgical approach to the forearm. This was followed by Eichler and Lipscomb (Eichler and Lipscomb 1967), Matsen and Clawson (Matsen and Clawson 1975), Eaton and Green (Eaton and Green 1972), Gelberman (Gelberman, Zakaib et al. 1978) and Henry (Henry 1973).

While initial fasciotomy technique descriptions came from the studies of the forearm, Reneman undertook the discussion of the proper surgical exposures during fasciotomy in the lower limb (Reneman 1975). Almost concurrently, Mubarak (Mubarak and Owen 1977) and Matsen (Sheridan and Matsen 1976) also released detailed descriptions of their techniques for leg fasciotomy; all of these are still in use today.

1.3.2.1 Surgical Techniques for Fasciotomy

In the leg, three surgical techniques for complete fascial release are most commonly described: two-incision fasciotomy (Mubarak and Owen 1977), single incision perifibular fasciotomy (Matsen 1979), and fibulectomy (Ernst and Kaufer 1971) (Appendix I). In the forearm, most CS cases can be adequately treated by the release of superficial volar compartment (Matsen, Winquist et al. 1980, Ronel, Mtui et al. 2004); however, to avoid the possibility of missed CS, most surgeons prefer the technique where both volar and dorsal compartments are released at the same time (Jones, Santamarina et al. 2010) (Appendix I).

1.3.3 Complications of Fasciotomy

Fasciotomy, although being a gold standard therapy for CS (independent of its etiology), is not without complications – it is a procedure that carries significant risks, affecting patient morbidity and mortality. Possible complications include skin sloughing, infection, nerve, blood vessel and muscle damage, as well as scarring (Schmidt 2007). Some patients have been found to develop

deep chronic venous insufficiency (Bermudez, Knudson et al. 1998); 15-40% of patients reported wound healing complications (Johnson, Weaver et al. 1992, Heemskerk and Kitslaar 2003), neurological and vascular injury; 35% of patients suffered excessive bleeding, infection, scarring.

In their retrospective study of trauma patients undergoing fasciotomy, Dover et al found that 20% of patients developed early post-operative complications; of these, severe symptoms were found in 80% of patients. After recovery, 70% of patients suffered persistent symptoms, which severely limited them either occupationally or socially (Dover, Marafi et al. 2011, Dover, Memon et al. 2012)

Fitzgerald et al retrospectively assessed complications of fasciotomy in both upper and lower extremities over an 8-year period (Fitzgerald, Gaston et al. 2000). They found that 77% had neurologic symptoms, such as altered sensation of wounds, and one in every ten patients had chronic pain associated with their fasciotomy wounds. Other frequent complications included dry skin, pruritus, and discolouration of wounds. Chronic swelling, tethering of tendons and scars, recurrent ulceration, and muscle herniation were also reported. The effect on patient's life was also detrimental, as 28% changed hobbies and 12% changed occupation secondary to the complications of their fasciotomy. More than 20% of patients covered their scars due to the aesthetic appearance of the wound (Fitzgerald, Gaston et al. 2000).

CS may recur in as many as 3 to 20% of cases after fasciotomy (Barr 2008). Causes include excessive formation of scar tissue and inadequate initial release of the fascia (Schmidt 2007).

Timing of fasciotomy is absolutely critical in acute CS, as delay to treatment is associated with increased complications and negative outcomes (Finkelstein, Hunter et al. 1996). Delay to fasciotomy of greater than 12 hours was found to increase the rate of infection to 28% versus 7.3% in patients treated early (Williams, Luchette et al. 1997). In one of the largest series in the literature, Ritenour et al (Ritenour, Dorlac et al. 2008) found significant complications secondary to fasciotomy revision surgery in military combat casualties. In their retrospective study of 336 patients who underwent 643 fasciotomies, they found an association between fasciotomy revision and increased rates of muscle excision, as well as a three-fold increase in mortality. Furthermore, delayed fasciotomies doubled the rate of amputation and increased the mortality rate fourfold, as compared with patients who underwent early fasciotomies (Ritenour, Dorlac et al. 2008).

Thus, despite being the most effective treatment for CS, fasciotomy is not trivial to patient outcomes, and techniques, timing, and alternate therapies need to be further investigated.

1.3.4 Non-Operative Treatment of CS

Before 1911, CS treatments had placed emphasis on managing the sequelae of ischemic contracture. Thus, non-operative methods consisted mainly

of limb mobilization and muscle stretching. Today, in a patient who presents with a tight-fitting cast or occlusive dressing with increased neuritic and vascular symptoms, the removal of a cast or occlusive splints is the initial treatment of impending CS; if symptoms persist, fasciotomy is indicated.

Non-operative management of CS is used only in the cases where fasciotomy is absolutely contraindicated: in cases where the affected limb is non-viable due to multiple injuries or severe ischemia, which would almost certainly lead to multiple organ dysfunction (i.e. reperfusion syndrome) (Schmidt 2007). Thus, fasciotomy is the only gold standard therapy for CS. Given the fact that the consequences of delaying the fasciotomy are severe, non-operative measures are restricted to an adjunctive role supplemental to fasciotomy, and may be employed to attempt the prolongation of the window between the onset of CS and the time when irreversible changes occur.

Lately, several non-operative options have been researched in various animal models of CS: mannitol (Daniels, Reichman et al. 1998), vitamin C (Kearns, Daly et al. 2004), N-acetyl cysteine (NAC) (Kearns, O'Briain et al. 2010), non-steroidal anti-inflammatory drugs (Manjoo, Sanders et al. 2010), tissue ultrafiltration (Odland, Schmidt et al. 2005), and hyperbaric oxygen therapy (Strauss, Hargens et al. 1983, Strauss, Hargens et al. 1986).

Thus, while clinical trials are needed to assess their efficacy, these additional medical techniques may prove to be effective in patients presenting with an impending CS.

1.3.4.1 Mannitol

Mannitol is a sugar alcohol that is clinically used as an osmotherapeutic agent, to reduce intracranial pressure after head trauma (Cruz, Minoja et al. 2001), and in oliguric renal failure (Alvarez, Chatwin et al. 2000), among other things. Mannitol has been shown to increase water and sodium excretion, thereby decreasing extracellular fluid volume.

Mannitol has been administered in canine and rabbit models of CS, where it appeared to act, presumably, by reducing the edema (Ricci, Corbisiero et al. 1990, Better, Zinman et al. 1991, Oredsson, Plate et al. 1994, Daniels, Reichman et al. 1998). Case studies have been reported where fasciotomy was successfully averted following the use of mannitol in the context of clinically diagnosed CS (Daniels, Reichman et al. 1998, Gold, Barish et al. 2003).

1.3.4.2 L-Ascorbic Acid

L-ascorbic acid (vitamin C) is an essential nutrient produced by many animals and plants. Although a requirement of normal nutrition, humans and some other vertebrates lack the ability to produce it, and thus must obtain it from their diet (Sorice, Guerriero et al. 2014).

Vitamin C is necessary for the formation of collagen within tissues; its deficiency causes scurvy (Sorice, Guerriero et al. 2014). In addition, it is a potent anti-oxidant that appears to play an important role in immune function (Jacob and Sotoudeh 2002).

It has been demonstrated that the administration of vitamin C in a rat cremaster muscle model of CS was capable of preserving muscle function, decreasing myeloperoxidase (MPO) activity and tissue edema (Kearns, Daly et al. 2004).

1.3.4.3 N-Acetyl Cysteine

N-acetyl cysteine (NAC) is a precursor to glutathione, a potent anti-oxidant. NAC has been shown to have many clinical uses: protection of hepatocytes in acetaminophen overdose (Williamson, Wahl et al. 2013), nephroprotective agent in radiocontrast-induced nephropathy (Anderson, Park et al. 2011), as a mucolytic agent (emphysema, bronchitis, bronchiectasis, pneumonia, chronic obstructive pulmonary disease, pulmonary fibrosis) (Santus, Corsico et al. 2014), alleviation of cyclophosphamide-induced hemorrhagic cystitis (Palma, Villaca Junior et al. 1986), and interstitial lung disease (Santus, Corsico et al. 2014).

Administration of NAC in a rat cremaster muscle model of CS has been shown to preserve muscle contractility (Kearns, O'Briain et al. 2010).

1.3.4.4 NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that interfere with arachidonic acid metabolism, via inhibition of cyclooxygenase (COX) enzyme. Two isoforms have been identified: the constitutively expressed

COX-1, and inducible COX-2. COX-2 expression can be upregulated in response to inflammatory stimuli and pro-inflammatory cytokines (Jan and Lowry 2009).

Activation of leukocytes appears to play a significant role in the generation of tissue injury seen in CS (Lawendy, Bihari et al. 2015). As such, anti-inflammatory medications may prove to be of benefit. It has been demonstrated that administration of indomethacin, a COX-2 inhibitor, in a rat model of CS led to an improvement in tissue perfusion and viability (Manjoo, Sanders et al. 2010).

1.3.4.5 Tissue Ultrafiltration

Tissue ultrafiltration has been used to reduce ICP by reducing fluid volume, thus decreasing the magnitude of tissue swelling (Odland, Schmidt et al. 2005). Odland, Schmidt et al (Odland, Schmidt et al. 2005) have found that application of tissue ultrafiltration resulted in significant decrease in tissue injury in a porcine model of limb CS, presumably by lowering the actual fluid volume, hence tissue pressure, within the affected compartment.

1.3.4.6 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy involves medical use of oxygen at levels higher than atmospheric pressure. The equipment consists of a hyperbaric pressure chamber and a means of delivering 100% oxygen (normal atmospheric oxygen content is approximately 21%). In the context of CS, the mechanism of action is thought to reduce edema within the affected compartment by inducing vasoconstriction in response to high oxygen, while maintaining oxygen perfusion

at lower perfusion pressure (Nylander, Nordstrom et al. 1987). Some case studies reported success in averting fasciotomy in patients with CS (Strauss, Hargens et al. 1983, Wattel, Mathieu et al. 1998, Gold, Barish et al. 2003). Unfortunately, this method has limited availability, due to the need for very specialized equipment.

Hyperbaric oxygen has also been shown to improve wound healing, thereby reducing the need for amputation and unnecessary surgical procedures (Roeckl-Wiedmann, Bennett et al. 2005).

1.3.5 Outcomes

It is very difficult to determine the consequences of CS, since the injury itself might contribute to adverse outcomes. Very little published materials are available that specifically address the influence of fasciotomy on patient outcome. Mortality rates of 11-15% and amputation rates of 11-21% have been previously reported (Heemskerk and Kitslaar 2003). The outcome of patients with tibial fractures appeared to depend on the timing of fasciotomy: delayed fasciotomy resulted in muscle weakness and contractures, and higher risk of fracture healing complications (McQueen, Christie et al. 1996), as well as increased chance of death and amputations (Finkelstein, Hunter et al. 1996).

In their review of lower limb fasciotomy, Heemskerk and Kitslaar (2003) found that 45% of patients had good limb function, 28% had successfully salvaged limbs with diminished function, 12% had to have a limb amputation and 15% died. While all of the patients had serious overall morbidity, the only factor

predictive of poor outcome was patient age greater than 50 years; the underlying diagnosis did not contribute to the results after fasciotomy (Heemskerk and Kitslaar 2003).

While they might be considered non-life threatening, the scars caused by fasciotomies are not negligible to patients' functional outcomes: the study by Giannoudis et al (2000) found that patients who find their wounds aesthetically unappealing reported significantly poorer health-related quality of life as compared to patients who had no problem with the appearance of the wound. Rate of wound closure and need for skin graft were also associated with increased pain and discomfort (Giannoudis, Nicolopoulos et al. 2002).

1.4 PATHOPHYSIOLOGY OF CS

In order to improve diagnosis and treatment of CS, a much better understanding of its pathophysiology is needed; this would allow for the development of methods that would lessen (or prevent) tissue damage by targeting the specific mechanisms that contribute to the development and consequences of this condition. One common 'prerequisite' for the formation of CS is the rigidity of fascia; its unyielding nature (Gratz 1931) prevents expansion of tissue volume to compensate for an increase in fluid within the tissue. While multiple explanations for the complex pathophysiology of CS exist, the final commonality to all appears to be cellular anoxia and its consequences.

1.4.1 Historical Progression of Understanding CS Pathophysiology

Historically, it was Volkmann, in 1881, who first suggested that limb paralysis due to CS was secondary to the interruption in arterial blood supply, producing ischemia (von Volkmann 1881). Following Volkmann, Leser in 1884 designed animal models to test the idea; his experiments were crucial in confirmation of the muscle necrosis as a part of the condition (Leser 1884). Building on these studies, Hildebrand in 1890 demonstrated nerve involvement in addition to necrosis. Hildebrand was one of the first to coin the term “Volkmann’s contracture” (Hildebrand 1906). In 1909, based on his extensive retrospective review, Thomas also felt that neural tissue involvement was paramount to the understanding of the clinical presentation of CS; his findings were supported by the fact the deformity in Volkmann’s ischemic contracture was a claw hand, often caused by nerve damage to the upper extremity, although the observed deformity in Volkmann’s contracture was seen far more rapidly than what was observed with nerve compromise (Thomas 1909). In 1900, Bernays provided detailed description of the pathology of ischemic muscle and associated contracture, also making a reference to potential litigation and physician liability in these cases (Bernays 1900).

In contrast to the previous ideas of arterial and nerve involvement, Murphy drew attention to the venous obstruction as a possible factor in contracture formation (Murphy 1914). His idea was then followed by Brooks, with the concept of increased pressure (as initially described by Volkmann) remaining as the early consistent feature and the cause of muscle damage in CS (Brooks 1922). In

1926, Jepson demonstrated that the occlusion of an extremity by the means of a tourniquet was always followed by an edema (Jepson 1926). Both Brooks and Jepson believed that the re-establishment of circulation to compromised tissue could inevitably contribute to the CS pathology; this is in full agreement with today's understanding of ischemia-reperfusion injury.

Subsequently, Leriche (Leriche 1928), Griffiths (Griffiths 1940) and Foisie (Foisie 1942) all noticed the correlation between the 'arterial spasm' and tissue injury, and thus tried to restore the notion of Volkmann that it was the arterial injury that caused ischemic paralysis. Although erroneously interpreting the cause of CS, Griffiths advocated that the treatment should be aimed at exploring the artery in an impending Volkmann's contracture, inadvertently leading to secondary application of fasciotomy as a necessity of the surgical dissection (Griffiths 1940).

The biggest shift in the paradigm of understanding the CS pathophysiology came during the World War II: in 1941, Bywaters and Beall noticed that the revascularization of injured limbs in otherwise stable patients led to decreased urine output, systemic deterioration, multi-organ failure and eventual death, even when the injured limbs had been amputated (Bywaters and Beall 1941). This led to the discovery of 'crush syndrome', defining the clinical findings associated with what is known today as a severe 'reperfusion syndrome'.

Up to this point, few case reports regarding lower extremity involvement had been documented. Hughes (Hughes 1948), and later Mavor (Mavor 1956) were among the first to present clinical cases of atraumatic onset of ischemic

necrosis in the lower extremity secondary to strenuous exercise. The link between elevation of ICP and surrounding fascia, and its effects on vascularity was an important milestone and observation in defining the pathophysiology of Volkmann's ischemia. Mavor also independently demonstrated that with decompression of the fascial compartments, clinical resolution of symptoms was obtained (Mavor 1956). Both Hughes and Mavor brought forward the understanding that Volkmann's ischemia is not isolated to the upper extremity; they stressed that it was equally as important that fascial release, independent of the underlying mechanism, may be a critical part of treating this disorder.

Benjamin first correlated increased tissue pressure with the extent of injury seen in CS (Benjamin 1957). His idea was then later reinforced by Reneman, who also emphasized the importance of fasciotomy and discussed the proper surgical exposures during fasciotomy (Reneman 1975). Thus, by the mid-1970s, increased tissue pressure became accepted as the common basis of the CS disease process.

Matsen, in his paper on the unified concept of CS, synthesized the state of knowledge of CS up to that point (Matsen 1975). He confirmed that not only was CS not isolated to the upper extremity, but also that an increase in tissue pressure was a critical feature in the pathophysiology of the condition – fasciotomy provided an effective treatment. Once the increased pressure became accepted as the cause, research could then focus on the pressure measurement and definition of the threshold for the treatment.

Whitesides et al (Whitesides, Haney et al. 1975) developed the methodology for measuring intra-compartmental tissue pressure, and suggested thresholds for fasciotomy. The methodology of ICP measurement was further improved upon by Mubarak et al (Mubarak, Hargens et al. 1976), Matsen et al (Matsen, Mayo et al. 1977) and Rorabeck (Rorabeck and Clarke 1978, Rorabeck, Castle et al. 1981). Rorabeck then confirmed that the absolute tissue pressures in CS might be quite variable, producing clinical symptoms in a range of pressures as low as 28mmHg to as high as 47mmHg; he suggested that the post-operative outcomes would most likely be dependent on the magnitude of tissue pressure the time of CS onset (Rorabeck 1984).

1.4.2 Current Understanding

While all previous research had focused on defining compartment pressure thresholds and diagnostics of CS, microscopic changes to the tissue and microvasculature were also being studied. Increased pressure within the closed compartment had been shown to create tissue ischemia, restricting the oxygen and nutrient delivery, thus failing to meet the metabolic demands of the affected tissue. Ischemia-generated anoxia was then shown to lead to microvascular perfusion derangements and severe inflammation (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015).

Thus, while the current understanding of CS pathophysiology is still not complete, microcirculatory dysfunction due to ongoing ischemia-reperfusion injury, early leukocyte activation and the ensuing inflammation appear to be the

driving forces behind generation of the CS, and the detrimental outcomes of CS injury.

1.5 MICROCIRCULATION IN CS

Normal skeletal muscle microcirculation is comprised of arterioles, capillaries and venules (Fig 1.3). Terminal vascular beds bring flowing blood into close proximity with parenchymal cells, where adequate exchange of materials between blood and tissues is met by large numbers of closely spaced capillaries. A single layer of vascular endothelial cells lines all vessels, sitting upon a condensed layer of extracellular matrix (basement membrane); the outer portions of the vessels are largely comprised of ordered layers of contractile mesenchymal cells (i.e. smooth muscle). While arterioles are comprised of smooth muscle cell-containing wall and have a divergent branching pattern, the post-capillary venules have no smooth muscle, and are instead lined by pericytes. Venules collect the blood from the capillaries, and have a larger cross sectional area than the corresponding arterioles, resulting in a lower flow velocity and wall shear stress. Thus, leukocyte adhesion (and inflammation) is normally restricted to venules; this is also augmented by the selective expression of adhesion molecules on venular (but not arteriolar or capillary) endothelium (Ley 2008).

As the ICP rises, generating CS, there is a disruption in microvascular perfusion, reducing oxygen and nutrient delivery to the tissue to a point where

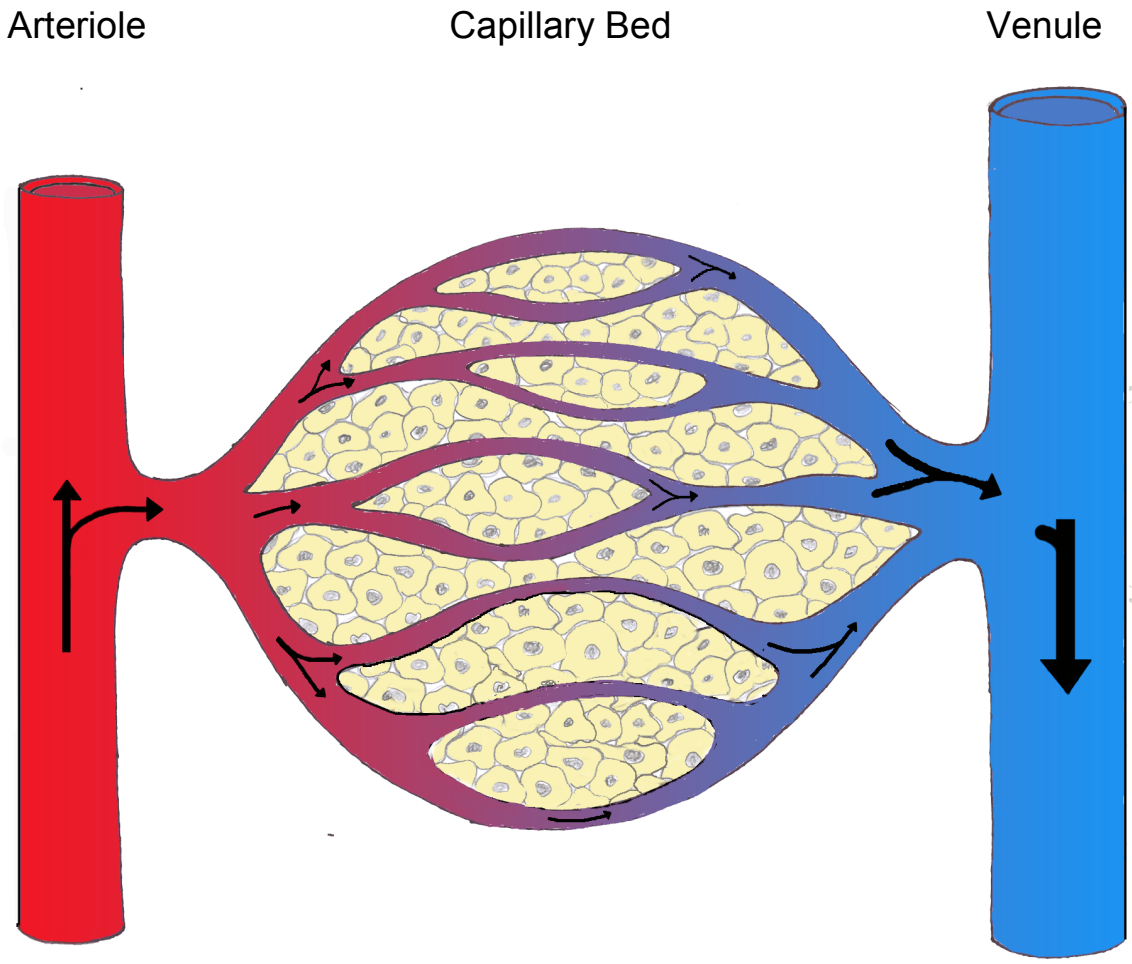


Figure 1.3. **Microcirculation of the skeletal muscle**. Normal microvascular unit is made up of an arteriole that branches into capillaries. Oxygenated blood flows from arterioles (red) into capillary beds; deoxygenated blood from the capillary beds is then collected in the post-capillary venules (blue).

the remaining perfusion can no longer meet the tissue demand (Lawendy, Sanders et al. 2011). This creates an ischemic insult, resulting in the production of reactive oxygen metabolites, oxidation of iron and membrane lipid peroxidation, causing significant damage to the affected tissues. Machinery to repair the damage is then initiated, triggering inflammatory response at both local and systemic levels.

Thus, CS-associated microvascular dysfunction and the resulting tissue damage appear to be attributable to at least two mechanisms stemming from the elevated ICP: tissue ischemia due to disruption of the normal microvasculature, followed by reperfusion-induced inflammatory reaction due to neutrophil activation (Figure 1.4).

1.5.1 Tissue Ischemia

While the actual pathophysiological mechanism generating tissue ischemia is not known, three major theories have attempted to explain the microvascular dysfunction and ischemia, as they relate to tissue pressure and CS: critical closing pressure, microvascular occlusion, and arterio-venous gradient.

1.5.1.1 Critical Closing Pressure Theory

The main assumption in the critical closing pressure theory is that the active closure of arterioles would occur at a critical pressure secondary to a drop in transmural pressure (i.e. the difference between the intravascular and tissue

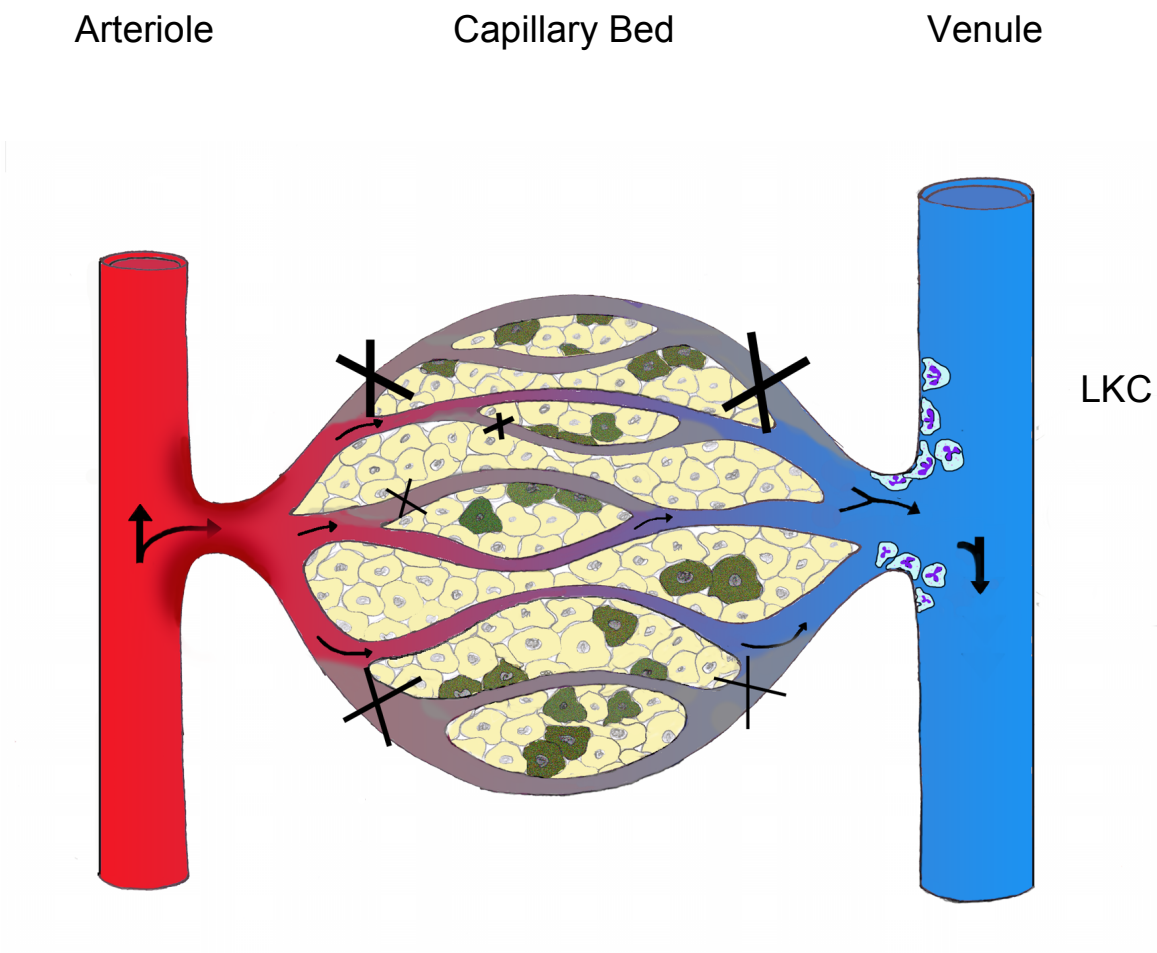


Figure 1.4. Microcirculatory dysfunction in compartment syndrome. Hypo-perfusion of the capillary beds (i.e. low-flow ischemia) generates tissue injury (brown cells), which, in turn, activates leukocytes in post-sinusoidal venules and results in inflammation. *LKC*, leukocytes.

pressures) (Burton 1951). Arterioles, due to their small size, would experience high tension, and thus require high arteriolar tissue pressure gradient to maintain patency. Thus, CS would be caused by either extreme elevation of pressure, or a significant reduction in the arteriolar tissue pressure gradient, producing arteriolar collapse (Ashton 1975), thereby rendering the tissue ischemic.

No supporting evidence for the theory, however, was produced when Vollmar et al (1999) tested the response of arterioles, capillaries and post-capillary venules of various diameters to graded pressure elevation. Using the skinfold chamber in Syrian gold hamsters, the study was not able to demonstrate any signs of arteriolar spasm or collapse (Vollmar, Westermann et al. 1999).

1.5.1.2 Microvascular Occlusion Theory

The main assumption of the microvascular occlusion theory is that CS results from the capillary occlusion as a consequence to an increase in the absolute compartment pressure (Hargens, Akeson et al. 1978). Thus, in response to an increase in the tissue pressure above the normal resting capillary pressure, there should be a concomitant reduction in capillary blood flow, producing muscle ischemia and subsequent tissue necrosis. Therefore, even a modest increase in pressure would result in critical impairment of capillary patency, leading to microvascular compromise.

The theory was, at least partially, discredited when the direct observation of rodent cremaster muscle subjected to sequential elevation of pressure did not produce vessel collapse, even when the pressure had been increased to a level

causing complete arrest of capillary flow; instead intraluminal pressure had also appeared to have increased to prevent the collapse of the vessel (Hartsock, O'Farrell et al. 1998).

1.5.1.3 Arterio-Venous Gradient Theory

The main prediction of arterio-venous (AV) theory is that an increase in tissue pressure, as seen in CS, would result in a net decrease of blood flow due to concomitant reduction of the AV pressure gradient (Matsen, Wyss et al. 1980). An increase in pressure would result in a rise in the intraluminal pressure – and the flow from arteries (i.e. high pressure) to veins (i.e. low pressure) depends on the pressure gradient maintenance. Thus, an elevation of ICP would diminish the AV gradient, reducing muscle blood flow and damaging the muscle. In addition, the decrease in AV gradient would also diminish the rate of clearance of the venous blood, causing fluid leakage into the interstitium, with edema ensuing, thereby elevating compartment pressure (Matsen and Krugmire 1978).

The theory appears to be supported, at least in part, by the experiments on hamster striated muscle by Vollmar et al (Vollmar, Westermann et al. 1999). Using graded external pressure changes, Vollmar found that minimal increase in external pressure could halt the flow through capillaries and venules while maintaining arterial blood flow. Venules responded by decreasing diameter and flow; reestablishment of a pressure gradient, by relieving the external pressure, was able to restore blood flow. The study demonstrated the vulnerability of the

microvasculature to pressure fluctuation, and the need for pressure gradient to generate flow from capillaries to venules.

The drawback of the AV gradient theory is that it relies on the assumption that the microvasculature passively responds to pressure. It does not account for the local adaptive responses of vasodilation, shunting of blood, endothelial structural changes, or the role of inflammation in the process (Gourgiotis, Villias et al. 2007).

1.5.2 Reperfusion and Inflammation

Reperfusion of previously ischemic tissue has been shown to elicit a strong inflammatory response in the microvasculature, characterized by vasodilation, production of inflammatory cytokines/chemokines, activation of complement cascade and leukocyte infiltration (Harris, Walker et al. 1986, Carden, Smith et al. 1990, Potter, Dietrich et al. 1993, Forbes, Carson et al. 1995, Gute, Ishida et al. 1998, Ley 2008, Gillani, Cao et al. 2011). As a result, there is an increase in the production of reactive oxygen species (ROS) and soluble inflammatory mediators, enhanced adhesion of leukocytes and platelets to vascular endothelium and increased microvascular permeability. All of these then culminate in tissue damage and can lead to impaired organ function.

Leukocyte recruitment to the site of injury (i.e. post-ischemic tissue), particularly neutrophils, occurs within the post-capillary venules (Forbes, Harris et al. 1996, Harris and Skalak 1996). A number of different cells are activated when ischemic tissues reperfuse with well-oxygenated blood: cells within the blood

vessel wall (endothelial cells) (Lefer, Tsao et al. 1991, Sabido, Milazzo et al. 1994), those of perivascular compartment (macrophages) (Gute, Ishida et al. 1998, Ley, Laudanna et al. 2007), and neutrophils (a class of polymorphonuclear (PMN) leukocytes) (Carden, Smith et al. 1990, Ley, Laudanna et al. 2007). Endothelial cells assume inflammatory phenotype: increased production of ROS, release of inflammatory cytokines, and changes in adhesion molecule expression to bind leukocytes (Seekamp, Warren et al. 1993, Schlag, Harris et al. 2001, Ley, Laudanna et al. 2007, Gillani, Cao et al. 2011).

1.5.2.1 Endothelial Activation

Resting endothelial cells are largely un-interactive with leukocytes and actually maintain leukocyte quiescence (Ley, Laudanna et al. 2007), probably due to the fact that adhesion molecules found at sites of inflammation are not expressed (like E-selectin or VCAM-1), or expressed at very low levels (like ICAM-1), and sequestered internally (like P-selectin).

Activation of the endothelial cells in response to reperfusion injury consists of three stages: immediate (within minutes), acute (within hours) and chronic (within days) (Ley and Reutershan 2006). Each step serves a different function, with the ultimate goal being the repair of the damaged tissue.

Immediate endothelial activation is triggered by many inflammatory chemokines, and results in endothelial degranulation, as well as endothelial cell contraction (Maier and Bulger 1996). P-selectin, normally stored within the Weibel-Palade bodies within the cytoplasm, is brought to the endothelial surface.

Its function is to facilitate leukocyte recruitment (Weibel and Palade 1964), by interacting with the P-selectin glycoprotein ligand-1 (PSGL-1) on white blood cells.

Acute endothelial activation is characterized by increased gene transcription and production of E-selectin as well as ICAM-1 (Kurose, Anderson et al. 1994, Gute, Ishida et al. 1998, Ley, Laudanna et al. 2007). Pro-inflammatory cytokines, particularly TNF-α and IL-1β are known triggers for this step.

Chronic endothelial activation serves as a remodelling process. While no intravital video microscopy data on leukocyte behaviour or blood flow during this phase exists, the process appears to be reversible once the cause of inflammation is resolved (Ley, Laudanna et al. 2007).

1.5.2.2 Increased Vascular Permeability

Endothelial cell activation triggers massive endothelial cell contraction, producing gaps between the adjacent endothelial cells. This results in increased vascular permeability for plasma proteins, producing protein-rich exudate in the extravascular tissue (Michel and Curry 1999). While it may augment the release of antibodies into the affected site, it also causes tissue edema and swelling, resulting in (temporary) loss of function and pain; this effect is most likely mediated by bradykinin, histamine and leukotrienes release (McDonald, Thurston et al. 1999).

1.5.2.3 Vasomotor Response

Strong vasodilatory response usually accompanies acute inflammation, resulting in several-fold increase in the blood flow to the inflamed tissue (Ley 2008). The principal mediator appears to be nitric oxide (NO), derived from the endothelium (Moncada, Radomski et al. 1988) In addition to increased oxygen delivery, vasodilation also increases the delivery of nutrients, glucose and leukocytes themselves. Leukocytes use glycolysis to produce energy (Ley and Reutershan 2006), not oxidative phosphorylation; thus vasodilation may also serve as a means of glucose delivery, to supply the neutrophil requirements. Moreover, vasodilation-triggered blood flow alterations most likely produce changes in shear, which may then facilitate better leukocyte-endothelial interaction.

1.5.2.4 Cytokine Release

Inflammation is regulated by the release of chemokines and cytokines (a subset of chemokines), both at tissue level and systemically (Jan and Lowry 2009). Of the 45 or so known chemokines, some 20 have been shown to have pro-inflammatory effects; a majority of them appear to promote neutrophil infiltration. Of the inflammatory mediators, TNF-α, IL-1β and IL-6 are three of the most important cytokines playing a role in reperfusion injury (Seekamp, Warren et al. 1993, Jan and Lowry 2009).

TNF-α is a cell signalling protein that is produced by many cell types, particularly by activated macrophages and neutrophils. Although the circulating

half-life of TNF-α is brief (Beutler, Milsark et al. 1985), its activity elicits many metabolic and immunomodulatory functions. Upon binding to TNF-α receptor, the cytokine is able to induce inflammation and apoptosis by controlling the expression of transcription factor NF-κB, MAPK (particularly the stress-related JNK group) and proteolytic caspases (Seekamp, Warren et al. 1993, Roebuck, Carpenter et al. 1999, Ley 2008). While there appears to be a lot of extensive cross-talk among the different pathways, such complicated signalling probably ensures that various cells with vastly diverse functions and conditions can all respond appropriately to inflammation.

IL-1β is produced by activated macrophages, monocytes, endothelial cells, fibroblasts, and appears to be involved in cell proliferation, differentiation and apoptosis. It mediates an inflammatory sequence similar to that of TNF-α (Yi and Ulich 1992).

IL-6 has pleiotropic functions in different organs and tissues. It is produced by macrophages and T-cells to stimulate immune response after trauma (Gebhard, Pfetsch et al. 2000), by osteoblasts to stimulate osteoclast formation (Hashizume and Mihara 2011), as well as by the muscle in response to contraction (Munoz-Canoves, Scheele et al. 2013), in response to inflammatory mediators, such as TNF-α and IL-1. It is a very long-lived cytokine that can be considered both pro- and anti-inflammatory, depending on the type of injury and the involved tissue. Its counter-regulatory function on the inflammatory cascade appears to be through the inhibition of TNF-α and IL-1 (Song and Kellum 2005).

1.5.2.5 Leukocyte Activation

Activated leukocytes display a very specific multistep behavioural sequence of leukocyte adhesion cascade, first described by Rudolph Wagner, a prominent pathologist of the nineteenth century (Wagner 1839). Both activated leukocytes and activated endothelium mediate this process by expressing different classes of adhesion molecules (selectins, integrins, Ig superfamily) in a temporally-coordinated fashion. The current paradigm consists of several steps: (1) capture or tethering by the endothelium, where leukocyte makes the first contact with the endothelium; (2) rolling along the endothelium, during which leukocytes sample chemokines presented by the endothelium (slow rolling); (3) chemokine release, which then triggers (4) firm adhesion; (5) adhesion strengthening, which then leads to (6) integrin clustering; and in the presence of appropriate endothelial/leukocyte stimuli, (7) transmigration occurs; the final result is (8) extravasation into the affected tissue (Ley, Laudanna et al. 2007) (Figure 1.5).

Initial leukocyte capture and rolling appear to be mediated by selectins (L-selectin on leukocytes, P-selectin and E-selectin on endothelial cells); slow rolling by integrins; lastly, firm adhesion and extravasation by the adhesion molecules of Ig superfamily: ICAM-1 (binds CD11/CD18, Mac-1 and LFA-1) on endothelium, VCAM-1 (binds VLA-4) on the endothelial surface, PECAM-1 on neutrophils (Albelda, Muller et al. 1991, Barreiro, Yanez-Mo et al. 2002, Yang, Froio et al. 2005). Neutrophil accumulation appears to be the cause (not a consequence) of ischemia-reperfusion-induced endothelial barrier failure.

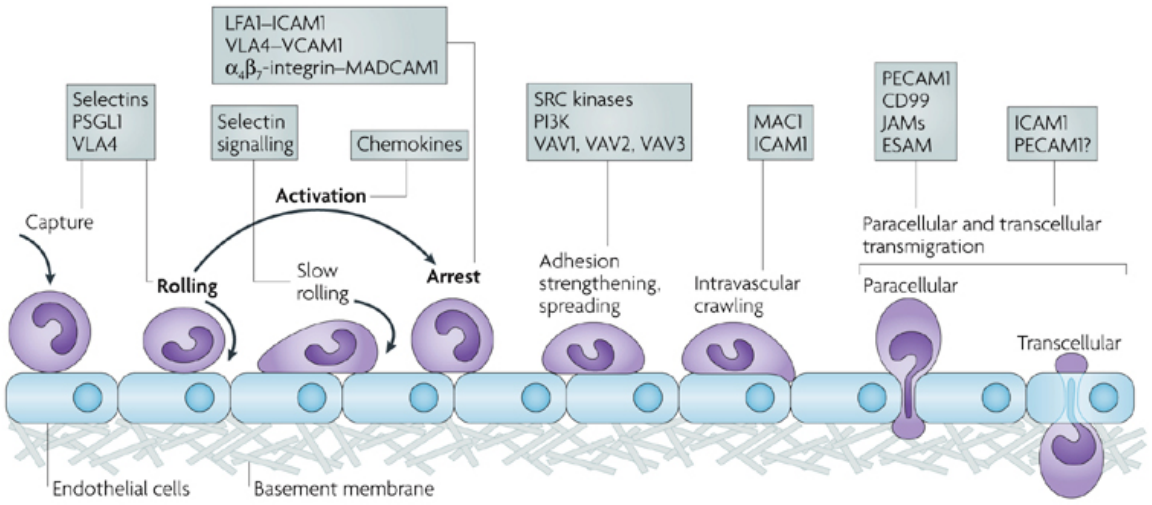


Figure 1.5. Leukocyte activation sequence in inflammation. Activated leukocyte paradigm consists of leukocyte capture/tethering, leukocyte rolling, firm adhesion, arrest and extravasation. The sequence is mediated by various adhesion molecules (selectins, integrins, Ig superfamily).

Adapted from Ley, Laudanna et al (2007).

Leukocyte rolling serves to sufficiently reduce the velocity of leukocyte movement along the endothelium, to allow for firm adhesion. Thus, the upregulation of P-selectin expression is very rapid (within 15-30 minutes of activation), as this molecule is normally stored in the pre-formed pools within the Weibel-Palade bodies of the endothelium, from where it can be rapidly mobilized to the cell surface in response to stimuli (e.g. reactive oxygen species, leukotrienes, histamine). E-selectin, on the other hand, is under transcriptional control, and as such, requires up to 3 hours to achieve peak expression. L-selectin is constitutively expressed on the leukocytes on the microvillus cell surface protrusions. Upon activation, leukocyte L-selectin interacts with endothelial cell P- and E-selectins, mediating leukocyte rolling (Ley, Laudanna et al. 2007). As leukocyte activation progresses, L-selectin is then rapidly shed from the cell surface via a protease-dependent mechanism.

The transition of rolling to firm arrest can be triggered by arrest chemokines (e.g. IL-8, GRO, MCP-1, MIP-1α). Firm adhesion and/or transendothelial migration are mediated by ICAM-1, VCAM-1, and PECAM-1 expressed on the surface of the endothelium, under the control of transcription factors NFκB and AP-1, resulting in increased expression of ICAM-1 and VCAM-1 within 4 to 6 hours. These molecules engage with leukocyte counter-receptors (i.e. CD11/CD18, Mac-1, LFA-1 and VLA-4) to mediate firm adhesion and/or transendothelial migration. CD11 (a and b) are constitutively expressed within most leukocytes, where they are stored in granules, and can be rapidly (i.e. within minutes) mobilized to the surface of leukocytes. The simultaneous rapid

up-regulation of CD11/CD18 and shedding of L-selectin on leukocytes upon activation enables leukocytes to rapidly transition between the rolling and firmly adherent states (Ley 2008).

Leukocyte transmigration can be triggered by chemoattractant transendothelial gradient. The whole process can take up to 25 minutes. Emigrating leukocytes encounter three distinct barriers: endothelial cells, endothelial basement membrane and pericytes. While the migration through the endothelial cell barrier can be rapid (within less than 2 minutes), penetration of the endothelial basement membrane takes much longer (upwards of 5-15 minutes). The process is driven by differential expression of adhesion molecules on the leukocytes and endothelial junctions. These include PECAM-1, ICAM-1, ICAM-2, JAM-A, JAM-B and JAM-C. Different molecules mediate leukocyte transmigration in either a stimulus-specific or leukocyte specific manner (Ley 2008).

1.5.2.6 Reactive Oxygen Species (ROS)

ROS are small molecules that are highly reactive due to the presence of unpaired outer orbit electrons. Oxygen radicals are produced as a byproduct of oxygen metabolism, and by anaerobic processes. The main areas of ROS production include mitochondrial electron transport chain, peroxisomal fatty acid metabolism, cytochrome P450 and the respiratory burst of phagocytic cells (Jan and Lowry 2009). Under normal conditions, host cells are protected from the

damaging effects of ROS by endogenous anti-oxidants such as superoxide dismutase, catalase and glutathione peroxidase.

Ischemia results in many changes to normal intracellular metabolism. One of these is the accumulation of hypoxanthine due to inadequate oxidative phosphorylation of ATP (Smith, Carden et al. 1989, Idstrom, Soussi et al. 1990); another is the conversion of xanthine dehydrogenase (normally kept oxidized by NAD^+-dependent mechanism) into xanthine oxidase (Granger 1988). Upon re-introduction of oxygen (i.e. reperfusion), xanthine oxidase will convert molecular oxygen into ROS, such as superoxide and hydroxyl radicals; additionally, superoxide will react with nitric oxide, producing peroxynitrites. ROS are known to attack cell membrane lipids (lipid peroxidation), proteins and glycosaminoglycans causing further tissue damage, which, in turn, triggers the inflammatory cascade by bringing leukocytes (neutrophils) to the affected tissue.

Neutrophils themselves are equipped with enzymatic machinery that is capable of producing respiratory burst: oxygen-, nitrogen- and chlorine-derived free radicals (Bellavite 1988, Weiss 1989, Hampton, Kettle et al. 1998). Activated neutrophil accumulation, and their subsequent degranulation (which leads to the release of myeloperoxidase, a free-radical producing enzyme) will then ultimately be the cause of endothelial barrier failure.

Unfortunately, while inflammation and ROS production serve a useful function (i.e. clean up of the diseased/dead cells and tissue repair), *overwhelming* inflammation will contribute to the extensive tissue and organ damage.

1.6 HEME METABOLISM AND OXIDATIVE STRESS

As multicellular life developed the ability to sustain itself by the use of oxygen gas (which is inherently toxic), it also had to evolve mechanisms that would allow it to survive oxidative stress. One of the most ubiquitous means to do this is that of heme oxygenase (HO), an enzyme whose presence and function is absolutely critical to the living organisms. HO deficiency in mammals is lethal – only one case of a human child deficient in this enzyme had been identified; the child did not survive beyond the age of 3 years (Yachie, Niida et al. 1999).

1.6.1 Heme Oxygenase

HO degrades heme by cleaving the heme ring at the α-methene bridge to form equimolar amounts of biliverdin (which is immediately converted into bilirubin by the enzyme biliverdin reductase), free iron and carbon monoxide (CO) (Ryter, Alam et al. 2006) (Figure 1.6). The correlation of endogenous CO found in the blood with hemoglobin-derived heme degradation, and the α-carbon selectivity of this process, predates the discovery of HO by several decades (Hallberg 1955).

It was Tenhunen et al who first characterized HO, a distinct enzyme system responsible for heme degradation (Tenhunen, Marver et al. 1968). Subsequently, three separate isoforms have since been defined: the constitutively expressed HO-2 and HO-3, and the inducible HO-1 (Maines, Trakshel et al. 1986, McCoubrey, Huang et al. 1997).

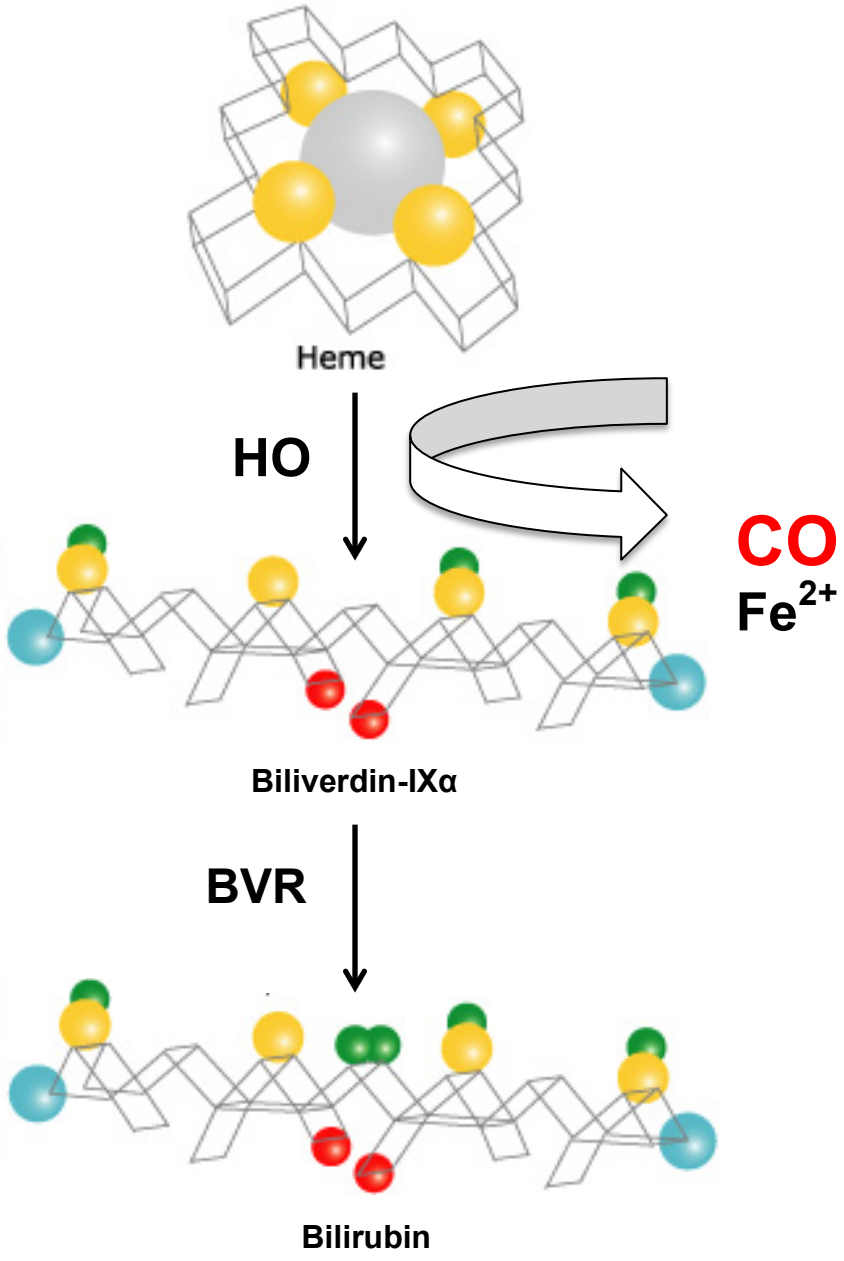


Figure 1.6. **Heme degradation pathway**. Hemoglobin-derived heme is broken down into biliverdin by heme oxygenase (HO), which is then rapidly converted into bilirubin by biliverdin reductase (BVR). Carbon monoxide (CO) and free iron (Fe^{2+}) are generated by this reaction.

Normally, stress-induced proteins are generated or activated by an organism in response to stressors such as fever, heat, infection, toxins or radiation (Choi and Otterbein 2002). Not only do they protect cells from immediate stress, but also fortify the organism to withstand future stresses originating from a different source. In the 1980s, a 32kD mammalian stress protein, known as the heat shock protein-32 (HSP32), had been identified (Shibahara, Muller et al. 1987). HSP32 was later proven to be HO-1, the inducible form of HO (Keyse and Tyrrell 1989).

Several studies have demonstrated that upregulation of HO, either by the use of various protoporphyrins (heme PPIX, cobalt PPIX) (Maines and Kappas 1977), or by transfecting animals with adenovirus containing HO gene construct, can be beneficial in protecting tissues from ischemia-reperfusion injury (Otterbein, Kolls et al. 1999, Otterbein, Lee et al. 1999, McCarter, Badhwar et al. 2004). Conversely, inhibition of HO (by pharmacological or gene knockout means) was found to be detrimental, leading to the conclusion that upregulation of HO provides protective benefits to the tissue (Dungey, Badhwar et al. 2006). Clinically speaking, however, upregulation of HO by adenoviral transfer may not be a feasible method to be employed in patients; therefore, the downstream byproducts of the HO-catalyzed heme degradation pathway have been examined for their contribution to the observed protective effects. Since HO activity results in production of bilirubin and CO, research has focused on the examination of the potential beneficial role of these compounds, particularly CO.

1.6.2 Carbon Monoxide

CO is a low-molecular weight diatomic molecule that occurs in nature as an odourless gas. CO is considered a ubiquitous pollutant, arising primarily from the partial combustion of organic molecules by oxidation of natural hydrocarbon pools or burning fossil fuels, automobile emissions, catastrophic events (e.g. volcanic emissions and forest fires), plant metabolism and oceanic activity.

CO is relatively stable in biological systems. It functions as heme iron ligand, and forms complexes with a number of hemoproteins and metalloenzymes (Coburn 1979, Maines 1997), binding only to reduced (ferrous) iron centres (Omura and Sato 1964).

1.6.2.1 CO Toxicity

Due to its invisibility and lack of odour, CO presents an especially dangerous inhalation hazard. Common causes of exposure are due to improper use of furnaces, engines, heaters, or incomplete combustion (i.e. in inadequately ventilated areas). Clinical manifestations of CO poisoning include dizziness, drowsiness, headache, vomiting and loss of motor coordination; prolonged exposure causes respiratory difficulty, disorientation, chest pain, loss of consciousness, coma and death (Weaver 1999).

While symptoms of hypoxic CO poisoning begin to appear at 20% carboxyhemoglobin (COHb) levels, death likely occurs in the range of 50-80% COHb (Weaver 1999). Inhalation studies have revealed that CO can cause oxidative damage in the brain, as demonstrated by an increase in lipid

peroxidation (Thom 1990) and apoptotic cell death (Piantadosi, Zhang et al. 1997).

In 1857, Claude Bernard first realized that CO is capable of binding heme within hemoglobin, leading to formation of COHb (Bernard 1857). In 1912, Douglas demonstrated that the binding of CO to hemoglobin is reversible (Douglas, Haldane et al. 1912). The affinity of CO for heme within hemoglobin has been shown to be approximately 240 times that of oxygen (Weaver 1999). There are four oxygen-binding sites within one molecule of hemoglobin at which CO competes for occupancy. It has been demonstrated that half saturation (i.e. the partial occupation of the binding sites by two CO molecules) inhibits the release of oxygen from the remaining heme groups. This results in a left shift of the oxy-hemoglobin dissociation curve, reducing the oxygen-carrying capacity of the blood, thus generating the anemic hypoxia that appears to account for the asphyxiating properties of CO (Weaver 1999).

The formation of COHb complex is reversible by the out-competition of CO in favour of oxygen. In some severe cases, hyperbaric oxygen therapy has been successfully applied as an antidote to CO poisoning (Weaver 1999).

1.6.2.2 Endogenous Sources of CO

A considerable amount of CO arises endogenously, as a byproduct of metabolism. At least 86% originates from heme metabolism, while the remaining fractions may arise from other metabolic processes, including lipid oxidation and xenobiotic metabolism (Vreman, Wong et al. 2000, Archakov, Karuzina et al.

2002). It was Coburn in 1967, who first demonstrated that CO in the body is derived from metabolic conversion of hemoglobin (Coburn, Williams et al. 1967), and estimated the rate of endogenous CO production at around 0.42ml/h (Coburn 1967).

Under normal conditions, in the absence of significant ambient CO, the majority of blood COHb comes from endogenous production, and corresponds to blood CO levels of 0.4-1% (Vreman, Wong et al. 2000). The values increase in the presence of CO in the environmental background: for example, cigarette smokers display, on average, 3-8% COHb (Vreman, Wong et al. 2000).

Certain pathological or toxicological conditions have been shown to lead to upregulation of HO-1 expression, which would produce increased blood CO levels: inflammation, physical stress, and environmental exposure to a variety of agents. As an example, several studies have demonstrated that the exhaled breath of patients suffering from asthma or COPD (i.e. pro-inflammatory conditions) contains increased levels of CO (Zayasu, Sekizawa et al. 1997, Kawane 2002).

1.6.3 Biological Effects of Carbon Monoxide

It appears that CO has profound influence on intracellular signaling processes, which culminate in anti-inflammatory, anti-proliferative, anti-apoptotic, and anti-coagulative effects. The physiological outcomes of CO action have been related to its endogenous production by both the constitutive (HO-2 and HO-3) and inducible HO (HO-1) activity. While it remains unclear whether exogenous

application of CO represents true physiological levels, it has been demonstrated that it can produce the effects similar to those that can be achieved by the induction of HO-1 protein.

1.6.3.1 Cellular Signalling

CO, as a gaseous molecule, has a very low reactivity; therefore, it can reach various cellular and molecular targets with great ease. Nevertheless, to-date, very few mechanisms have been defined over which CO has particular influence. CO has high binding affinity for transition metals and heme proteins containing iron in ferrous form (Fe^{2+}) (Kajimura, Fukuda et al. 2010), particularly hemoglobin and myoglobin. The selectivity depends on the intrinsic reactivity of ferrous heme, the chemical nature and geometry of the ligand, as well as steric constraint and electrostatic interactions of the bound ligand on the distal side of heme. The most common mode of action of CO in the biological systems appears to be the modulation of soluble guanylate cyclase (sGC) and the subsequent production of cGMP (Ryter and Otterbein 2004). The binding of heme iron within sGC by CO stimulates its activity, which, in turn, leads to several-fold increase in cGMP. For example, direct treatment of vascular smooth muscle cells with CO resulted in an increase in cellular levels of cGMP; hypoxia (which would cause an induction of HO-1, thus endogenous production of CO) also produced the same effect (Morita, Perrella et al. 1995). However, unlike NO (another potent activator of sGC), CO causes only a minor increase in the activity of this enzyme, due to the formation of a 6-coordinate complex (rather than 5-

coordinate complex, as is the case with NO – which causes more than 100-fold activation of the enzyme). When both CO and NO are present simultaneously at the site, NO will bind sGC with much greater affinity than CO; in addition, CO will modestly but significantly attenuate the activation of NOS, thus serving as a partial antagonist to NO-induced sGC effects (Kajimura, Fukuda et al. 2010).

Although sGC appears to be the most common cellular signalling pathway implicated in CO action, other intermediaries may also be involved. While these represent a downstream rather than primary target, as they do not bind CO directly, they include the modulation of various MAPK activation and stimulation of calcium-dependent potassium channel activity (Ryter, Otterbein et al. 2002).

Large conductance calcium and voltage-activated potassium channel (BK_{Ca}), implicated in the control of hypoxic response in the carotid body, the control of vessel relaxation and neuronal activity (Jaggar, Li et al. 2005), is another target for CO action. Additionally, other ion channels have also been found to be regulated by CO: epithelial sodium channels (Althaus, Fronius et al. 2009), ligand-gated P2X receptors (Wilkinson, Gadeberg et al. 2009), L-type calcium channels (Scragg, Dallas et al. 2008) and tandem P domain potassium channels (Dallas, Scragg et al. 2008).

Studies suggest that cystathionine β-synthase, one of the enzymes responsible for the synthesis of endogenously-produced hydrogen sulfide (another biologically active signalling gaseous molecule) may act as a CO sensor *in vivo* (Kajimura, Fukuda et al. 2010). There appears to be a lot of cross-talk between CO and other gas-transducing biologic systems (i.e. nitric oxide, oxygen

and hydrogen sulfide), although the exact purpose, control and mechanisms of this action still remain to be fully elucidated.

1.6.3.2 Vasodilation

CO appears to exert a variable and multimodal effect on vasodilation, which involves at least several mechanisms. The dilatory effects are usually attributed to the direct, endothelium-independent effects on vascular smooth muscle cells (including the modulation of sGC and cGMP, as well as the effect on the potassium channels), while indirect effects appear to affect the expression of endothelial-derived vasoconstrictors and myogenic factors (Motterlini and Otterbein 2010).

In 1978, Sylvester at al demonstrated that application of CO resulted in dilatation of pulmonary artery and a reversal of hypoxia-induced vasoconstriction in isolated perfused porcine lung (Sylvester and McGowan 1978). McFaul and McGrath demonstrated that CO was capable of reversing methoxamine-induced vasoconstriction in rat coronaries (McFaul and McGrath 1987). These effects have since been confirmed in rat thoracic aorta, pig, rabbit and dog coronary arteries (Lin and McGrath 1988, Graser, Vedernikov et al. 1990, Furchgott and Jothianandan 1991). Intact endothelium was not required in any of the above; tissue hypoxia caused by CO was also excluded as the driving force behind the observed effects.

Although activation of sGC and subsequent increase in cGMP plays a major role in CO-induced vasodilation in aorta, cGMP-independent mechanisms

of vasodilation have also been demonstrated in peripheral vasculature in some experiments, where CO appeared to directly activate BK_{Ca}, calcium-dependent potassium channels (Wang, Wang et al. 1997); inhibition studies using ryanodine, a known calcium release channel blocker, have shown that CO-induced vasodilation could be inhibited by this treatment (Jaggar, Leffler et al. 2002). Limited evidence has also been found that would suggest the direct association of HO-2 with large-capacity potassium channels in the carotid body (Williams, Wootton et al. 2004); however, it still remains to be confirmed whether the same mechanism would occur in peripheral vasculature.

Additionally, neural CO may also play an indirect role in vasoregulation by signalling in the autonomous nervous system (Verma, Hirsch et al. 1993).

1.6.3.3 Anti-Inflammatory Effects

Many studies, both *in vitro* and *in vivo*, have demonstrated that exogenous application of CO produces potent anti-inflammatory effects. For example, in an *in vitro* model of sepsis, stimulation of macrophages with LPS led to an increase in production of pro-inflammatory cytokines, particularly TNF-α (Otterbein, Bach et al. 2000). Exogenous administration of low dose CO inhibited this response, and when given as a pre-treatment, it also inhibited the expression of additional pro-inflammatory cytokines (IL-1β, MIP-1β) while augmenting the expression of anti-inflammatory cytokine IL-10.

Beneficial effects of exogenous application of CO in systemic inflammation have also been documented *in vivo*. Similar to HO-1 overexpression, Ott et al

(2005) and Scott et al (2009) demonstrated that low-dose inhaled CO was able to prevent microvascular dysfunction in the liver and small intestine following ischemia-reperfusion-induced systemic inflammatory response syndrome (SIRS), respectively (Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009). Song et al demonstrated anti-inflammatory effects of CO in orthotopic lung transplant (Song, Kubo et al. 2003).

1.6.3.4 Anti-Apoptotic Effects

Cell death can be classified according to its morphological appearance, enzymological criteria, functional aspects or immunological characteristics. Apoptosis describes a specific morphological aspect, characterized by rounding-up of the cell, retraction of pseudopods, pyknosis, chromatin condensation, karyorrhexis, little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing (although its integrity is maintained until the final stages of the process), and engulfment by resident phagocytes (Kroemer, Galluzzi et al. 2009). Apoptosis is mediated by the activation of proteolytic caspases. Under inflammatory conditions, increased recruitment of neutrophils to the site of injury contributes to the induction of apoptotic signalling through oxidative and proteolytic stress.

Many studies have demonstrated the anti-apoptotic effects of CO to-date, both *in vivo* and *in vitro*. Similar to overexpression of HO-1, TNF-α-induced apoptosis has been shown to be abolished in an *in vitro* model in mouse fibroblasts and endothelial cells (Petrache, Otterbein et al. 2000). In the

endothelial cell model, p38 MAPK pathway appeared to be involved (Brouard, Otterbein et al. 2000).

In several *in vivo* models of disease and/or tissue injury (e.g. ischemia-reperfusion, lung transplantation), a low dose of CO pretreatment led to net anti-apoptotic effects (Ryter, Alam et al. 2006), although higher concentrations of CO (i.e. at CO poisoning levels) produced pro-apoptotic brain injury in a rat (Piantadosi, Zhang et al. 1997).

1.6.3.5 Anti-Proliferative Effects

The inhibitory effects of CO on cell growth were examined by Morita et al (Morita, Perrella et al. 1995). They found that HO-1-induced endogenous CO production, triggered by hypoxia, inhibited vascular smooth muscle cell proliferation. The effect appeared to be due to cGMP-dependent downregulation of the expression of endothelial-derived mitogens, such as platelet-derived growth factor and endothelin-1. These investigators were also the first to suggest that CO inhibited cell growth by influencing the expression and/or activation of cell cycle-related factors, particularly transcription factor E2F (Morita, Perrella et al. 1995).

1.6.4 Carbon Monoxide Releasing Molecules (CO-RMs)

Given the observed beneficial effects of HO and a possible therapeutic application of exogenous CO gas, the need for a better method of CO delivery had, by this point, become apparent. The first breakthrough occurred when

Motterlini et al synthesized a novel class of transition metal carbonyls, capable of releasing CO on demand (Motterlini, Clark et al. 2002). These carbon monoxide-releasing molecules (CO-RMs) are capable of delivering CO to the tissues in a controlled manner, and they do so without the detrimental formation of COHb (Motterlini, Clark et al. 2002), thus providing an alternative approach to CO/HO delivery (rather than adenoviral transfer or inhalational CO). In addition to providing a much better method of CO delivery, these compounds also provide the means for further mechanistic insight into the behaviour of CO in biological systems.

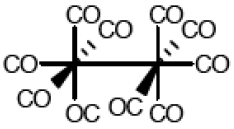
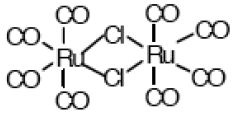
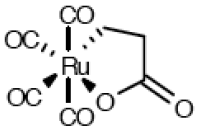
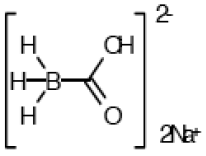
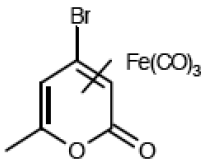
Most CO-RMs contain a central metal core (manganese, ruthenium, iron, boron) to which carbonyl groups are attached (Table 1.1). The general formula for a CO-RM is $[M_x(CO)_y]_n$.

The first CO-RM to be synthesized, CORM-1 (formula $[Mn_2(CO)_{10}]$), is a rapid CO releaser (Motterlini 2007). The molecule contains manganese in its centre, but is hydrophobic and requires photo-activation to initiate the CO release, thereby making its applicability limited to *in vitro* use only. The second CO-RM prototype, CORM-2 (formula $[Ru(CO_3Cl_2)]$, contains ruthenium metal dimer at its centre. CO is rapidly released by dissolving CORM-2 in an organic solvent (e.g. DMSO), by ligand substitution; again, hydrophobic character or the molecule makes its use in the clinical setting rather limited (Motterlini 2007).

The first *water-soluble* CO-RM to be synthesized was CORM-3 (formula $[Ru(CO)_3Cl(glycinate)]$), also a ruthenium-based compound. CORM-3 is relatively stable in water, but promptly releases CO by ligand substitution when it comes in

Table 1.1. Most common carbon monoxide-releasing molecules (CO-RMs). CORM-1 and CORM-2 are fast CO releasers, but not water-soluble. CORM-3 and CORM-A1 are water-soluble.

Adapted from Motterlini et al (2009).

Name	Chemical Structure	Solubility	CO Release
CORM-1		DMSO	Light-dependent Fast ($t_{1/2}$<1min) 1M CO/mole CO-RM
CORM-2		DMSO	Ligand substitution Fast ($t_{1/2}$=1min) 0.7M CO/mole CO-RM
CORM-3		Water	Ligand substitution Fast ($t_{1/2}$=1min) 1M CO/mole CO-RM
CORM-A1		Water	pH dependent Slow ($t_{1/2}$=21min) 1M CO/mole CO-RM
CORM-F3		DMSO	Metal oxidation Slow ($t_{1/2}$=55min) 0.25M CO/mole CORM

contact with biological stimuli directly interacting with ruthenium metal (Motterlini 2007). It is stable at an acidic pH (i.e. less than pH 5), and rapidly releases equimolar amounts of CO in physiological conditions.

In parallel, a second class of CO-RM chemicals able to generate CO in aqueous solutions was soon manufactured: CORM-A1 (formula [$Na_2H_3BCO_2$]) and CORM-401. Unlike CORM-3, CORM-A1 does not contain transition metal at its centre, but a boron-bound carboxylic group that can slowly liberate CO through hydrolysis under physiological conditions; the release of CO is initiated by a pH shift, rather than by ligand substitution. CORM-A1, however, is a very slow CO releaser, thus giving it a very long half-life ($t_{1/2}$=21min); this property makes its clinical use rather limited. CORM-401 contains manganese at its centre, instead of ruthenium (Crook, Mann et al. 2011). Upon activation by deprotonation in physiological solution, it releases three moles of CO per one mole of CORM-401 (Crook, Mann et al. 2011). While its long half-life is similar to that of CORM-1A (i.e. approximately 21 minutes) (Fayad-Kobeissi, Ratovonantenaina et al. 2016), making it a slow CO releaser, the fact that it does not contain any heavy metals not normally found in the body, and that it releases three times as much CO makes it an attractive alternative option for future clinical explorations.

The release of CO from each CO-RM has been validated spectroscopically, by monitoring the conversion of myoglobin into carboxymyoglobin (Motterlini 2007), while the biological effects have been

confirmed in numerous experiments by observing lack of effect with the deactivated form of the compound.

1.6.5 CORM-3

Carbon monoxide releasing molecule-3 (CORM-3) is a water-soluble CO donor. Originally, the molecule was synthesised by Motterlini et al by glycination of the water-insoluble CORM-2. CORM-3 is stable at an acidic pH, and rapidly releases equimolar amounts of CO in physiological conditions.

Beneficial effects of CORM-3 have been demonstrated in multiple *in vitro* and *in vivo* studies. For example, vascular inflammation in endothelial cells, particularly that involving oxidative burst of PMNs (Masini, Vannacci et al. 2008, Song, Bergstrasser et al. 2009, Bergstraesser, Hoeger et al. 2012), bacterial activity (Davidge, Sanguinetti et al. 2009, Desmard, Davidge et al. 2009), leukocyte-endothelial interaction under flow in acute pancreatitis (Urquhart, Rosignoli et al. 2007) and inflammatory response induced by LPS and/or IFN-γ (Sawle, Foresti et al. 2005, Bani-Hani, Greenstein et al. 2006, Bani-Hani, Greenstein et al. 2006) were all reduced by the application of CORM-3 in many *in vitro* experiments. Additionally, CORM-3 has been demonstrated to affect multiple cell types and pathways that coordinate the inflammatory cascade: production of TNF-α, fibrinogen/fibrin, cellular infiltration, ICAM-1 expression and activation of transcription factors (NFκB, MAPK) have been reported to be significantly diminished in *in vivo* models of vascular thrombosis (Kramkowski, Leszczynska et al. 2012), hemorrhagic stroke (Yabluchanskiy, Sawle et al.

2012), polymicrobial sepsis (Lancel, Hassoun et al. 2009, Tsoyi, Lee et al. 2009), I/R injury (Katada, Bihari et al. 2009, Katada, Bihari et al. 2010, Caumartin, Stephen et al. 2011), arthritis (Ibanez, Alcaraz et al. 2012), xenotransplantation (Vadori, Seveso et al. 2009), neuropathic pain due to nerve injury (Hervera, Leanez et al. 2012), post-operative ileus (De Backer, Elinck et al. 2009) and cutaneous wound healing (Ahanger, Prawez et al. 2011) in animals treated with CORM-3.

Interestingly, in relation to the pathology studied, the mode of action and efficacy of CORM-3 appear to depend on the timing of administration. CORM-3 pretreatment (5 minutes) or post-treatment (3 days) after the onset of hemorrhage in the rat model of hemorrhagic stroke provided protective effects. On the contrary, administration of CORM-3 three hours after the stroke (the timing corresponds to the acute phase of the disease process) resulted in exacerbated damage (Yabluchanskiy, Sawle et al. 2012).

The mechanisms of CORM-3 action appear to be linked not only to its anti-inflammatory properties, but also stimulation of mitochondrial biogenesis and the control of oxidative stress. CORM-3 administration was able to conserve cardiac mitochondrial function by preserving membrane potential and respiration; it also led to the induction of mitochondrial biogenesis in sepsis-mediated cardiac damage and metabolic syndrome-like disorder (Lancel, Hassoun et al. 2009, Lancel, Montaigne et al. 2012), while inhibiting NADPH activity and overproduction of superoxide anion (Taille, El-Benna et al. 2005, Lo Iacono, Boczkowski et al. 2011).

1.7 AIM OF THIS THESIS

While the pathophysiology of CS is not well understood, ischemia-reperfusion injury appears to be a major driving force behind the observed detrimental outcomes. The effects of complete ischemia (where the onset is known, as all circulation is cleanly cut off) and subsequent reperfusion injury have been extensively described in the literature; we borrow from these studies in our attempt to design rational therapeutic approaches to CS.

In response to the initial limb trauma, tissue edema develops. Rigidity of fascia prevents muscle compartments from expanding, resulting in the elevation of ICP and compression of the microvasculature, with tissue ischemia as the end result. However, unlike complete ischemia, CS appears in the face of patent vessels – after all, distal pulses are present in the majority of CS patients. Thus, the *macro*circulation within the involved compartments is still intact, while the *micro*circulation becomes dysfunctional. Although some degree of microvascular perfusion may still be maintained, the metabolic demands for oxygen and nutrients cannot be fully met; 'low-flow' ischemic state leads to ROS and toxic metabolite formation, initiating local inflammatory response much earlier than that seen in complete ischemia. Subsequent cytokine release leads to early local leukocyte activation (particularly neutrophils) (Lawendy, Bihari et al. 2015), also triggering systemic inflammatory response coupled with remote organ injury (Lawendy, Bihari et al. 2016). In CS, ischemia and reperfusion phases happen almost concurrently, not only making it impossible to pinpoint the exact timing of CS onset, but initiating the reperfusion injury much earlier than that of complete

ischemia, with the degree of severity correlated to the duration of CS (Lawendy, Sanders et al. 2011).

While different tissues have different tolerance for the survival of hypoxia, irreversible damage occurs within 6-8 hours, making it crucial that fasciotomy is carried out within that surgical window. Given the unclear nature of the CS onset and severe consequences of missed CS, the necessity of developing therapy aimed at prolongation of surgical interval becomes obvious.

Exogenous application of CO appears to be beneficial in animal models of ischemia-reperfusion injury, but it has never been tried in the context of low-flow ischemia (i.e. CS). The purpose of this thesis was to test CO as a possible therapeutic agent for the treatment of CS.

We hypothesized that exogenous application of carbon monoxide (either in inhalational form, or as CORM-3 derived CO) would be of immense benefit in acute compartment syndrome. We believe that CO could be used as an adjunct to fasciotomy; it may have the capacity to at least prolong the surgical window, if not dispense with the need for fasciotomy altogether.

CORM-3 as CO donor was chosen due to its water solubility and rapid CO release upon activation. It is also important to note that, for the purpose of this thesis, the terms ‘elevated ICP’ and ‘CS’ are used interchangeably; however, CS is a clinical definition, and as such, can only be applied to humans.

1.8 REFERENCES

Ahanger AA, Prawez S, Kumar D, R. Prasad R, Amarpal, Tandan SK and Kumar D (2011). Wound healing activity of carbon monoxide liberated from CO-releasing molecule (CO-RM). Naunyn Schmiedebergs Arch Pharmacol **384**(1): 93-102.

Albelda SM, Muller WA, Buck CA and Newman PJ (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol **114**(5): 1059-1068.

Althaus M, Fronius M, Buchäeckert Y, Vadász I, Clauss WG, Seeger W, Motterlini R, Morty RE (2009). Carbon monoxide rapidly impairs alveolar fluid clearance by inhibiting epithelial sodium channels. Am J Respir Cell Mol Biol **41**(6): 639-650.

Alvarez JM, Chatwin C and Fahrer C (2000). Prophylactic intravenous mannitol and normal saline in patients with poor renal function prior to cardiac surgery: time for a multicentre trial? Heart Lung Circ **9**(2): 74-77.

Anderson SM, Park ZH and Patel RV (2011). Intravenous N-acetylcysteine in the prevention of contrast media-induced nephropathy. Ann Pharmacother **45**(1): 101-107.

Arbabi S, Brundage SI and Gentilello LM (1999). Near-infrared spectroscopy: a potential method for continuous, transcutaneous monitoring for compartmental syndrome in critically injured patients. J Trauma **47**(5): 829-833.

Archakov AI, Karuzina II, Petushkova NA, Lisitsa AV and Zgoda VG (2002). Production of carbon monoxide by cytochrome P450 during iron-dependent lipid peroxidation. Toxicol In Vitro **16**(1): 1-10.

Ashton H (1975). The effect of increased tissue pressure on blood flow. Clin Orthop Relat Res (113): 15-26.

Bae DS, Kadiyala RK and Waters PM (2001). Acute compartment syndrome in children: contemporary diagnosis, treatment, and outcome. J Pediatr Orthop **21**(5): 680-688.

Bani-Hani MG, Greenstein D, Mann BE, Green CJ and Motterlini R (2006). A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia. Pharmacol Rep **58 Suppl**: 132-144.

Bani-Hani MG, Greenstein D, Mann BE, Green CJ and Motterlini R (2006). Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. J Pharmacol Exp Ther **318**(3): 1315-1322.

Bardenheuer L (1911). Die entstehung und behandlung der ischamischen muskelkontractur und gangran. Dtsch Z Chir **108**: 44.

Barr KB (2008). Compartment Syndrome. Essentials of Physical Medicine and Rehabilitation. W. R. Frontera, J. K. Silver and T. Rizzo. Philadelphia, Saunders, Elsevier.

Barreiro O, Yanez-Mo M, Serrador JM, Montoya MC, Vicente-Manzanares M, Tejedor R, Furthmayr H and Sanchez-Madrid F (2002). Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. J Cell Biol **157**(7): 1233-1245.

Bellavite P (1988). The superoxide-forming enzymatic system of phagocytes. Free Radic Biol Med **4**(4): 225-261.

Benjamin A (1957). The relief of traumatic arterial spasm in threatened Volkmann's ischaemic contracture. J Bone Joint Surg Br **39-B**(4): 711-713.

Bergstraesser C, Hoeger S, Song H, Ermantraut L, Hottenrot M, Czymai T, Schmidt M, Goebeler M, Ponelies N, Stich C, Loesel R, Molema G, Seelen M, van Son W, Yard BA and Rafat N (2012). Inhibition of VCAM-1 expression in endothelial cells by CORM-3: the role of the ubiquitin-proteasome system, p38, and mitochondrial respiration. Free Radic Biol Med **52**(4): 794-802.

Bermudez K, Knudson MM, Morabito D and Kessel O (1998). Fasciotomy, chronic venous insufficiency, and the calf muscle pump. Arch Surg **133**(12): 1356-1361.

Bernard C (1857). Le Cons Sur les Effets des Substances Toxiques et Médicamenteuses. Paris, Bailliere.

Bernays AC (1900). On ischemic paralysis and contracture of muscles. Boston Med Surg J **142**(21): 539-542.

Better OS, Zinman C, Reis DN, Har-Shai Y, Rubinstein I and Abassi Z (1991). Hypertonic mannitol ameliorates intracompartmental tamponade in model compartment syndrome in the dog. Nephron **58**(3): 344-346.

Beutler BA, Milsark IW and Cerami A (1985). Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. J Immunol **135**(6): 3972-3977.

Bhattacharyya T and Vrahas MS (2004). The medical-legal aspects of compartment syndrome. J Bone Joint Surg Am **86-A**(4): 864-868.

Born CT (2005). Blast trauma: the fourth weapon of mass destruction. Scand J Surg **94**(4): 279-285.

Brooks B (1922). Pathologic changes in muscle as a result of disturbances of circulation: an experimental study of Volkmann's ischemic paralysis. Arch Surg **5**(1): 188.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM and Soares MP (2000). Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med **192**(7): 1015-1026.

Brumback RJ (1990). Traumatic rupture of the superior gluteal artery, without fracture of the pelvis, causing compartment syndrome of the buttock. A case report. J Bone Joint Surg Am **72**(1): 134-137.

Burton AC (1951). On the physical equilibrium of small blood vessels. Am J Physiol **164**(2): 319-329.

Bywaters EG, and Beall D (1941). Crush Injuries with Impairment of Renal Function. Br Med J **1**(4185): 427-432.

Carden DL, Smith JK and Korthuis RJ (1990). Neutrophil-mediated microvascular dysfunction in postischemic canine skeletal muscle. Role of granulocyte adherence. Circ Res **66**(5): 1436-1444.

Cascio BM, Wilckens JH, Ain MC, Toulson C and Frassica FJ (2005). Documentation of acute compartment syndrome at an academic health-care center. J Bone Joint Surg Am **87**(2): 346-350.

Caumartin Y, Stephen J, Deng JP, Lian D, Lan Z, Liu W, Garcia B, Jevnikar AM, Wang H, Cepinskas G and Luke PP (2011). Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. Kidney Int **79**(10): 1080-1089.

Chautems RC, Irmay F, Magnin M, Morel P and Hoffmeyer P (1997). Spontaneous anterior and lateral tibial compartment syndrome in a type I diabetic patient: case report. J Trauma **43**(1): 140-141.

Choi AM and Otterbein LE (2002). Emerging role of carbon monoxide in physiologic and pathophysiologic states. Antioxid Redox Signal **4**(2): 227-228.

Coburn RF (1967). Endogenous carbon monoxide production and body CO stores. Acta Med Scand Suppl **472**: 269-282.

Coburn RF (1979). Mechanisms of carbon monoxide toxicity. Prev Med **8**(3): 310-322.

Coburn RF, Williams WJ, White P and Kahn SB (1967). The production of carbon monoxide from hemoglobin in vivo. J Clin Invest **46**(3): 346-356.

Crook SH, Mann BE, Meijer AJ, Adams H, Sawle P, Scapens D and Motterlini R (2011). [Mn(CO)4{S2CNMe(CH2CO2H)}], a new water-soluble CO-releasing molecule. Dalton Trans **40**(16): 4230-4235.

Cruz J, Minoja G and Okuchi K (2001). Improving clinical outcomes from acute subdural hematomas with the emergency preoperative administration of high doses of mannitol: a randomized trial. Neurosurgery **49**(4): 864-871.

Daniels M, Reichman J and Brezis M (1998). Mannitol treatment for acute compartment syndrome. Nephron **79**(4): 492-493.

Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R and Poole PK (2009). Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. J Biol Chem **284**(7): 4516-4524.

De Backer O, Elinck E, Blanckaert B, Leybaert L, Motterlini R and Lefebvre RA (2009). Water-soluble CO-releasing molecules reduce the development of postoperative ileus via modulation of MAPK/HO-1 signalling and reduction of oxidative stress. Gut **58**(3): 347-356.

Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R and Boczkowski J (2009). A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteraemia. FASEB J **23**(4): 1023-1031.

Douglas CG, Haldane JS and Haldane JB (1912). The laws of combination of haemoglobin with carbon monoxide and oxygen. J Physiol **44**(4): 275-304.

Dover M, Marafi H and Quinlan JF (2011). Long-term sequelae following fasciotomy in trauma patients. J Bone Joint Surg Br **93-B**(Supp II): 180.

Dover M, Memon AR, Marafi H, Kelly G and Quinlan JF (2012). Factors associated with persistent sequelae after fasciotomy for acute compartment syndrome. J Orthop Surg (Hong Kong) **20**(3): 312-315.

Dungey AA, Badhwar A, Bihari A, Kvietys PR, Harris KA, Forbes TL and Potter RF (2006). Role of heme oxygenase in the protection afforded skeletal muscle during ischemic tolerance. Microcirculation **13**(2): 71-79.

Eaton RG, and Green WT (1972). Epimysiotomy and fasciotomy in the treatment of Volkmann's ischemic contracture. Orthop Clin North Am **3**(1): 175-186.

Eichler GR, and Lipscomb PR (1967). The changing treatment of Volkmann's ischemic contractures from 1955 to 1965 at the Mayo Clinic. Clin Orthop Relat Res **50**: 215-223.

Elliot K (2014). Intramuscular pH: diagnosing acute compartment syndrome with confidence. Proceedings of the 2014 London Effort Conference Trauma Session.

Elliott KG, and Johnstone AJ (2003). Diagnosing acute compartment syndrome. J Bone Joint Surg Br **85**(5): 625-632.

Ernst CB, and Kaufer H (1971). Fibulectomy-fasciotomy. An important adjunct in the management of lower extremity arterial trauma. J Trauma **11**(5): 365-380.

Fayad-Kobeissi S, Ratovonantenaina J, Dabire H, Wilson JL, Rodriguez AM, Berdeaux A, Dubois-Rande JL, Mann BE, Motterlini R and Foresti R (2016). Vascular and angiogenic activities of CORM-401, an oxidant-sensitive CO-releasing molecule. Biochem Pharmacol **102**: 64-77.

Finkelstein JA, Hunter GA and Hu RW (1996). Lower limb compartment syndrome: course after delayed fasciotomy. J Trauma **40**(3): 342-344.

Fitzgerald AM, Gaston P, Wilson Y, Quaba A and McQueen MM (2000). Long-term sequelae of fasciotomy wounds. Br J Plast Surg **53**(8): 690-693.

Foisie PS (1942). Volkmann's ischemic contracture. An analysis of its proximate mechanism. N Engl J Med **226**(17): 671-679.

Forbes TL, Carson M, Harris KA, DeRose G, Jamieson WG and Potter RF (1995). Skeletal muscle injury induced by ischemia-reperfusion. Can J Surg **38**(1): 56-63.

Forbes TL, Harris KA, Jamieson WG, DeRose G, Carson M and Potter RF (1996). Leukocyte activity and tissue injury following ischemia-reperfusion in skeletal muscle. Microvasc Res **51**(3): 275-287.

Furchgott RF and Jothianandan D (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels **28**(1-3): 52-61.

Gebhard F, Pfetsch H, Steinbach G, Strecker W, Kinzl L and Bruckner UB (2000). Is interleukin 6 an early marker of injury severity following major trauma in humans? Arch Surg **135**(3): 291-295.

Gelberman RH, Garfin SR, Hergenroeder PT, Mubarak SJ and Menon J (1981). Compartment syndromes of the forearm: diagnosis and treatment. Clin Orthop Relat Res(161): 252-261.

Gelberman RH, Zakaib GS, Mubarak SJ, Hargens AR and Akeson WH (1978). Decompression of forearm compartment syndromes. Clin Orthop Relat Res(134): 225-229.

Gentilello LM, Sanzone A, Wang L, Liu PY and Robinson L (2001). Near-infrared spectroscopy versus compartment pressure for the diagnosis of lower extremity compartmental syndrome using electromyography-determined measurements of neuromuscular function. J Trauma **51**(1): 1-8, discussion 8-9.

Giannoudis PV, Nicolopoulos C, Dinopoulos H, Ng A, Adedapo S and Kind P (2002). The impact of lower leg compartment syndrome on health related quality of life. Injury **33**(2): 117-121.

Gillani S, Cao J, Suzuki T and Hak DJ (2012). The effect of ischemia reperfusion injury on skeletal muscle. Injury **43**(6): 670-5.

Gold BS, Barish RA, Dart RC, Silverman RP and Bochicchio GV (2003). Resolution of compartment syndrome after rattlesnake envenomation utilizing non-invasive measures. J Emerg Med **24**(3): 285-288.

Gourgiotis S, Villias C, Germanos S, Foukas A and Ridolfini MP (2007). Acute limb compartment syndrome: a review. J Surg Educ **64**(3): 178-186.

Granger DN (1988). Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. Am J Physiol **255**(6 Pt 2): H1269-1275.

Graser T, Vedernikov YP and Li DS (1990). Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. Biomed Biochim Acta **49**(4): 293-296.

Gratz CM (1931). Tensile strength and elasticity tests on human fascia lata. J Bone Joint Surg Am **13**(2): 334-340.

Gray H (2000). Anatomy of the Human Body. Philadelphia, Lea & Frebiger.

Griffiths DV (1940). Volkmann's ischaemic contracture. Br J Surg **28**(110): 239-260.

Gute DC, Ishida T, Yarimizu K and Korthuis RJ (1998). Inflammatory responses to ischemia and reperfusion in skeletal muscle. Mol Cell Biochem **179**(1-2): 169-187.

Hallberg L (1955). Blood volume, hemolysis and regeneration of blood in pernicious anemia; studies based on the endogenous formation of carbon monoxide and determinations of the total amount of hemoglobin. Scand J Clin Lab Invest **7 Suppl. 16**: 1-127.

Hampton MB, Kettle AJ and Winterbourn CC (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood **92**(9): 3007-3017.

Hargens AR, Akeson WH, Mubarak SJ, Owen CA, Evans KL, Garetto LP, Gonsalves MR and Schmidt DA (1978). Fluid balance within the canine anterolateral compartment and its relationship to compartment syndromes. J Bone Joint Surg Am **60**(4): 499-505.

Hargens AR, Akeson WH, Mubarak SJ, Owen CA, Gershuni DH, Garfin SR, Lieber RL, Danzig LA, Botte MJ and Gelberman RH (1989). Kappa Delta Award paper. Tissue fluid pressures: from basic research tools to clinical applications. J Orthop Res **7**(6): 902-909.

Hargens AR and Ballard RE (1995). Basic principles for measurement of intramuscular pressure. Oper Tech Sports Med **3**(4): 237-242.

Harrington P, Bunola J, Jennings AJ, Bush DJ and Smith RM (2000). Acute compartment syndrome masked by intravenous morphine from a patient-controlled analgesia pump. Injury **31**(5): 387-389.

Harris AG and Skalak TC (1996). Effects of leukocyte capillary plugging in skeletal muscle ischemia-reperfusion injury. Am J Physiol **271**(6 Pt 2): H2653-2660.

Harris K, Walker PM, Mickle DA, Harding R, Gatley R, Wilson GJ, Kuzon B, McKee N and Romaschin AD (1986). Metabolic response of skeletal muscle to ischemia. Am J Physiol **250**(2 Pt 2): H213-220.

Hartsock LA, O'Farrell D, Seaber AV and Urbaniak JR (1998). Effect of increased compartment pressure on the microcirculation of skeletal muscle. Microsurgery **18**(2): 67-71.

Hashizume M and Mihara M (2011). The roles of interleukin-6 in the pathogenesis of rheumatoid arthritis. Arthritis **2011**: 765624.

Heckman MM, Whitesides TE, Jr., Grewe SR and Rooks MD (1994). Compartment pressure in association with closed tibial fractures. The relationship between tissue pressure, compartment, and the distance from the site of the fracture. J Bone Joint Surg Am **76**(9): 1285-1292.

Heemskerk J and Kitslaar P (2003). Acute compartment syndrome of the lower leg: retrospective study on prevalence, technique, and outcome of fasciotomies. World J Surg **27**(6): 744-747.

Henry AK, (1973). Extensile exposure. London, Churchill Livingstone.

Hervera A, Leanez S, Negrete R, Motterlini R and Pol O (2012). Carbon monoxide reduces neuropathic pain and spinal microglial activation by inhibiting nitric oxide synthesis in mice. PLoS One **7**(8): e43693.

Hildebrand O (1906). Die Lehre von den ischemische Muskellahmungen und Kontrakturen. Samml Klin Vortage **122**: 437.

Hope MJ and McQueen MM (2004). Acute compartment syndrome in the absence of fracture. J Orthop Trauma **18**(4): 220-224.

Hsu SI, Thadhani RI and Daniels GH (1995). Acute compartment syndrome in a hypothyroid patient. Thyroid **5**(4): 305-308.

Hughes JR (1948). Ischaemic necrosis of the anterior tibial muscles due to fatigue. J Bone Joint Surg Br **30B**(4): 581-594.

Ibanez L, Alcaraz MJ, Maicas N, Guede D, Caeiro JR, Motterlini R and Ferrandiz ML (2012). Downregulation of the inflammatory response by CORM-3 results in protective effects in a model of postmenopausal arthritis. Calcif Tissue Int **91**(1): 69-80.

Idstrom JPSoussi B, Elander A and Bylund-Fellenius AC (1990). Purine metabolism after in vivo ischemia and reperfusion in rat skeletal muscle. Am J Physiol **258**(6 Pt 2): H1668-1673.

Jacob RA and Sotoudeh G (2002). Vitamin C function and status in chronic disease. Nutr Clin Care **5**(2): 66-74.

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova D, E S and Cheng X (2002). Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca2+ sparks to Ca2+-activated K+ channels. Circ Res **91**(7): 610-617.

Jaggar JH, Li A, Parfenova H, Liu J, Umstot ES, Dopico AM, Leffler CW (2005). Heme is a carbon monoxide receptor for large-conductance Ca2+-activated K+ channels. Circ Res **97**(8): 805-812.

Jan BV and Lowry SF (2009). Systemic Response to Injury and Metabolic Support. Schwatz's Principles of Surgery. F. C. Brunicardi, D. K. Andersen, T. R. Billiar et al., McGraw-Hill Professional.

Jepson PN (1926). Ischaemic Contracture: Experimental Study. Ann Surg **84**(6): 785-795.

Johnson SB, Weaver FA, Yellin AE, Kelly R and Bauer M (1992). Clinical results of decompressive dermotomy-fasciotomy. Am J Surg **164**(3): 286-290.

Jones MD, Santamarina R and Warhold LG (2010). Surgical Decompression of the Forearm, Hand and Digits for Compartment Syndrome. Philadelphia, PA, Wolters Kluwer Health | Lippincott Williams & Wilkins.

Jorge J (1925). Retraction ischemique de Volkmann. Rapport d'Albert monchet. Bull Mem Soc Nat Chir **51**: 884.

Jose RM, Viswanathan N, Aldlyami E, Wilson Y, Moiemen N and Thomas R (2004). A spontaneous compartment syndrome in a patient with diabetes. J Bone Joint Surg Br **86**(7): 1068-1070.

Kajimura M, Fukuda R, Bateman RM, Yamamoto T and Suematsu M (2010). Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H2S gas biology. Antioxid Redox Signal **13**(2): 157-192

Katada K, Bihari A, Badhwar A, Yoshida N, Yoshikawa T, Potter RF and Cepinskas G (2009). Hindlimb ischemia/reperfusion-induced remote injury to the small intestine: role of inducible nitric-oxide synthase-derived nitric oxide. J Pharmacol Exp Ther **329**(3): 919-927.

Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF and Cepinskas G (2010). Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. Inflammation **33**(2): 92-100.

Katz LM, Nauriyal V, Nagaraj S, Finch A, Pearlstein K, Szymanowski A, Sproule C, Rich PB, Guenther BD and Pearlstein RD (2008). Infrared imaging of trauma patients for detection of acute compartment syndrome of the leg. Crit Care Med **36**(6): 1756-1761.

Kawane H (2002). Exhaled carbon monoxide in COPD. Chest **121**(5): 1723; author reply 1723.

Kearns SR, Daly AF, Sheehan K, Murray P, Kelly C and Bouchier-Hayes D (2004). Oral vitamin C reduces the injury to skeletal muscle caused by compartment syndrome. J Bone Joint Surg Br **86**(6): 906-911.

Kearns SR, O'Briain DE, Sheehan KM, Kelly C and Bouchier-Hayes D (2010). N-acetylcysteine protects striated muscle in a model of compartment syndrome. Clin Orthop Relat Res **468**(8): 2251-2259.

Keyse SM and Tyrrell RM (1989). Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci U S A **86**(1): 99-103.

Kramkowski K, Leszczynska A, Mogielnicki A, Chlopicki S, Fedorowicz A, Grochal E, Mann B, Brzoska T, Urano T, Motterlini R and Buczko W (2012). Antithrombotic Properties of Water-Soluble Carbon Monoxide-Releasing Molecules. Arterioscler Thromb Vasc Biol **32**(9): 2149-57.

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009). Classification of cell death:

recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ **16**(1): 3-11.

Kurose I, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR and Granger DN (1994). Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. Circ Res **74**(2): 336-343.

Lancel S, Hassoun SM, Favory R, Decoster B, Motterlini R and Neviere R (2009). Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis. J Pharmacol Exp Ther **329**(2): 641-648.

Lancel S, Montaigne D, Marechal X, Marciniak C, Hassoun SM, Decoster B, Ballot C, Blazejewski C, Corseaux D, Lescure B, Motterlini R and Neviere R (2012). Carbon monoxide improves cardiac function and mitochondrial population quality in a mouse model of metabolic syndrome. PLoS One **7**(8): e41836.

Lawendy AR, Bihari A, Sanders D, Badhwar A and Cepinskas G (2016). Compartment syndrome causes systemic inflammation in a rat. Bone Joint J **98-B**(8): 1132-7.

Lawendy A and Sanders D (2010). Operative Techniques: Orthopaedic Trauma Surgery, Saunders.

Lawendy AR, Bihari A, Sanders D, McGarr G, Badhwar A and Cepinskas G (2015). Contribution of inflammation to cellular injury in compartment syndrome in an experimental rodent model. Bone Joint J **97-B**(4): 539-543.

Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D and Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Lefer AM, Tsao PS, Lefer DJ and Ma XL (1991). Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. FASEB J **5**(7): 2029-2034.

Leriche R (1928). Surgery of the Sympathetic System. Indications and Results. Ann Surg **88**(3): 449-469.

Leser E (1884). Untersuchungen uber ischamische Muskellahumungen und Muskelcontracturen. Samml Klin Vortage **3**: 2087.

Ley K (2008). The Microcirculation in Inflammation. Handbook of Physiology: Microcirculation. R. F. Tuma, W. N. Duran and K. Ley. Oxford, UK, Academic Press (Elsevier).

Ley K, Laudanna C, Cybulsky MI and Nourshargh S (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol **7**(9): 678-689.

Ley K and Reutershan J (2006). Leucocyte-endothelial interactions in health and disease. Handb Exp Pharmacol(176 Pt 2): 97-133.

Lin H and McGrath JJ (1988). Vasodilating effects of carbon monoxide. Drug Chem Toxicol **11**(4): 371-385.

Lo Iacono L, Boczkowski J, Zini R, Salouage I, Berdeaux A, Motterlini R and Morin D (2011). A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species. Free Radic Biol Med **50**(11): 1556-1564.

Maier RV and Bulger EM (1996). Endothelial changes after shock and injury. New Horiz **4**(2): 211-223.

Maines MD (1997). The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol **37**: 517-554.

Maines MD and Kappas A (1977). Enzymatic oxidation of cobalt protoporphyrin IX: observations on the mechanism of heme oxygenase action. Biochemistry **16**(3): 419-423.

Maines MD, Trakshel GM and Kutty RK (1986). Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J Biol Chem **261**(1): 411-419.

Manjoo A, Sanders D, Lawendy A, Gladwell M, Gray D, Parry N and Badhwar A (2010). Indomethacin reduces cell damage: shedding new light on compartment syndrome. J Orthop Trauma **24**(9): 526-529.

Mars M and Hadley GP (1998). Raised compartmental pressure in children: a basis for management. Injury **29**(3): 183-185.

Masini E, Vannacci A, Failli P, Mastroianni R, Giannini L, Vinci MC, Uliva C, Motterlini R and Mannaioni PF (2008). A carbon monoxide-releasing molecule (CORM-3) abrogates polymorphonuclear granulocyte-induced activation of endothelial cells and mast cells. FASEB J **22**(9): 3380-3388.

Massart R (1935). La maladie de Volkmann. Rev Orthop **22**: 385.

Mathews PV, Perry JJ and Murray PC (2001). Compartment syndrome of the well leg as a result of the hemilithotomy position: a report of two cases and review of literature. J Orthop Trauma **15**(8): 580-583.

Matsen FA, 3rd (1975). Compartmental syndrome. An unified concept. Clin Orthop Relat Res(113): 8-14.

Matsen FA, 3rd (1979). Compartmental syndromes. N Engl J Med **300**(21): 1210-1211.

Matsen FA, 3rd and Clawson DK (1975). The deep posterior compartmental syndrome of the leg. J Bone Joint Surg Am **57**(1): 34-39.

Matsen FA, 3rd and Krugmire RB, Jr. (1978). Compartmental syndromes. Surg Gynecol Obstet **147**(6): 943-949.

Matsen FA, 3rd, Mayo KA, Sheridan GW and Krugmire RB, Jr (1977). Continuous monitoring of intramuscular pressure and its application to clinical compartmental syndromes. Bibl Anat(15 Pt 1): 112-115.

Matsen FA, 3rd, Winquist RA and Krugmire RB, Jr (1980). Diagnosis and management of compartmental syndromes. J Bone Joint Surg Am **62**(2): 286-291.

Matsen FA, 3rd, Wyss CR, Krugmire RB, Jr, Simmons CW and King RV (1980). The effects of limb elevation and dependency on local arteriovenous gradients in normal human limbs with particular reference to limbs with increased tissue pressure. Clin Orthop Relat Res(150): 187-195.

Mavor GE (1956). The anterior tibial syndrome. J Bone Joint Surg Br **38-B**(2): 513-517.

McCarter SD, Badhwar A, Scott JR, Akyea TG, Bihari A, Dungey AA, Harris KA and Potter RF (2004). Remote liver injury is attenuated by adenovirus-mediated gene transfer of heme oxygenase-1 during the systemic inflammatory response syndrome. Microcirculation **11**(7): 587-595.

McCoubrey WK, Jr, Huang TJ and Maines MD (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem **247**(2): 725-732.

McDermott AG, Marble AE and Yabsley RH (1984). Monitoring acute compartment pressures with the S.T.I.C. catheter. Clin Orthop Relat Res(190): 192-198.

McDonald DM, Thurston MG and Baluk P (1999). Endothelial gaps as sites for plasma leakage in inflammation. Microcirculation **6**(1): 7-22.

McFaul SJ and McGrath JJ (1987). Studies on the mechanism of carbon monoxide-induced vasodilation in the isolated perfused rat heart. Toxicol Appl Pharmacol **87**(3): 464-473.

McQueen MM, Christie J and Court-Brown CM (1996). Acute compartment syndrome in tibial diaphyseal fractures. J Bone Joint Surg Br **78**(1): 95-98.

McQueen MM and Court-Brown CM (1996). Compartment monitoring in tibial fractures. The pressure threshold for decompression. J Bone Joint Surg Br **78**(1): 99-104.

McQueen MM, Gaston P and Court-Brown CM (2000). Acute compartment syndrome. Who is at risk? J Bone Joint Surg Br **82**(2): 200-203.

Michel CC and Curry FE (1999). Microvascular permeability. Physiol Rev **79**(3): 703-761.

Moed BR and Thorderson PK (1993). Measurement of intracompartmental pressure: a comparison of the slit catheter, side-ported needle, and simple needle. J Bone Joint Surg Am **75**(2): 231-235.

Moncada S, Radomski MW and Palmer RM (1988). Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem Pharmacol **37**(13): 2495-2501.

Morita T, Perrella MA, Lee ME and Kourembanas S (1995). Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc Natl Acad Sci U S A **92**(5): 1475-1479.

Motterlini R (2007). Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities." Biochem Soc Trans **35**(Pt 5): 1142-1146.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE and Green CJ (2002). Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res **90**(2): E17-24.

Motterlini R and Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nat Rev Drug Discov **9**(9): 728-743.

Moulonquet P and Seneque J (1928). Syndrome de Volkmann. Bull Mem Soc Nat Chir **54**: 1094.

Mubarak SJ, Hargens AR, Owen CA, Garetto LP and Akeson WH (1976). The wick catheter technique for measurement of intramuscular pressure. A new research and clinical tool. J Bone Joint Surg Am **58**(7): 1016-1020.

Mubarak SJ and Owen CA (1977). Double-incision fasciotomy of the leg for decompression in compartment syndromes. J Bone Joint Surg Am **59**(2): 184-187.

Mubarak SJ, Owen CA, Hargens AR, Garetto LP and Akeson WH (1978). Acute compartment syndromes: diagnosis and treatment with the aid of the wick catheter. J Bone Joint Surg Am **60**(8): 1091-1095.

Mubarak SJ and Wilton NC (1997). Compartment syndromes and epidural analgesia. J Pediatr Orthop **17**(3): 282-284.

Munoz-Canoves P, Scheele C, Pedersen BK and Serrano AL (2013). Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? FEBS J **280**(17): 4131-4148.

Murphy JB (1914). Myositis. JAMA **63**(15): 1249-1255.

Nylander G, Nordstrom H, Lewis D and Larsson J (1987). Metabolic effects of hyperbaric oxygen in postischemic muscle. Plast Reconstr Surg **79**(1): 91-97.

Odland R, Schmidt AH, Hunter B, Kidder L, Bechtold JE, Linzie BM, Pedowitz RA and Hargens AR (2005). Use of tissue ultrafiltration for treatment of compartment syndrome: a pilot study using porcine hindlimbs. J Orthop Trauma **19**(4): 267-275.

Omura T and Sato R (1964). The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. J Biol Chem **239**: 2370-2378.

Oredsson S, Plate G and Qvarfordt P (1994). The effect of mannitol on reperfusion injury and postischaemic compartment pressure in skeletal muscle. Eur J Vasc Surg **8**(3): 326-331.

Ott MC, Scott JR, Bihari A, Badhwar A, Otterbein LE, Gray DK, Harris KA and Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hind limb ischemia/reperfusion. FASEB J **19**(1): 106-108.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA and Choi AM (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med **6**(4): 422-428.

Otterbein LE, Kolls JK, Mantell LL, Cook JL, Alam J and Choi AM (1999). Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. J Clin Invest **103**(7): 1047-1054.

Otterbein LE, Lee PJ, Chin BY, Petrache I, Camhi SL, Alam J and Choi AM (1999). Protective effects of heme oxygenase-1 in acute lung injury. Chest **116**(1 Suppl): 61S-63S.

Palma PC, Villaca CJ, Jr and Netto NR, Jr (1986). N-acetylcysteine in the prevention of cyclophosphamide induced haemorrhagic cystitis. Int Surg **71**(1): 36-37.

Petrache I, Otterbein LE, Alam J, Wiegand GW and Choi AM (2000). Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. Am J Physiol Lung Cell Mol Physiol **278**(2): L312-319.

Piantadosi CA, Zhang AJ, Levin ED, Folz RJ and Schmechel DE (1997). Apoptosis and delayed neuronal damage after carbon monoxide poisoning in the rat. Exp Neurol **147**(1): 103-114.

Potter RF Dietrich HH, Tyml K, Ellis CG, Cronkwright J and Groom AC (1993). Ischemia-reperfusion induced microvascular dysfunction in skeletal muscle: application of intravital video microscopy. Int J Microcirc Clin Exp **13**(3): 173-186.

Reneman RS (1975). The anterior and the lateral compartmental syndrome of the leg due to intensive use of muscles. Clin Orthop Relat Res(113): 69-80.

Ricci MA, Corbisiero RM, Mohamed F, Graham AM and Symes JF (1990). Replication of the compartment syndrome in a canine model: experimental evaluation of treatment. J Invest Surg **3**(2): 129-140.

Ritenour AE, Dorlac WC, Fang R, Woods T, Jenkins DH, Flaherty SF, Wade CE and Holcomb JB (2008). Complications after fasciotomy revision and delayed compartment release in combat patients. J Trauma **64**(2 Suppl): S153-161; discussion S161-152.

Roebuck KA, Carpenter LR, Lakshminarayanan V, Page SM, Moy JN and Thomas LL (1999). Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-kappaB. J Leukoc Biol **65**(3): 291-298.

Roeckl-Wiedmann I, Bennett M and Kranke P (2005). Systematic review of hyperbaric oxygen in the management of chronic wounds. Br J Surg **92**(1): 24-32.

Ronel DN, Mtui E and Nolan WB, 3rd (2004). Forearm compartment syndrome: anatomical analysis of surgical approaches to the deep space. Plast Reconstr Surg **114**(3): 697-705.

Rorabeck CH (1984). The treatment of compartment syndromes of the leg. J Bone Joint Surg Br **66**(1): 93-97.

Rorabeck CH, Castle GS, Hardie R and Logan J (1981). Compartmental pressure measurements: an experimental investigation using the slit catheter. J Trauma **21**(6): 446-449.

Rorabeck CH and Clarke KM (1978). The pathophysiology of the anterior tibial compartment syndrome: an experimental investigation. J Trauma **18**(5): 299-304.

Ryter SW, Alam J and Choi AM (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev **86**(2): 583-650.

Ryter SW and Otterbein LE (2004). Carbon monoxide in biology and medicine. Bioessays **26**(3): 270-280.

Ryter SW, Otterbein LE, Morse D and Choi AM (2002). Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol Cell Biochem **234-235**(1-2): 249-263.

Sabido F, Milazzo VJ, Hobson RW, 2nd and Duran WN (1994). Skeletal muscle ischemia-reperfusion injury: a review of endothelial cell-leukocyte interactions. J Invest Surg **7**(1): 39-47.

Sanghavi R, Aneman A, Parr M, Dunlop L and Champion D (2006). Systemic capillary leak syndrome associated with compartment syndrome and rhabdomyolysis. Anaesth Intensive Care **34**(3): 388-391.

Santus P, Corsico A, Solidoro P, Braido F, Di Marco F and Scichilone N (2014). Oxidative stress and respiratory system: pharmacological and clinical reappraisal of N-acetylcysteine. COPD **11**(6): 705-717.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ and Motterlini R (2005). Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. Br J Pharmacol **145**(6): 800-810.

Schlag MG, Harris KA and Potter RF (2001). Role of leukocyte accumulation and oxygen radicals in ischemia-reperfusion-induced injury in skeletal muscle. Am J Physiol Heart Circ Physiol **280**(4): H1716-1721.

Schmidt AH (2007). Acute Compartment Syndrome. Surgical Treatment of Orthopaedic Trauma. J. P. Stannards, A. H. Schmidt and P. J. Kregor. New York, NY, Thieme Medical Publisher**:** 44-57.

Schnall SB, Holtom PD and Silva E (1994). Compartment syndrome associated with infection of the upper extremity. Clin Orthop Relat Res(306): 128-131.

Schwartz JT, Jr, Brumback RJ, Lakatos R, Poka A, Bathon GH and Burgess AR (1989). Acute compartment syndrome of the thigh. A spectrum of injury. J Bone Joint Surg Am **71**(3): 392-400.

Scragg JL, Dallas ML, Wilkinson JA, Varadi G, Peers C (2008). Carbon monoxide inhibits L-type Ca2+ channels via redox modulation of key cysteine residues by mitochondrial reactive oxygen species. J Biol Chem **283**(36): 24412-24419.

Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray DK, Harris KA, Parry NG and Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol **296**(1): G9-G14.

Seekamp A, Warren JS, Remick DG, Till GO and Ward PA (1993). Requirements for tumor necrosis factor-alpha and interleukin-1 in limb ischemia/reperfusion injury and associated lung injury. Am J Pathol **143**(2): 453-463.

Shears E and Porter K (2006). Acute compartment syndrome of the limb. Trauma **8**: 261-266.

Sheridan GW, Matsen FA, 3rd (1976). Fasciotomy in the treatment of the acute compartment syndrome. J Bone Joint Surg Am **58**(1): 112-115.

Shibahara S, Muller RM and Taguchi H (1987). Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem **262**(27): 12889-12892.

Smith JK, Carden DL and Korthuis RJ (1989). Role of xanthine oxidase in postischemic microvascular injury in skeletal muscle. Am J Physiol **257**(6 Pt 2): H1782-1789.

Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R and Yard BA (2009). "The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression." Br J Pharmacol **157**(5): 769-780.

Song M and Kellum JA (2005). Interleukin-6. Crit Care Med **33**(12 Suppl): S463-465.

Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR and Choi AM (2003). Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. Am J Pathol **163**(1): 231-242.

Sorice A, Guerriero E, Capone F, Colonna G, Castello G and Costantini S (2014). Ascorbic acid: its role in immune system and chronic inflammation diseases. Mini Rev Med Chem **14**(5): 444-452.

Strauss MB, Hargens AR, Gershuni DH, Greenberg DA, Crenshaw AG, Hart GB and Akeson WH (1983). Reduction of skeletal muscle necrosis using intermittent hyperbaric oxygen in a model compartment syndrome. J Bone Joint Surg Am **65**(5): 656-662.

Strauss MB, Hargens AR, Gershuni DH, Hart GB and Akeson WH (1986). Delayed use of hyperbaric oxygen for treatment of a model anterior compartment syndrome. J Orthop Res **4**(1): 108-111.

Sylvester JT and McGowan C (1978). The effects of agents that bind to cytochrome P-450 on hypoxic pulmonary vasoconstriction. Circ Res **43**(3): 429-437.

Taille C, El-Benna J, Lanone S, Boczkowski J and Motterlini R (2005). Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. J Biol Chem **280**(27): 25350-25360.

Tenhunen R, Marver HS and Schmid R (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A **61**(2): 748-755.

Thom SR (1990). Carbon monoxide-mediated brain lipid peroxidation in the rat. J Appl Physiol (1985) **68**(3): 997-1003.

Thomas JJ (1909). Nerve involvement in the ischemic paralysis and contracture of Volkmann. Ann Surg **49**(3): 330-370.

Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH and Chang KC (2009). Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo. Mol Pharmacol **76**(1): 173-182.

Ulmer T (2002). The clinical diagnosis of compartment syndrome of the lower leg: are clinical findings predictive of the disorder? J Orthop Trauma **16**(8): 572-577.

Urquhart P, Rosignoli G, Cooper D, Motterlini R and Perretti M (2007). Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. J Pharmacol Exp Ther **321**(2): 656-662.

Vadori M, Seveso M, Besenzon F, Bosio E, Tognato E, Fante F, Boldrin M, Gavasso S, Ravarotto L, Mann BE, Simioni P, Ancona E, Motterlini R and Cozzi E (2009). In vitro and in vivo effects of the carbon monoxide-releasing molecule, CORM-3, in the xenogeneic pig-to-primate context. Xenotransplantation **16**(2): 99-114.

Verma D, Hirsch DJ, Glatt CE, Ronnett GV and Snyder SH (1993). Carbon monoxide: a putative neural messenger. Science **259**(5093): 381-384.

Vigasio A, Battiston B, De Filippo G, Brunelli G and Calabrese S (1991). Compartmental syndrome due to viper bite. Arch Orthop Trauma Surg **110**(3): 175-177.

Vollmar B, Westermann S and Menger MD (1999). Microvascular response to compartment syndrome-like external pressure elevation: an in vivo fluorescence microscopic study in the hamster striated muscle. J Trauma **46**(1): 91-96.

von Volkmann R (1881). Die Ischaemischen Muskellahmungen und Kontrakturen. Zentralbl Chir **8**: 801-803.

Vreman HJ, Wong RJ and Stevenson DK (2000). Carbon monoxide in breath, blood, and other tissues. Carbon Monoxide Toxicity. D. G. Penney. Boca Raton, FL, CRC**:** 19-60.

Wagner R (1839). Erlauterungstaflen zur Physiologie und Entwicklungsgeschichte. Leipzig, Germany, Leopold Voss.

Wang R, Wang Z and Wu L (1997). Carbon monoxide-induced vasorelaxation and the underlying mechanisms. Br J Pharmacol **121**(5): 927-934.

Wattel F, Mathieu D, Neviere R and Bocquillon N (1998). Acute peripheral ischaemia and compartment syndromes: a role for hyperbaric oxygenation. Anaesthesia **53 Suppl 2**: 63-65.

Weaver LK (1999). Carbon monoxide poisoning. Crit Care Clin **15**(2): 297-317, viii.

Weaver LK (1999). Hyperbaric oxygen in carbon monoxide poisoning. BMJ **319**(7217): 1083-1084.

Weibel ER and Palade GE (1964). New Cytoplasmic Components in Arterial Endothelia. J Cell Biol **23**: 101-112.

Weiss SJ (1989). Tissue destruction by neutrophils. N Engl J Med **320**(6): 365-376.

West H (2007). Rhabdomyolysis associated with compartment syndrome resulting in acute renal failure. Eur J Emerg Med **14**(6): 368-370.

Whitesides TE, Haney TC, Morimoto K and Harada H (1975). Tissue pressure measurements as a determinant for the need of fasciotomy. Clin Orthop Relat Res(113): 43-51.

Whitesides TE and Heckman MM (1996). Acute Compartment Syndrome: Update on Diagnosis and Treatment. J Am Acad Orthop Surg **4**(4): 209-218.

Whitesides TE Jr, Haney TC, Harada H, Holmes HE and Morimoto K (1975). A simple method for tissue pressure determination. Arch Surg **110**(11): 1311-1313.

Wilkinson WJ, Gadeberg HC, Harrison AW, Allen ND, Riccardi D, Kemp PJ (2009). Carbon monoxide is a rapid modulator of recombinant and native P2X(2) ligand-gated ion channels. Br J Pharmacol **158**(3): 862-871.

Williams AB, Luchette FA, Papaconstantinou HT, Lim E, Hurst JM, Johannigman JA and Davis K Jr. (1997). The effect of early versus late fasciotomy in the management of extremity trauma. Surgery **122**(4): 861-866.

Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C and Kemp PJ (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. Science **306**(5704): 2093-2097.

Williamson KM, Wahl MS and Mycyk MB (2013). Direct comparison of 20-hour IV, 36-hour oral, and 72-hour oral acetylcysteine for treatment of acute acetaminophen poisoning. Am J Ther **20**(1): 37-40.

Willy C, Gerngross H and Sterk J (1999). Measurement of intracompartmental pressure with use of a new electronic transducer-tipped catheter system. J Bone Joint Surg Am **81**(2): 158-168.

Wright JG, Bogoch ER and Hastings DE (1989). The 'occult' compartment syndrome. J Trauma **29**(1): 133-134.

Yabluchanskiy A, Sawle P, Homer-Vanniasinkam S, Green CJ, Foresti R and Motterlini R (2012). CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of hemorrhagic stroke. Crit Care Med **40**(2): 544-552.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y and Koizumi S (1999). Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest **103**(1): 129-135.

Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R and Luscinskas FW (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood **106**(2): 584-592.

Yi ES and Ulich TR (1992). Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. Am J Pathol **140**(3): 659-663.

Zayasu K, Sekizawa K, Okinaga S, Yamaya M, Ohrui T and Sasaki H (1997). Increased carbon monoxide in exhaled air of asthmatic patients. Am J Respir Crit Care Med **156**(4 Pt 1): 1140-1143.

CHAPTER 2

THE EFFECT OF EXOGENOUS APPLICATION OF CARBON MONOXIDE ON SKELETAL MUSCLE MICROCIRCULATION IN THE RAT MODEL OF ACUTE LIMB COMPARTMENT SYNDROME.

A portion of this chapter formed the basis for the patent application titled 'Therapeutic Use of Carbon Monoxide in Acute Limb Compartment Syndrome', awarded to Dr. Lawendy and his Trauma lab research team.

CHAPTER 2: THE EFFECT OF EXOGENOUS APPLICATION OF CARBON MONOXIDE ON SKELETAL MUSCLE MICROCIRCULATION IN THE RAT MODEL OF ACUTE LIMB COMPARTMENT SYNDROME.

2.1 INTRODUCTION

Acute limb compartment syndrome (CS), a complication of musculoskeletal trauma, results from an increase in pressure within a closed osseofascial compartment, leading to muscle-threatening and limb-threatening ischemia (Matsen 1975, Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen 1980, Hartsock, O'Farrell et al. 1998). Emergency fasciotomy, to fully decompress all the tissues in the involved compartments, remains the only effective treatment and current gold-standard surgical therapy, but it must be performed in a timely manner, before the injury to the tissues becomes permanent. Despite ongoing research dedicated to understanding the pathophysiology of CS, the mechanisms of CS-induced tissue damage are still poorly understood, making therapeutic targets rather limited.

CS, as a form of ischemia-reperfusion (I/R) injury, results in significant microvascular dysfunction within the affected muscle (Lawendy, Sanders et al. 2011). Leukocytes appear to play a major role in the pathophysiology of this condition (Lawendy, Bihari et al. 2015). However, unlike complete ischemia, CS occurs in the face of patent vessels (i.e. low-flow ischemia), making it impossible

to pinpoint the exact time when the metabolic demand of the tissue exceeds the actual supply of oxygen, thereby triggering oxidative stress, cytokine release, inflammation and consequent tissue damage.

Previously, the upregulation of heat shock proteins, particularly heme oxygenase (HO), has been shown to be beneficial in various models of reperfusion injury (Nie, McCarter et al. 2002, McCarter, Scott et al. 2003, Akamatsu, Haga et al. 2004, McCarter, Badhwar et al. 2004, Lee, Gao et al. 2007). HO is a rate-limiting enzyme involved in catabolic degradation of heme into biliverdin and carbon monoxide (CO) (Tenhunen, Marver et al. 1968). Numerous studies have demonstrated potent cytoprotective and anti-inflammatory effects of HO/CO in various models (Motterlini and Otterbein 2010), making HO a potential therapeutic target. While systemic upregulation of HO (e.g. by the use of adenoviral transfer) may not be clinically feasible, administration of its byproducts, particularly CO, on the other hand, can be accomplished with relative ease.

Several studies have demonstrated that exogenous administration of low-dose CO by inhalation offers both protection to microvascular perfusion, and anti-inflammatory benefits during systemic inflammation (Nakao, Kimizuka et al. 2003, Hegazi, Rao et al. 2005, Mazzola, Forni et al. 2005, Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009), sepsis (Mazzola, Forni et al. 2005, Koulouras, Li et al. 2011), and organ preservation for transplantation (Neto, Nakao et al. 2004, Hanto, Maki et al. 2010). However, the possible therapeutic application and efficacy of CO have never been assessed in CS.

The purpose of this study was to test the effect of inhalational CO on the severity of microvascular dysfunction following CS. It was hypothesized that application of CO may be beneficial by reducing the degree of microvascular perfusion deficits, inflammation, and tissue injury normally seen in CS. The ultimate goal of this study is the development of pharmacological adjunctive therapy that would allow the prolongation of the fasciotomy surgical window, or completely eliminate the need for it.

2.2 MATERIALS AND METHODS

2.2.1 Animal Preparation

The experimental protocol was approved by the Council on Animal Care of the University of Western Ontario (Appendix III), and has been previously described in detail (Lawendy, Sanders et al. 2011). Briefly, male Wistar rats (body weight 180-250g) were anesthetized by inhalational isoflurane (5% induction, 2% maintenance) in 1:1 oxygen/nitrogen mixture. Left carotid artery was cannulated to allow for the monitoring of systemic blood pressure, fluid administration and blood sampling.

Compartment pressure monitoring probe (Synthes, Westchester PA) was inserted into the posterior compartment via gauge 16 angiocatheter (BD), while gauge 24 angiocatheter (BD) attached to an IV line was placed into the anterior compartment of the rat hind limb (Figure 2.1). CS was induced by an infusion of isotonic saline, leading to an elevation of intra-compartmental pressure (ICP) to

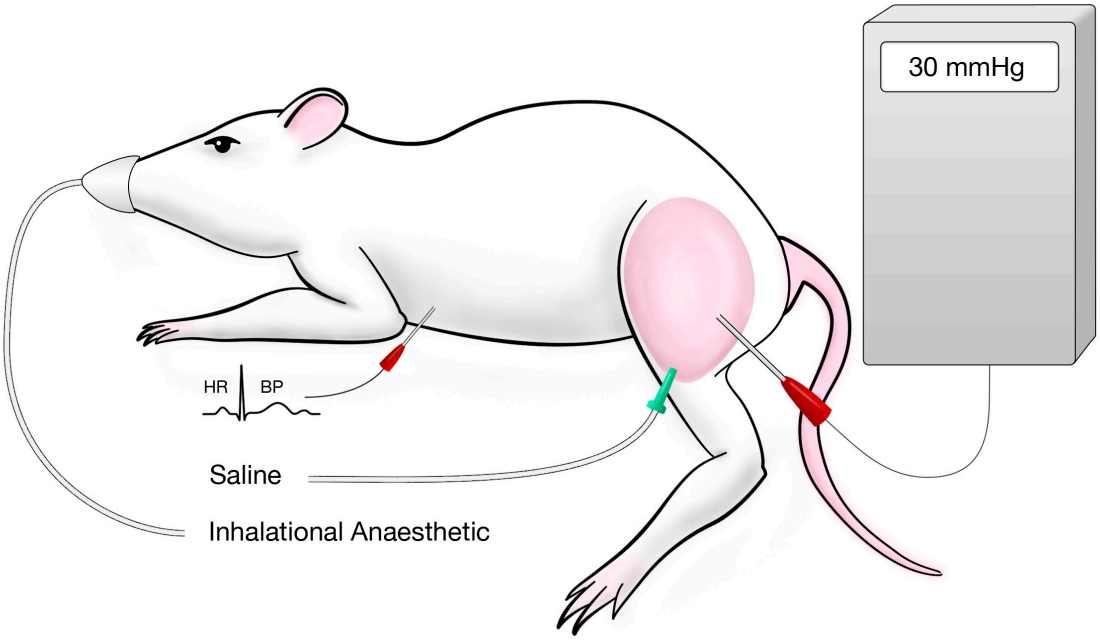


Figure 2.1 **Schematics of the experimental setup of a rat model of compartment syndrome**. Elevation of intra-compartmental pressure to 30-40mmHg was achieved by an infusion of isotonic saline into the anterior compartment of the rat hind limb. Elevated compartment pressure was continuously monitored and maintained for 2 hours, producing compartment syndrome-like conditions.

30-40 mmHg. Elevated ICP was maintained for 2 hours. Fasciotomy was performed to decompress the hind limb compartments; the muscles were allowed to reperfuse for 45 minutes, followed by intravital video microscopy (IVVM).

2.2.2 Exogenous Application of CO

CO was exogenously applied to animals at fasciotomy, by inhalation. The inhaled gas mixture contained 250ppm CO in medical air, pumped into the facemask through the anesthesia circuit. Inhalation of CO to animals commenced right before fasciotomy; animals were subjected to inhalational CO during the whole reperfusion period of 45 minutes.

2.2.3 Experimental Groups

Animals were randomly assigned into one of the four groups: (1) sham (n=4), (2) sham under CO inhalation (n=4), (3) CS under normal air (n=4) and (4) CS followed by CO inhalation (n=4). Sham animals underwent all procedures as CS animals, but the ICP was maintained at baseline level (0mmHg).

At the conclusion of the experiment, systemic levels of carboxyhemoglobin (COHb) were assessed in all animals.

2.2.4 Intravital Video Microscopy

The extensor digitorum longus (EDL) muscle was dissected to the level of its distal tendon, which was tied with a suture and cut from its bony insertion. The animal was transferred onto the stage of an inverted microscope (Nikon); the

EDL was reflected into a saline bath containing 5μg/ml each of the fluorescent vital dyes bisbenzimide (BB; exc. 343nm, em. 483nm) and ethidium bromide (EB; exc. 482nm, em. 616nm). BB stains the nuclei of all cells while EB stains the nuclei of only those cells with damaged cell membrane; thus, EB/BB ratio provided an index of tissue injury.

Microvascular perfusion and leukocytes within the post-capillary venules were recorded by translumination with 20x and 40x objectives, respectively, in five adjacent 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 anesthetic agent.

2.2.5 Offline Video Analysis

Capillary perfusion was assessed by counting the number of continuously-perfused (CPC), intermittently-perfused (IPC) and non-perfused (NPC) capillaries that crossed three parallel lines drawn perpendicular to the capillary axis on the video monitor, and was expressed as % of total capillaries. Tissue injury was assessed by counting the number of EB- and BB-labelled nuclei, and expressed as EB/BB ratio. Leukocyte activation was assessed by counting the numbers of rolling and adherent leukocytes in post-capillary venules and expressed per unit area (i.e. 1000μm^2). Venular area was measured using ImageJ (NIH, Bethesda, MD). A leukocyte was considered adherent if it remained stationary for at least

30 seconds, and a cell was considered rolling if it remained in contact with the wall of the vessel during its movement.

2.2.6 Statistical Analysis

All parameters were expressed as mean ± SEM and analyzed using one-way ANOVA, with Bonferroni post-hoc test as needed. $p<0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Microvascular Perfusion

Elevation of ICP led to significant changes in microvascular perfusion, as shown in Figure 2.2. The number of continuously-perfused capillaries decreased from 74±6% in sham to 22±3% in CS group ($p<0.0001$), while the number of non-perfused capillaries increased from 12±3% in sham to 54±3% in CS group. Inhalation of CO, commenced upon fasciotomy, was able to restore the number of perfused capillaries to 57±3% ($p<0.001$). Inhalational CO had no effect on capillary perfusion in sham animals (76±2%, not significant).

2.3.2 Tissue Injury

Elevation of ICP resulted in a significant increase in muscle tissue injury, as measured by EB/BB ratio, from 0.05±0.03 in sham to 0.31±0.05 in CS group ($p<0.001$). Inhalation of CO significantly decreased tissue injury to 0.02±0.02

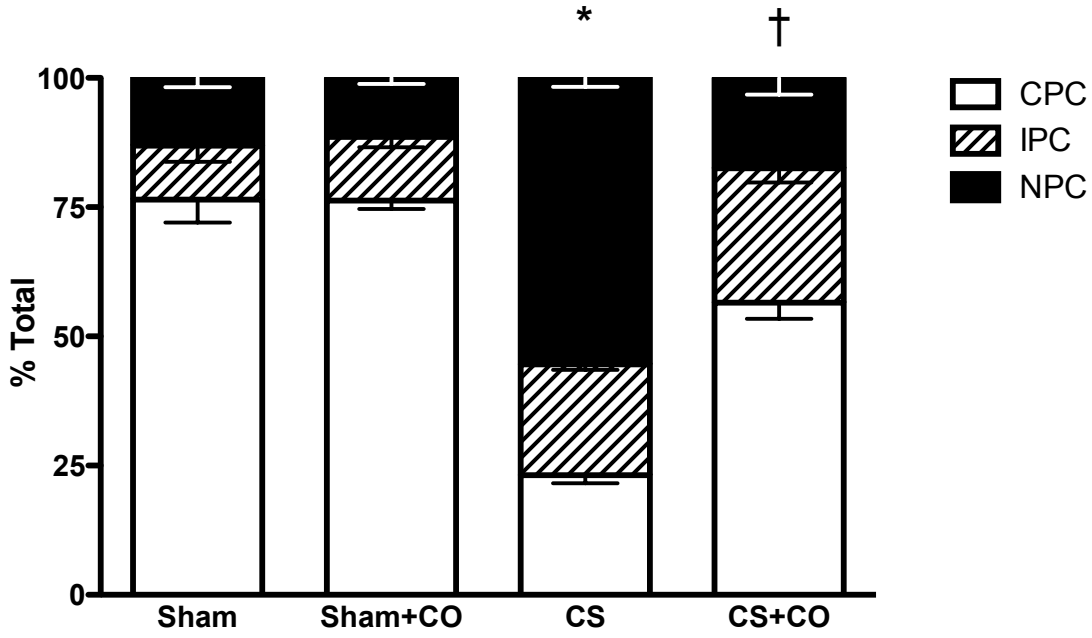


Figure 2.2. The effect of CO inhalation on skeletal muscle microvascular perfusion following CS. Two hours of elevated ICP were followed by fasciotomy, 45min reperfusion coupled with CO inhalation, and IVVM. *CS-associated perfusion changes were partially reversed by CO inhalation* (*p<0.001 from sham and sham+CO; †p<0.001 from CS; see the text for additional details). *CPC*, continuously-perfused capillaries; *IPC*, intermittently-perfused capillaries; *NPC*, non-perfused capillaries.

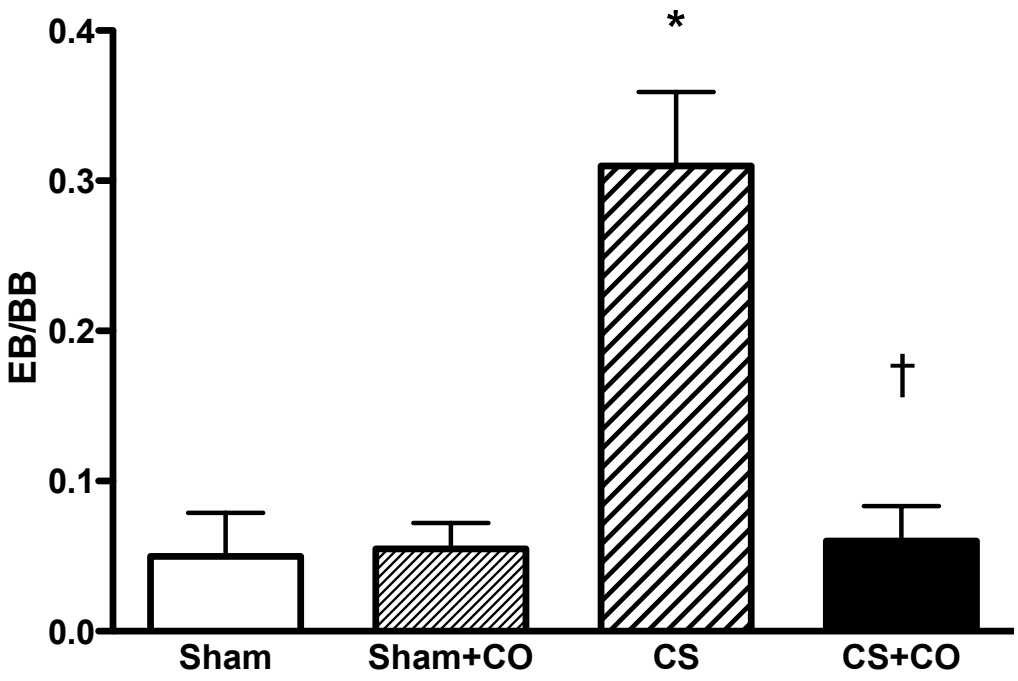


Figure 2.3. The effect of CO inhalation on skeletal muscle tissue injury following CS. Two hours of elevated ICP were followed by fasciotomy, 45min reperfusion coupled with CO inhalation, and IVVM. *CS-associated tissue injury was reversed by CO inhalation* (*p<0.001 from sham and sham+CO; †p<0.001 from CS).

($p<0.0001$) (Figure 2.3). CO by itself had no effect on tissue injury (0.02±0.02, not significant).

2.3.3 Inflammation

Elevation of ICP resulted in leukocyte activation, as demonstrated by an increase in adhesion to the vascular endothelium. Leukocyte adherence in the post-capillary venules of the skeletal muscle was significantly increased from 1.9±0.5 in sham to 17.5±0.9 leukocytes/30s/1000μm^2 in CS group ($p<0.0001$). Leukocyte rolling only moderately increased from 1.7±0.7 to 3.3±0.7 leukocytes/30s/1000μm^2. Inhalation of CO led to a significant, 10-fold decrease in CS-triggered leukocyte adherence (0.6±0.2 adherent leukocytes/30s/1000μm^2, $p<0.001$), while having no statistically significant effect on leukocyte rolling (2.0±0.6 rolling leukocytes/30s/1000μm^2, not significant) (Figure 2.4).

2.3.4 Systemic COHb Level

Inhalation of CO led to significant increase in carboxyhemoglobin (COHb) levels in both sham and CS animals (7.6±1.6% in CS+CO and 7.4±1.3% in sham+CO, respectively versus 0.3±0.1% in sham, $p<0.0001$) (Figure 2.5). Elevation of ICP by itself did not produce any changes in COHb (0.4±0.2%, not significant).

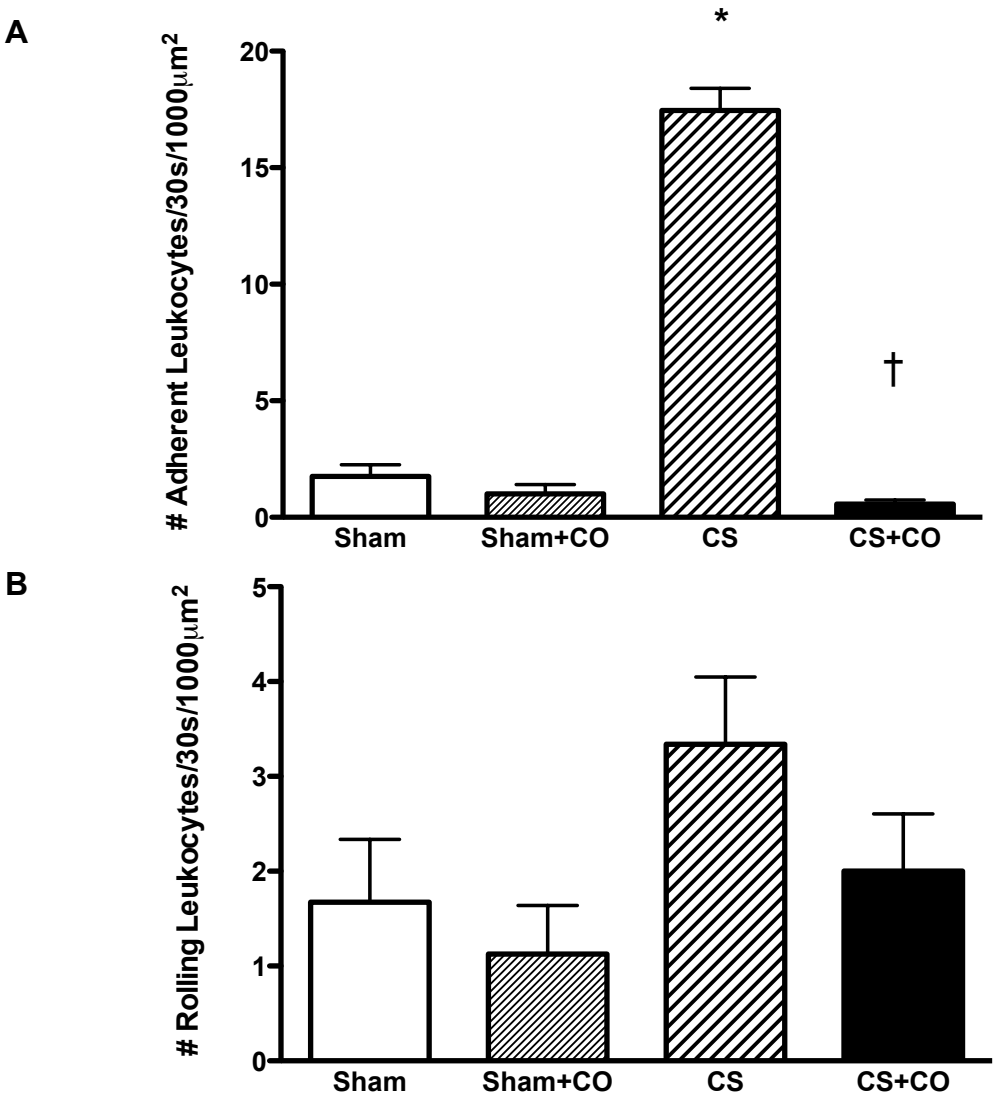


Figure 2.4. The effect of CO inhalation on leukocyte adhesive interactions with endothelium of skeletal muscle microvasculature in CS-challenged rat. Two hours of elevated ICP were followed by fasciotomy and 45min reperfusion coupled with CO inhalation. Leukocyte adhesion (A) and rolling (B) were assessed by IVVM. *CO inhalation was able to prevent leukocyte adhesion within the post-capillary venules* (*$p<0.001$ from sham and sham+CO; †$p<0.001$ from CS).

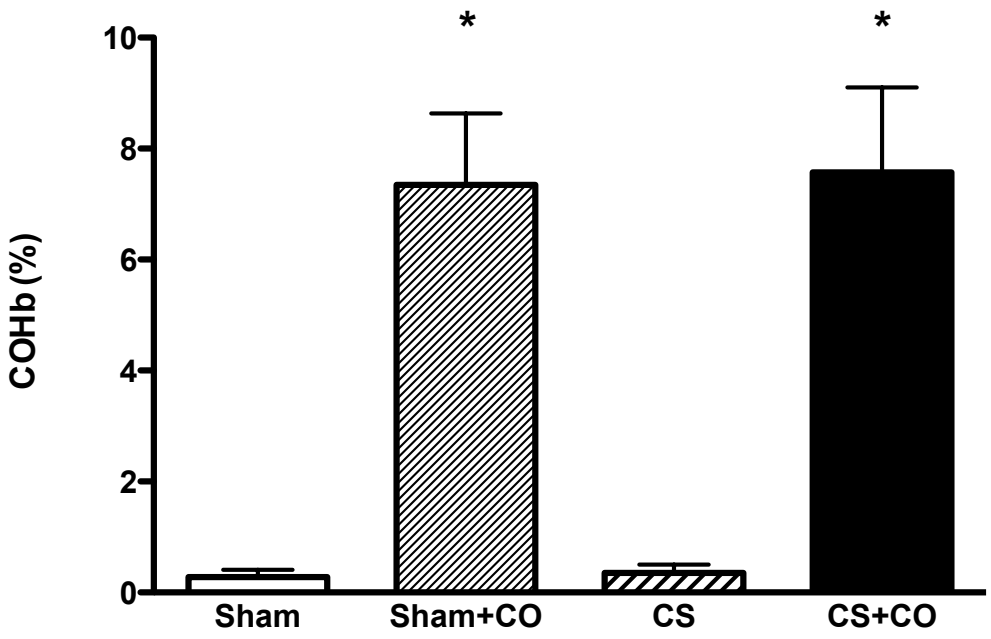


Figure 2.5. The effect of inhaled CO on systemic COHb levels. Two hours of elevated ICP were followed by fasciotomy and 45min reperfusion under CO inhalation. *Inhalation of CO caused a significant increase in COHb, while elevation of ICP by itself had no effect* (*p<0.0001 from sham and CS).

2.4 DISCUSSION

Compartment syndrome is a challenging complication of musculoskeletal trauma. Therapeutic applications, aside from the gold-standard fasciotomy, are limited, and only address the sequelae of CS rather than pathophysiological mechanisms leading to CS formation (Olson and Glasgow 2005). In the present study, we demonstrated the benefit of inhalational carbon monoxide on improving capillary perfusion, reducing cellular damage and leukocyte recruitment following compartment syndrome and fascial release.

CO is a signalling molecule produced endogenously by the degradation of heme, a reaction catalyzed by inducible and constitutively expressed heme oxygenases (HO-1 and HO-2, respectively) (Otterbein 2009). CO has been shown to generate vasodilatory effects, mitigate intracellular apoptosis and suppress inflammatory pathways in various models of inflammation, including ischemia-reperfusion (Otterbein 2002, Motterlini 2007). In our study, exogenous application of CO by inhalation demonstrated a beneficial effect by preserving microvascular flow in CS-challenged muscle.

Continuous perfusion is characterized by the non-stop passage of blood through the capillary bed, supplying the tissue with the necessary oxygen and nutrients. Any derangement in the blood movement (i.e. shift to intermittency or no flow) compromises gas exchange, and results in ischemia (Lawendy, Sanders et al. 2011). In our study, we demonstrated that CO inhalation was capable of improving the capillary perfusion, restoring the number of continuously-perfused capillaries almost to sham levels (Figure 2.2). Moreover, CO inhalation was very

effective in restoring tissue injury levels back to baseline levels, as those seen in the sham group (Figure 2.3). Thus, CO appeared to provide a substantial protective effect within the CS-challenged muscle. To the best of our knowledge, this study is the first to demonstrate such potent protective effects of CO in the context of acute CS.

CS pathophysiology is, at least in part, driven by I/R injury. The effects of complete ischemia on the skeletal muscle have been well documented in literature, with predictable alterations in the microcirculation (Harman 1948, Strock and Majno 1969, Labbe, Lindsay et al. 1987, Belkin, Brown et al. 1988, Lindsay, Liauw et al. 1990, Hickey, Hurley et al. 1992, Sabido, Milazzo et al. 1994). Ischemia results in a shift of the cellular metabolism into oxidative mode, producing pro-inflammatory environment (Gute, Ishida et al. 1998, Gillani, Cao et al. 2011). Upon the restoration of blood flow (i.e. reperfusion), pro-inflammatory mediators (e.g. reactive oxygen species (ROS), cytokines/chemokines, platelet activating factor (PAF), etc.) released from the activated vascular endothelial cells and leukocytes enter the circulation, leading to systemic activation of leukocytes and subsequent recruitment into the reperfused tissue (Hernandez, Grisham et al. 1987, Kubes, Suzuki et al. 1990, Schlag, Harris et al. 2001). During inflammation, leukocyte extravasation from the blood is controlled by well-coordinated adhesive interactions between leukocytes and vascular endothelial cells, which involves rolling (P-, E-, L-selectins), firm adhesion (integrins, ICAM-1, VCAM-1) and migration across the endothelium (PECAM-1, CD99, JAMs) (Ley, Laudanna et al. 2007).

Activated leukocytes produce ROS and proteolytic enzymes that cause further cellular damage and increased vascular permeability to plasma proteins, thereby contributing to the formation of progressive edema (Sexton, Korthuis et al. 1990, Forbes, Carson et al. 1995, Rubin, Romaschin et al. 1996, Kurose, Argenbright et al. 1997, Gute, Ishida et al. 1998). In the context of CS, fluid accumulation in the muscle will lead to an increase in the interstitial pressure, compressing the neighbouring capillaries and causing further shift to non-perfusion (Lawendy, Sanders et al. 2011).

In our rat model of CS, the blood flow through the capillary beds was not completely blocked; rather, the elevated ICP created a low-flow ischemic state, with reperfusion injury happening almost concurrently with ischemia. Upon fasciotomy, the full restoration of blood flow resulted in additional reperfusion damage, further accentuating the CS-induced microvascular dysfunction. We observed a substantial inflammatory response, characterized by an increase in leukocyte adhesion under conditions of flow, and recruitment of activated leukocytes into muscle vasculature in response to elevation of ICP and fasciotomy (Figure 2.4). CO inhalation led to a complete inhibition of leukocyte activation, with respect to suppressed leukocyte adhesion within post-capillary venules (Figure 2.4). This was coupled with more than 75% inhibition of tissue injury (Figure 2.3), implying that inflammation (i.e. overwhelming leukocyte recruitment) imparts a considerable pathological process driving the CS-associated parenchymal damage. Indeed, activated leukocytes have been shown to be one of the most significant contributors to the parenchymal injury seen in

CS (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2015); the findings parallel those obtained from the models of reperfusion injury after complete ischemia (Forbes, Harris et al. 1996, Gute, Ishida et al. 1998). Given the potent anti-inflammatory nature of CO (Otterbein, Bach et al. 2000, Otterbein 2002, Song, Kubo et al. 2003, Motterlini, Haas et al. 2012), our data provides further confirmation of the critical role of inflammation in the pathophysiology of CS.

Despite some key findings, it is important to note that while inhalation of CO significantly improved the overall microvascular perfusion in CS-challenged rats, some degree of perfusion derangement still remained (Figure 2.2). This suggests that the microvascular dysfunction and pathophysiology of CS may also be driven by other, not yet understood, factor(s).

Elevated levels of COHb may represent a serious challenge when considering inhaled CO as a potential therapy for treatment of CS. Affinity of CO for hemoglobin is approximately 240 times higher than that for oxygen (Weaver 1999); in our study, 45 minute inhalation of 250ppm CO resulted in an increase of COHb to 8%, in both sham and CS-challenged rats (Figure 2.5). It is important to note, however, that similar levels (i.e. 3-8% COHb) are detected in the blood of cigarette smokers (Vreman, Wong et al. 2000). While such level of COHb in normal, healthy subjects is unlikely to be toxic, it may not be negligible in trauma patients, majority of whom already experience insufficient levels of oxygenation.

This study, as a proof of concept, demonstrates that exogenous application of CO may be of benefit in patients at risk of acute CS. While an increase in COHb level may not be inconsequential, with the rapid advancements

in the development of transition metal carbonyls, pharmacological means of CO delivery might open further avenues for research into prolongation of the surgical window and tissue preservation.

2.5 REFERENCES

Akamatsu Y, Haga M, Tyagi S, Yamashita K, Graca-Souza AV, Ollinger R, Czismadia E, May GA, Ifedigbo E, Otterbein LE, Bach FH and Soares MP (2004). Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. FASEB J **18**(6): 771-772.

Belkin M, Brown RD, Wright JG, LaMorte WW and Hobson RW, 2nd (1988). A new quantitative spectrophotometric assay of ischemia-reperfusion injury in skeletal muscle. Am J Surg **156**(2): 83-86.

Forbes TL, Carson M, Harris KA, DeRose G, Jamieson WG and Potter RF (1995). "Skeletal muscle injury induced by ischemia-reperfusion." Can J Surg **38**(1): 56-63.

Forbes TL, Harris KA, Jamieson WG, DeRose G, Carson M and Potter RF (1996). Leukocyte activity and tissue injury following ischemia-reperfusion in skeletal muscle. Microvasc Res **51**(3): 275-287.

Gillani S, Cao J, Suzuki T and Hak DJ (2012). The effect of ischemia reperfusion injury on skeletal muscle. Injury **43**(6): 670-5.

Gute DC, Ishida T, Yarimizu K and Korthuis RJ (1998). Inflammatory responses to ischemia and reperfusion in skeletal muscle. Mol Cell Biochem **179**(1-2): 169-187.

Hanto DW, Maki T, Yoon MH, Csizmadia E, Chin BY, Gallo D, Konduru B, Kuramitsu K, Smith NR, Berssenbrugge A, Attanasio C, Thomas M, Wegiel B and Otterbein LE (2010). Intraoperative administration of inhaled carbon monoxide reduces delayed graft function in kidney allografts in Swine. Am J Transplant **10**(11): 2421-2430.

Harman JW (1948). The significance of local vascular phenomena in the production of ischemic necrosis in skeletal muscle. Am J Pathol **24**(3): 625-641.

Hartsock LA, O'Farrell D, Seaber AV and Urbaniak JR (1998). Effect of increased compartment pressure on the microcirculation of skeletal muscle. Microsurgery **18**(2): 67-71.

Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE and Plevy SE (2005). Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. J Exp Med **202**(12): 1703-1713.

Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM and Granger DN (1987). Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am J Physiol **253**(3 Pt 2): H699-703.

Hickey MJ, Hurley JV, Angel MF and O'Brien BM (1992). The response of the rabbit rectus femoris muscle to ischemia and reperfusion. J Surg Res **53**(4): 369-377.

Koulouras VP, Li R, Chen L and Hedenstierna GG (2011). Effects of inhaled carbon monoxide and glucocorticoids in porcine endotoxin sepsis. Int J Clin Exp Med **4**(1): 53-66.

Kubes P, Suzuki M and Granger DN (1990). Modulation of PAF-induced leukocyte adherence and increased microvascular permeability. Am J Physiol **259**(5 Pt 1): G859-864.

Kurose I, Argenbright LW, Wolf R, Lianxi L and Granger DN (1997). Ischemia/reperfusion-induced microvascular dysfunction: role of oxidants and lipid mediators. Am J Physiol **272**(6 Pt 2): H2976-2982.

Labbe R, Lindsay T and Walker PM (1987). The extent and distribution of skeletal muscle necrosis after graded periods of complete ischemia. J Vasc Surg **6**(2): 152-157.

Lawendy AR, Bihari A, Sanders D, McGarr G, Badhwar A and Cepinskas G (2015). Contribution of inflammation to cellular injury in compartment syndrome in an experimental rodent model. Bone Joint J **97-B**(4): 539-543.

Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D and Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Lee SS, Gao W, Mazzola S, Thomas MN, Csizmadia E, Otterbein LE, Bach FH and Wang H (2007). Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells. FASEB J **21**(13): 3450-3457.

LeyK, Laudanna C, Cybulsky MI and Nourshargh S (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol **7**(9): 678-689.

Lindsay TF, Liauw S, Romaschin AD and Walker PM (1990). The effect of ischemia/reperfusion on adenine nucleotide metabolism and xanthine oxidase production in skeletal muscle. J Vasc Surg **12**(1): 8-15.

Manjoo A, Sanders D, Lawendy A, Gladwell M, Gray D, Parry N and Badhwar A (2010). Indomethacin reduces cell damage: shedding new light on compartment syndrome. J Orthop Trauma **24**(9): 526-529.

Matsen FA, 3rd (1975). Compartmental syndrome. An unified concept. Clin Orthop Relat Res(113): 8-14.

Matsen FA, 3rd (1980). Compartmental syndromes. Hosp Pract **15**(2): 113-117.

Mazzola S, Forni M, Albertini A, Bacci ML, Zannoni A, Gentilini F, Lavitrano M, Bach FH, Otterbein LE and Clement MG (2005). Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. FASEB J **19**(14): 2045-2047.

McCarter SD, Badhwar A, Scott JR, Akyea TG, Bihari A, Dungey AA, Harris KA and Potter RF (2004). Remote liver injury is attenuated by adenovirus-mediated gene transfer of heme oxygenase-1 during the systemic inflammatory response syndrome. Microcirculation **11**(7): 587-595.

McCarter SD, Scott JR, Lee PJ, Zhang X, Choi AM, McLean A, Badhwar A, Dungey AA, Bihari A, Harris KA and Potter RF (2003). Cotransfection of heme oxygenase-1 prevents the acute inflammation elicited by a second adenovirus. Gene Therapy **10**(19): 1629-1635.

Motterlini R (2007). Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem Soc Trans **35**(Pt 5): 1142-1146.

Motterlini R, Haas B and Foresti R (2012). Emerging concepts on the anti-inflammatory actions of carbon monoxide-releasing molecules (CO-RMs). Med Gas Res **2**(1): 28.

Motterlini R and Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nat Rev Drug Discov **9**(9): 728-743.

Mubarak SJ, Owen CA, Hargens AR, Garetto LP and Akeson WH (1978). Acute compartment syndromes: diagnosis and treatment with the aid of the wick catheter. J Bone Joint Surg Am **60**(8): 1091-1095.

Nakao A, Kimizuka K, Stolz DB, Neto JS, Kaizu T, Choi AM, Uchiyama T, Zuckerbraun BS, Nalesnik MA, Otterbein LE and Murase N (2003). Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. Am J Pathol **163**(4): 1587-1598.

Neto JS, Nakao A, Kimizuka K, Romanosky AJ, Stolz DB, Uchiyama, Nalesnik MA, Otterbein LE and Murase N (2004). "Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide." Am J Physiol Renal Physiol **287**(5): F979-989.

Nie RG, McCarter SD, Harris KA, Lee PJ, Zhang X, Bihari A, Gray D, Wunder C, Brock RW and Potter RF (2002). The role of endogenous heme oxygenase in the initiation of liver injury following limb ischemia/reperfusion. J Hepatol **36**(5): 624-630.

Olson SA and Glasgow RR (2005). Acute compartment syndrome in lower extremity musculoskeletal trauma. J Am Acad Orthop Surg **13**(7): 436-444.

Ott MC, Scott JR, Bihari A, Badhwar, Otterbein LE, Gray DK, Harris KA and Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hind limb ischemia/reperfusion. FASEB J **19**(1): 106-108.

Otterbein LE (2002). Carbon monoxide: innovative anti-inflammatory properties of an age-old gas molecule. Antioxid Redox Signal **4**(2): 309-319.

Otterbein LE (2009). The evolution of carbon monoxide into medicine. Respir Care **54**(7): 925-932.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, R. J. Davis RJ, Flavell RA and Choi AM (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med **6**(4): 422-428.

Rorabeck CH and Clarke KM (1978). "The pathophysiology of the anterior tibial compartment syndrome: an experimental investigation." J Trauma **18**(5): 299-304.

Rubin BB, Romaschin A, Walker RM, Gute DC and Korthuis RJ (1996). Mechanisms of postischemic injury in skeletal muscle: intervention strategies. J Appl Physiol **80**(2): 369-387.

Sabido F, Milazzo VJ, Hobson RW, 2nd and Duran WN (1994). Skeletal muscle ischemia-reperfusion injury: a review of endothelial cell-leukocyte interactions. J Invest Surg **7**(1): 39-47.

Schlag MG, Harris KA and Potter RF (2001). Role of leukocyte accumulation and oxygen radicals in ischemia-reperfusion-induced injury in skeletal muscle. Am J Physiol Heart Circ Physiol **280**(4): H1716-1721.

Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray DK, Harris KA, Parry NG and Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol **296**(1): G9-G14.

Sexton WL, Korthuis RJ and Laughlin MH (1990). Ischemia-reperfusion injury in isolated rat hindquarters. J Appl Physiol **68**(1): 387-392.

Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR and Choi AM (2003). Carbon monoxide induces cytoprotection in rat

orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. Am J Pathol **163**(1): 231-242.

Strock PE and Majno G (1969). Microvascular changes in acutely ischemic rat muscle. Surg Gynecol Obstet **129**(6): 1213-1224.

Tenhunen R, Marver HS and Schmid R (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A **61**(2): 748-755.

Vreman HJ, Wong RJ and Stevenson DK (2000). Carbon monoxide in breath, blood, and other tissues. Carbon Monoxide Toxicity. D. G. Penney. Boca Raton, FL, CRC**:** 19-60.

Weaver LK (1999). Carbon monoxide poisoning. Crit Care Clin **15**(2): 297-317, viii.

Whitesides TE, Haney TC, Morimoto K and Harada H (1975). Tissue pressure measurements as a determinant for the need of fasciotomy." Clin Orthop Relat Res(113): 43-51.

CHAPTER 3

THE SEVERITY OF MICROVASCULAR DYSFUNCTION DUE TO COMPARTMENT SYNDROME IS DIMINISHED BY THE SYSTEMIC APPLICATION OF CO-RELEASING MOLECULE-3 (CORM-3).

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CHAPTER 3: THE SEVERITY OF MICROVASCULAR DYSFUNCTION DUE TO COMPARTMENT SYNDROME IS DIMINISHED BY THE SYSTEMIC APPLICATION OF CO-RELEASING MOLECULE-3 (CORM-3).

3.1 INTRODUCTION

Acute limb compartment syndrome (CS), a potentially devastating complication of musculoskeletal trauma, is characterized by an increase in pressure within a closed osseofascial compartment, resulting in muscle-threatening and ultimately limb-threatening ischemia (Matsen 1975, Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen 1980, Hartsock, O'Farrell et al. 1998). Fasciotomy, to fully decompress all the muscles in the involved compartments, remains the only effective treatment and current gold-standard surgical therapy. Despite a large body of literature dedicated to understanding the pathophysiology of CS, the mechanisms of CS-induced tissue damage are rather poorly understood.

Extremity CS occurs once swelling within a muscle compartment develops to such a degree that the tissue perfusion becomes compromised. The established view of the pathophysiological process of CS development is that increasing compartmental pressure compromises microcirculatory perfusion, thus restricting oxygen and nutrient delivery to vital tissues, ultimately resulting in cellular anoxia and severe tissue necrosis (Sheridan and Matsen 1975,

Whitesides, Haney et al. 1975, Rorabeck and Clarke 1978, Matsen, Winquist et al. 1980). Unlike complete ischemia, CS causes myonecrosis in the face of patent vessels. As such, the pathologic contribution of inflammation to the pathophysiology of CS is being increasingly recognized; studies from our group (Lawendy, Sanders et al. 2011) and others (Sadasivan, Carden et al. 1997, Kalns, Cox et al. 2011) have broadly implicated leukocytes as playing a primary role in both microvascular and parenchymal injury during CS.

Despite active investigation, few therapeutic options have been shown to be effective. Recently, carbon monoxide (CO), a byproduct of heme oxygenase (HO) activity has been shown to offer both protection to microvascular perfusion, and anti-inflammatory benefits during systemic inflammation (Nakao, Kimizuka et al. 2003, Hegazi, Rao et al. 2005, Mazzola, Forni et al. 2005, Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009). Although the exogenous administration of CO via inhalation (250ppm) has been shown beneficial during systemic inflammatory response syndrome (Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009), such method of administration results in increased carboxyhemoglobin (COHb) levels, thus presenting a potential threat to the host.

Lately, transitional metal carbonyls, CO-releasing molecules (CO-RMs) have been used to deliver CO in a controlled manner without significantly altering COHb (Motterlini, Clark et al. 2002, Clark, Naughton et al. 2003, Motterlini 2007). The major advantage of using CO-RMs versus inhaled CO is the ability to control CO delivery without significantly increasing COHb, and choice of various routes (intravenous, intraperitoneal, subcutaneous or tissue superfusion) of CO

administration to target specific organs/tissues. Consequently, CO-RMs have received an increased attention for the potential pharmaceutical application (Motterlini, Clark et al. 2002, Motterlini 2007, Motterlini and Otterbein 2010). CO-RMs have been shown to act pharmacologically in rat aortic and cardiac tissue, where liberation of CO produced vasorelaxant effects, decreased myocardial ischemia/reperfusion damage, and reduced inflammatory response in LPS-stimulated macrophages (Clark, Naughton et al. 2003, Cepinskas, Katada et al. 2008, Mizuguchi, Stephen et al. 2009, Katada, Bihari et al. 2010).

The purpose of this study was to investigate the effects of CO, liberated from a novel, water-soluble CO donor, CORM-3, on the microvascular function of CS-challenged muscle, using our clinically relevant rodent model of CS (Lawendy, Sanders et al. 2011). The ultimate goal is the development of a pharmacologic adjunctive treatment for compartment syndrome, which would reduce the morbidity and disability in patients.

3.2 MATERIALS AND METHODS

3.2.1 Animal Preparation

The experimental protocol was approved by the Council on Animal Care of the University of Western Ontario (Appendix III), and has been previously described in detail (Lawendy, Sanders et al. 2011). Briefly, male Wistar rats (body weight 180-250g) were anesthetized by inhalational isoflurane (5% induction, 2% maintenance) in 1:1 oxygen/nitrogen mixture. Left carotid artery

was cannulated to allow for the monitoring of systemic blood pressure, fluid administration and blood sampling.

Compartment pressure monitoring probe (Synthes, Westchester PA) was inserted into the posterior compartment via gauge 16 angiocatheter (BD), while gauge 24 angiocatheter (BD) attached to an IV line was placed into the anterior compartment of the rat hind limb. CS was induced by an infusion of isotonic saline, leading to an elevation of intra-compartmental pressure (ICP) to 30 mmHg. Elevated ICP was maintained for 2 hours. Fasciotomy was performed to decompress the hind limb compartments; the muscles were allowed to reperfuse for 45 minutes, followed by intravital video microscopy.

3.2.2 CORM-3 Synthesis

A water-soluble CORM-3 (tricarbonylchloro-glycinate-ruthenium(II), [$Ru(CO)_3Cl$-glycinate]; molecular weight 295 $gmol^{-1}$) was synthesized by us, in accordance with the previously-published method (Motterlini and Otterbein 2010). CORM-3 (10mg/ml stock solution) was prepared fresh by dissolving CORM-3 in isotonic saline just prior to injection. As a control, inactive CORM-3 (iCORM-3) was generated by dissolving CORM-3 in saline 72 hours prior to the experiment and allowing it to release all CO from the solution (Clark, Naughton et al. 2003).

3.2.3 Experimental Groups

Rats were randomly assigned to one of four experimental groups: sham (n=4), CS (n=4), CS+CORM-3 (n=8) and CS+iCORM-3 (n=8). CO-releasing

molecule-3 (CORM-3), or its inactive form (iCORM-3), was administered to animals undergoing CS upon fasciotomy at the dose of 10mg/kg, IP. Sham animals underwent all the procedures as CS groups, but they did not receive saline infusion into the anterior compartment of the hind limb, and the ICP was maintained at the baseline level (0mmHg).

3.2.4 Intravital Video Microscopy (IVVM)

The extensor digitorum longus (EDL) muscle was dissected to the level of its distal tendon, which was tied with a suture and cut from its bony insertion. The animal was transferred onto the stage of an inverted microscope (Nikon); the EDL was reflected into a saline bath containing 5μg/ml each of the fluorescent vital dyes bisbenzimide (BB; exc. 343nm, em. 483nm) and ethidium bromide (EB; exc. 482nm, em. 616nm). BB stains the nuclei of all cells while EB stains the nuclei of only those cells with damaged cell membrane. Thus, EB/BB ratio provided an index of tissue injury.

Microvascular perfusion and leukocytes within the post-capillary venules were recorded by translumination with 20x and 40x objectives, respectively, in five adjacent 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 anesthetic agent.

3.2.5 Offline Video Analysis

Capillary perfusion was assessed by counting the number of continuously-perfused (CPC), intermittently-perfused (IPC) and non-perfused (NPC) capillaries that crossed three parallel lines drawn perpendicular to the capillary axis on the video monitor, and was expressed as % of total capillaries. Tissue injury was assessed by counting the number of EB- and BB-labelled nuclei, and expressed as EB/BB ratio. Leukocyte activation was assessed by counting the numbers of rolling and adherent leukocytes in post-capillary venules and expressed per unit area (i.e. 1000μm^2). Venular area was measured using ImageJ (NIH, Bethesda, MD). A leukocyte was considered adherent if it remained stationary for at least 30 seconds, and a cell was considered rolling if it remained in contact with the wall of the vessel during its movement.

3.2.6 Serum Tumor Necrosis Factor Alpha (TNF-α) Measurements

TNF-α levels were measured from arterial blood samples drawn at 9 time points: (1) baseline, (2) 15 minutes into CS, (3) 45 minutes into CS, (4) 90 minutes into CS, (5) 2 hours into CS – just prior to fasciotomy and CORM-3 (or iCORM-3) injection, (6) 10 minutes post-fasciotomy, (7) 20 minutes post fasciotomy, (8) 30 minutes post fasciotomy, (9) 45 minutes post fasciotomy, just before IVVM. TNF-α was assessed using enzyme-linked immunosorbent assay (ELISA, Pierce Biotechnology, c/o Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The TNF-α ELISA was sensitive to less than 5 pg/mL.

3.2.7 Statistical Analysis

All parameters were expressed as mean ± SEM and analyzed using one-way ANOVA. $p<0.05$ was considered statistically significant.

3.3 RESULTS

3.3.1 Systemic Leukocyte Count and Carboxyhemoglobin (COHb)

Elevation of ICP, coupled with subsequent fasciotomy and 45 minutes of reperfusion, led to a small, but significant rise in leukocyte count; CORM-3 treatment was able to decrease the severity of this response (Table 3.1). Application of CORM-3 or iCORM-3 had no effect on COHb levels (Table 3.1).

3.3.2 Microvascular Perfusion

Elevation of ICP resulted in significant changes to microvascular perfusion, as shown in Figure 3.1. The number of continuously-perfused capillaries decreased from 76±4% in sham to 23±2% in CS+iCORM-3 ($p<0.0001$), while the number of non-perfused capillaries increased from 13±2% in sham to 55±2% in CS+iCORM-3 ($p<0.0001$). CORM-3 treatment was able to restore the number of continuously-perfused capillaries to 57±5% ($p<0.001$), while iCORM-3 had no effect.

Table 3.1. The effects of CORM-3 on systemic leukocyte count and COHb levels. Two hours of elevated ICP were followed by fasciotomy, injection of CORM-3 (or iCORM-3) and 45min reperfusion. *CS-associated rise in systemic leukocyte count was reversed by CORM-3 application* (*p<0.01 from sham; †p<0.05 from CS and CS+iCORM-3). *CORM-3 and iCORM-3 caused no changes in systemic levels of COHb*. *LKC*, leukocyte counts; COHb, carboxyhemoglobin.

	LKC (Units x10^9/L)	Hemoglobin (g/L)	COHb (%)
Sham	1.5 ± 0.2	125.0 ± 2.3	1.5 ± 0.2
CS+iCORM-3	4.1 ± 0.7*	124.8 ± 2.9	1.6 ± 0.1
CS+CORM-3	2.3 ± 0.2†	129.5 ± 2.7	1.6 ± 0.1

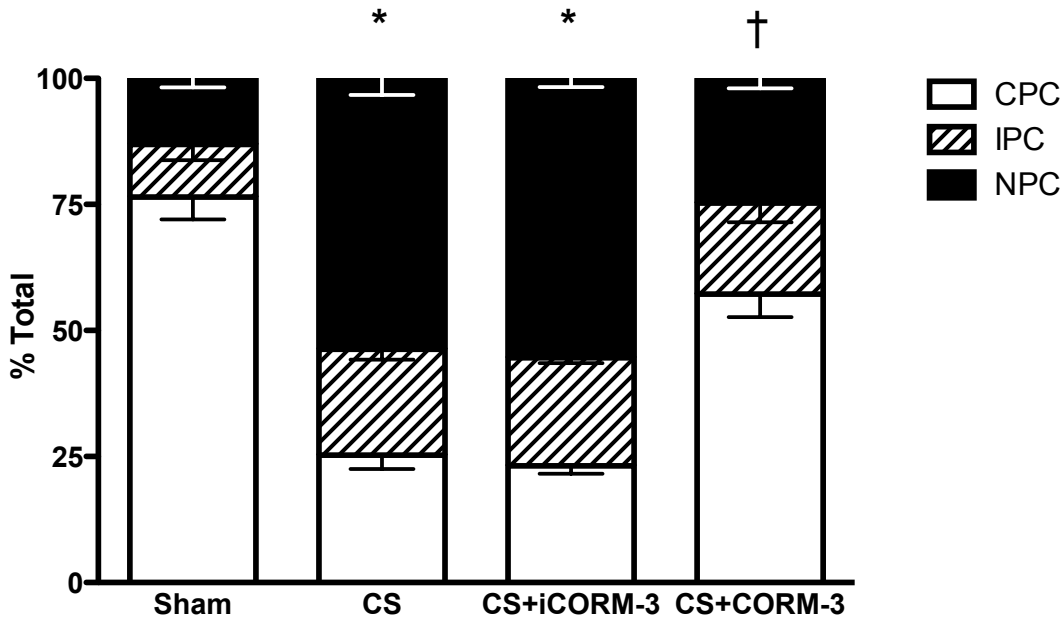

Figure 3.1. The effect of CORM-3 on skeletal muscle microvascular perfusion following CS. Two hours of elevated ICP were followed by fasciotomy, injection of CORM-3 (or its inactive form, iCORM-3), 45min reperfusion and IVVM. *CS-associated perfusion changes were reversed by CORM-3 application* (one-way ANOVA $p<0.05$; *$p<0.001$ from sham; †$p<0.001$ from CS and CS+iCORM-3; see the text for additional details). *CPC*, continuously-perfused capillaries; *IPC*, intermittently-perfused capillaries; *NPC*, non-perfused capillaries.

3.3.3 Tissue Injury

Muscle injury, as measured by EB/BB ratio, significantly increased from 0.05±0.03 in sham to 0.31±0.05 ($p<0.0001$) in CS+iCORM-3 group. CORM-3 treatment was able to diminish tissue injury to 0.07±0.01 ($p<0.001$) (Figure 3.2).

3.3.4 Serum TNF-α

Elevation of ICP led to a progressive serum TNF-α release, reaching its maximum level at 2 hours (just prior to fasciotomy; $p<0.01$) (Figure 3.3). TNF-α levels continued to rise in the post-fasciotomy/reperfusion period in animals treated with iCORM-3. In contrast, CORM-3 injection effectively prevented the latter response at 30 and 45 minutes post-fasciotomy ($p<0.001$) (Figure 3.3).

3.3.5 Inflammation

Elevation of ICP led to significant leukocyte activation, as demonstrated by the adhesive interactions with vascular endothelium. Leukocyte adherence in the post-capillary venules of the skeletal muscle was increased from 1.8±0.5 in sham to 13.7±0.9 leukocytes/30s/1000μm^2 in CS+iCORM-3 ($p<0.0001$). Leukocyte rolling, while not statistically significant, also increased from 1.7±0.6 to 3.3±0.7 leukocytes/30s/1000μm^2. CORM-3 treatment led to a significant, 8-fold decrease in leukocyte adherence, while having no effect on leukocyte rolling (0.6±0.3 adherent leukocytes/30s/1000μm^2, $p<0.001$ and 3.0±0.8 rolling leukocytes/30s/1000μm^2, not significant, respectively) (Figure 3.4).

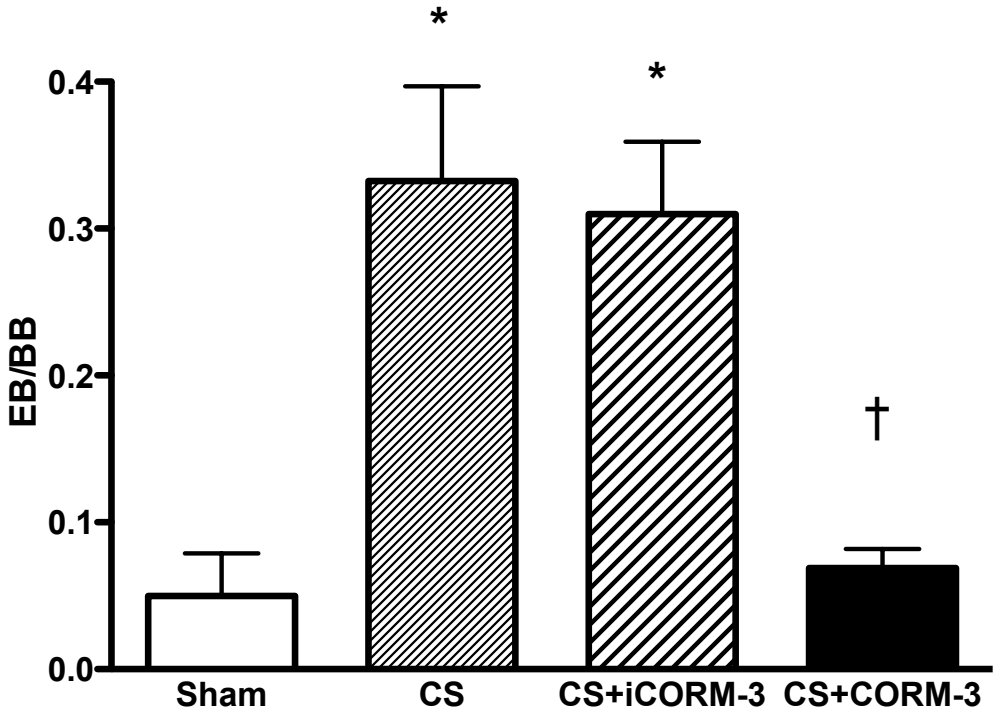

Figure 3.2. The effect of CORM-3 on skeletal muscle tissue injury following CS. Two hours of elevated ICP were followed by fasciotomy, injection of CORM-3 (or its inactive form, iCORM-3), 45min reperfusion, and IVVM. *CS-associated tissue injury was reversed by CORM-3 application* (one-way ANOVA $p<0.05$; *$p<0.001$ from sham; †$p<0.001$ from CS and CS+iCORM-3).

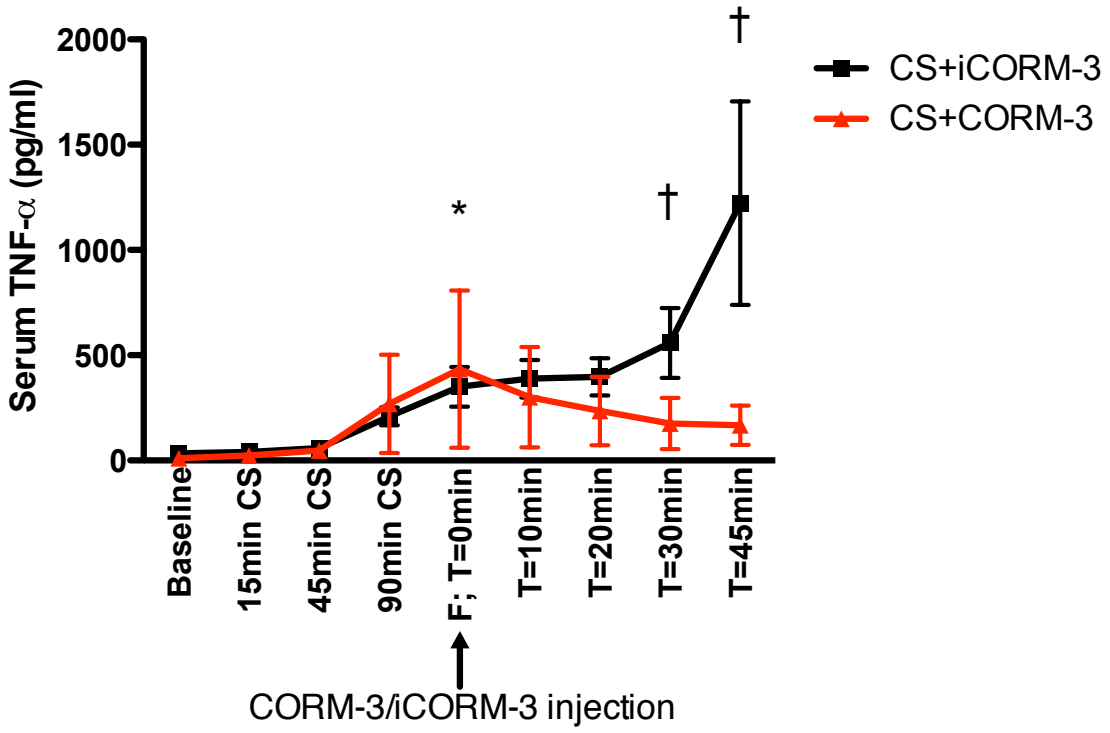

Figure 3.3. The effect of CORM-3 on serum TNF-α levels in CS. Two hours of elevated ICP were followed by fasciotomy, injection of CORM-3 (or its inactive form, iCORM-3) and 45min reperfusion. Serum TNF-α levels were assessed at each time point indicated. *Any further post-fasciotomy TNF-α elevation was reversed by CORM-3 application* (one-way ANOVA $p<0.05$; *$p<0.01$ from baseline; †$p<0.001$ from CS+iCORM-3). *F*, fasciotomy.

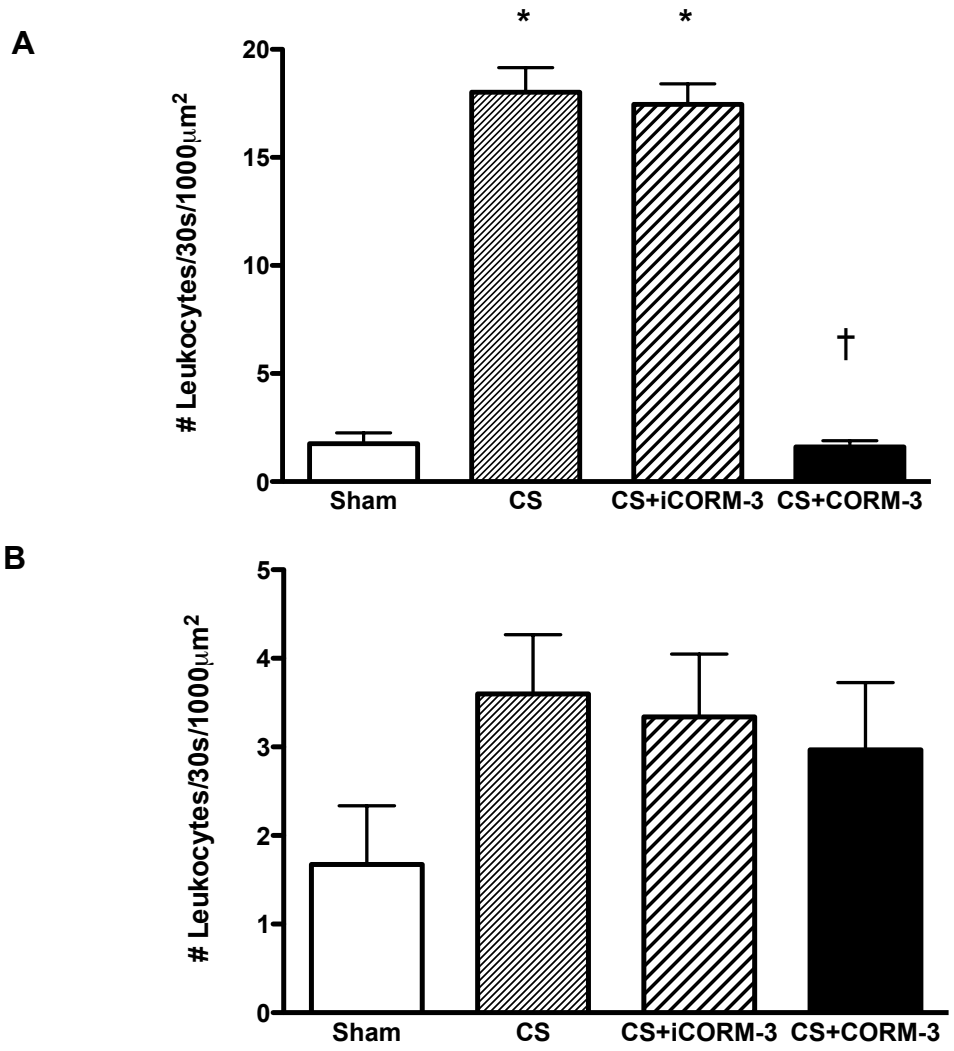

Figure 3.4. The effect of CORM-3 on modulation of leukocyte recruitment to the skeletal muscle vasculature following CS. (A) Leukocyte adhesion; (B) leukocyte rolling. Two hours of elevated ICP were followed by fasciotomy, injection of CORM-3 (or its inactive form, iCORM-3), 45min reperfusion and IVVM. *CORM-3 application was able to prevent leukocyte adhesion within the post-capillary venules.* (one-way ANOVA $p<0.05$; *$p<0.001$ from sham; †$p<0.001$ from CS and CS+iCORM-3).

3.4 DISCUSSION

Compartment syndrome poses a challenging problem. Aside from the gold standard of fasciotomy and supportive treatment to prevent other systemic sequelae during the recovery period, few other treatment modalities exist (Olson and Glasgow 2005). In the present study, we demonstrated the benefit of supraphysiologic levels of carbon monoxide in improving capillary perfusion, reducing cellular damage and leukocyte adherence following compartment syndrome and fascial release. While the benefits of this compound have been well described in the literature, finding a suitable method of administration to reduce additional risk to the host has posed a long-standing problem. CO delivery via transitional metal carbonyls is a recent discovery and has shown to provide the vasorelaxant, antiapoptotic, and anti-inflammatory effects without the risk of elevated carboxyhemoglobin seen previously through inhalation (Guo, Stein et al. 2004, Motterlini and Otterbein 2010). This method of CO delivery may provide a useful pharmacologic agent in the treatment of patients at risk for, or recovering from, compartment syndrome.

CO is a signalling molecule produced endogenously by the degradation of heme, catalyzed by heme oxygenases (HO-1 and HO-2) (Otterbein 2009). CO can exert vasodilatory effects, mitigate intracellular apoptosis, suppress inflammatory pathways and have anti-ischemic effects (Motterlini 2007). In our study, CORM-3 (a water-soluble formulation, administered IP) demonstrates a beneficial effect in preserving microvascular flow in CS-challenged muscle.

Continuous perfusion is defined as a physiologic flow through the capillary bed, whereas intermittent perfusion results from a marked decrease in red blood cell flow (Lawendy, Sanders et al. 2011). Non-perfused capillaries are seen when no red cell movement is observed. The change from continuous perfusion to a predominantly non-perfused profile demonstrates a pathologic shift in the microvascular bed in response to CS. In the current study, animals treated with CORM-3 had shown significant improvement in capillary perfusion rates, restoring the number of continuously-perfused capillaries to levels comparable to those of the sham group (Figure 3.1). Microvascular perfusion was virtually unchanged at 2 hours of elevated ICP in the presence of CORM-3, suggesting a substantial protective role of exogenously applied CO in the maintenance of skeletal muscle blood flow during CS. To the best of our knowledge, this study is the first to demonstrate such potent protective effects of CORM-3 in an acute and overwhelming inflammatory onset, such as CS. Moreover, administration of CORM-3 was very effective in restoring tissue injury levels back to baseline levels, as those seen in the sham group (Figure 3.2).

Ischemia-reperfusion injury, a major component of CS pathophysiology, produces a pro-inflammatory environment, resulting in the upregulation of cytokines and chemokines, both systemically and within the affected tissues (Forbes, Carson et al. 1995). Inflammation-relevant cytokines/chemokines, particularly those produced during the acute phase of inflammatory response (TNF-α, IL-1β, IL-6, IL-8), in turn, stimulate leukocyte activation (primarily polymorphonuclear leukocytes, PMN) and recruitment into the inflamed tissues.

Once activated, leukocytes produce reactive oxygen species (ROS) and release proteolytic enzymes that (individually or concurrently) cause cellular damage and contribute to the increased vascular permeability, as well as subsequent formation of edema. As a result, increased interstitial pressure compresses adjacent capillaries, creating non-perfused segments (Kurose, Anderson et al. 1994, Forbes, Harris et al. 1996, Gute, Ishida et al. 1998). In our study, we observed a marked increase in the levels of circulating TNF-α (one of the most potent pro-inflammatory cytokines) in CS-challenged animals, particularly post-fasciotomy. This was associated with overwhelming leukocyte recruitment to the CS-challenged muscle, as demonstrated by adherent leukocytes in the post-capillary venules (Figure 3.4). Interestingly, the increase in number of adherent leukocytes was completely prevented in animals treated with CORM-3, but not its inactive counterpart, iCORM-3. It is important to note that the decrease in leukocyte recruitment to CS-challenged muscle correlated with the CORM-3-dependent suppression of serum TNF-α levels (Figure 3.3).

The exact mechanisms of CORM-3-derived protection/anti-inflammatory effects in our CS model are, at the moment, unknown. However, given the complex and multi-component nature of CS pathophysiology (ischemic episode, oxidative stress, cytokine production, local and systemic responses), it is plausible to assume that the beneficial effects of CORM-3-derived CO are most likely due to its simultaneous action on multiple targets in various cell types. In this regard, our current findings are all in agreement with previous studies showing that CORM-3 is able to reduce pro-inflammatory cytokine production,

and, therefore, cytokine-dependent upregulation of leukocyte-endothelium adhesive interaction (Song, Bergstrasser et al. 2009, Vadori, Seveso et al. 2009, Katada, Bihari et al. 2010, Mizuguchi, Capretta et al. 2010, Bergstraesser, Hoeger et al. 2012). The identity of adhesion molecules expressed by the leukocytes and/or endothelial cells due to CS remain to be elucidated; however, our data (i.e. CORM-3 ability to reduce the leukocyte adhesion to post-capillary venule endothelium in CS-challenged animals) suggests that CORM-3 may directly or indirectly interfere with the expression of Ig-superfamily adhesion molecules (such as ICAM-1, VCAM-1, etc.) on endothelial cells. The lack of significant changes in leukocyte rolling in CORM-3-treated animals suggests that CORM-3 does not modulate expression/function of selectins (E-selectin, P-selectin or L-selectin), molecules responsible primarily for the leukocyte rolling but not adhesion to the vascular endothelium *per se* (Ley, Laudanna et al. 2007).

Previous studies have demonstrated that CORM-3 suppresses oxidative stress in vascular endothelial cells (Mizuguchi, Capretta et al. 2010), cardiac myocytes (Lancel, Hassoun et al. 2009) and inflammatory cells (PMN and macrophages) (Mizuguchi, Stephen et al. 2009); this effect may be responsible for our observation of CORM-3-dependent suppression of acute skeletal muscle cell injury during CS (Figure 3.2). In addition, CORM-3-dependents protection of vascular perfusion could also be attributed to the potent vasodilatory properties of CO (Foresti, Hammad et al. 2004), which could improve capillary flow of the affected tissue in the post-fasciotomy state. Previous studies have demonstrated

CO-dependent modulation of the blood vessel tone through the mechanisms involving soluble guanylate cyclase signalling (Motterlini and Otterbein 2010).

While the results of our experiments look promising, the study is not without limitations. Although a rodent model of compartment syndrome has many benefits (it is reliable, small and cost effective), with relatively simple regulation of both compartment pressure and blood pressure, its limitations are equally as important. First we employed an absolute compartment threshold of 30 mmHg, which may not be as severe an insult in a human model; thus the severity of our rat CS model may be relatively mild compared to a full-scale CS in humans. Given the differences in size and metabolic parameters, rats appear to be more sensitive to ischemic injury (unpublished observation); however, it remains to be seen how the severity of CS in rats compares to that in humans. In addition, the timing of CORM-3 administration may have to be optimized. In our study, CORM-3 was administered once, at fasciotomy; we did not study the effects of CORM-3 beyond 1 hour post-administration. It is highly unlikely that one injection of CORM-3 would prevent CS, but it might be able to prolong the surgical window, in case of delayed fasciotomy. Future studies are needed to examine the effect of CORM-3 administration on CS injury, but without fasciotomy. Unfortunately, direct method of visualization of microvasculature in rats requires fasciotomy; thus, any future studies will have to employ indirect means of assessing the tissue damage (e.g. functional gait assessment).

The potential toxicity of CORM-3 is yet to be established. While very little data exists on the safety of the systemic CORM-3 administration in large animals,

preclinical studies employing non-human primates suggest that there are few side effects associated with both single and repeated injections (Vadori, Seveso et al. 2009). In the future, CORM-3 may need to be tested in an animal more akin to humans. Larger animals appear to be more sensitive to the effects of CORM-3 (Vadori, Seveso et al. 2009); thus a lower therapeutic dose may be sufficient, minimizing potential side effects.

To our knowledge, this is the first study demonstrating the beneficial effects of carbon monoxide, delivered by a carbon-monoxide releasing molecule, in the treatment of acute compartment syndrome. While the exact mechanisms of CORM-3 protective action remain to be determined, the obtained data strongly indicate a potential therapeutic application of CORM-3 to patients at risk of developing CS.

3.5 REFERENCES

Bergstraesser C, Hoeger S, Song H, Ermantraut L, Hottenrot M, Czymal T, Schmidt M, Goebeler M, Ponelies N, Stich C, Loesel R, Molema G, Seelen M, van Son W, Yard BA, Rafat N (2012). Inhibition of VCAM-1 expression in endothelial cells by CORM-3: the role of the ubiquitin-proteasome system, p38, and mitochondrial respiration. Free Radic Biol Med **52**(4): 794-802.

Cepinskas G, Katada K, Bihari A, Potter RF (2008). Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. Am J Physiol Gastrointest Liver Physiol **294**(1): G184-191.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson BR, Mann TE, Foresti R, Motterlini R (2003). Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ Res **93**(2): e2-8.

Forbes TL, Carson M, Harris KA, DeRose G, Jamieson WG, Potter RF (1995). Skeletal muscle injury induced by ischemia-reperfusion. Can J Surg **38**(1): 56-63.

Forbes TL, Harris KA, Jamieson WG, DeRose G, Carson M, Potter RF (1996). Leukocyte activity and tissue injury following ischemia-reperfusion in skeletal muscle. Microvasc Res **51**(3): 275-287.

Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ, Motterlini R (2004). Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. Br J Pharmacol **142**(3): 453-460.

Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R, Bolli R (2004). Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. Am J Physiol Heart Circ Physiol **286**(5): H1649-1653.

Gute DC, Ishida T, Yarimizu K, Korthuis RJ (1998). Inflammatory responses to ischemia and reperfusion in skeletal muscle. Mol Cell Biochem **179**(1-2): 169-187.

Hartsock LA, O'Farrell D, Seaber AV, Urbaniak JR (1998). Effect of increased compartment pressure on the microcirculation of skeletal muscle. Microsurgery **18**(2): 67-71.

Hegazi RA, Rao KN, Mayle A Sepulveda AR, Otterbein LE, Plevy SE (2005). Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. J Exp Med **202**(12): 1703-1713.

Kalns J, Cox J, Baskin J, Santos A, Odland R, Fecura S, Jr. (2011) Threshold model for extremity compartment syndrome in swine. J Surg Res **167**(1): e13-19.

Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF, Cepinskas G (2010). Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. Inflammation **33**(2): 92-100.

Kurose I, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR, Granger DN (1994). Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. Circ Res **74**(2): 336-343.

Lancel S, Hassoun SM, Favory R, Decoster B, Motterlini R, Nevier R (2009).Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis. J Pharmacol Exp Ther **329**(2): 641-648.

Lawendy AR, Bihari A, Sanders DW, Parry N, Gray D, Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Ley K, Laudanna C, Cybulsky MI, Nourshargh S (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol **7**(9): 678-689.

Mazzola S, Forni M, Albertini M, Bacci ML, Zannoni A, Gentilini F, Lavitrano M, Bach FH, Otterbein LE, Clement MG (2005). Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. FASEB J **19**(14): 2045-2047.

Matsen, FA, 3rd (1975). Compartmental syndrome. An unified concept. Clin Orthop Rel Res (113): 8-14.

Matsen FA, 3rd (1980). Compartmental syndromes. Hosp Pract **15**(2): 113-117.

Matsen FA, 3rd, Winquist RA, Krugmire RB Jr (1980). Diagnosis and management of compartmental syndromes. J Bone Joint Surg Am **62**(2): 286-291.

Mizuguchi S, Stephen J, Bihari A, Markovic N, Suehiro S, Capretta A, Potter RF, Cepinskas G (2009). CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. Am J Physiol Heart Circ Physiol **297**(3): H920-929.

Mizuguchi S, Capretta A, Suehiro S, Nishiyama N, Luke P, Potter RF, Fraser DD, Cepinskas G (2010). Carbon monoxide-releasing molecule CORM-3 suppresses vascular endothelial cell SOD-1/SOD-2 activity while up-regulating the cell surface levels of SOD-3 in a heparin-dependent manner. Free Radic Biol Med **49**(10): 1534-1541.

Motterlini R (2007). Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem Soc Trans **35**(Pt 5): 1142-1146.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ (2002). Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res **90**(2): E17-24.

Motterlini R, Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nat Rev Drug Discov **9**(9): 728-743.

Mubarak SJ, Owen CA, Hargens AR, Garetto LP, Akeson WH (1978). Acute compartment syndromes: diagnosis and treatment with the aid of the wick catheter. J Bone Joint Surg Am **60**(8): 1091-1095.

Nakao A, Kimizuka K, Stolz DB, Neto JS, Kaizu T, Choi AM, Uchiyama T, Zuckerbraun BS, Nalesnik MA, Otterbein LE, Murase N (2003). Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. Am J Pathol **163**(4): 1587-1598.

Olson SA, Glasgow RR (2005). Acute compartment syndrome in lower extremity musculoskeletal trauma. J Am Acad Orthop Surg **13**(7): 436-444.

Ott MC, Scott JR, Bihari, A, Badhwar A, Otterbein LE, Gray D, Harris KA, Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hind limb ischemia/reperfusion. FASEB J **19**(1): 106-108.

Otterbein LE (2009). The evolution of carbon monoxide into medicine. Respir Care **54**(7): 925-932.

Rorabeck CH, Clarke KM (1978). The pathophysiology of the anterior tibial compartment syndrome: an experimental investigation. J Trauma **18**(5): 299-304.

Sadasivan KK, Carden DL, Moore MB, Korthuis RJ (1997). Neutrophil mediated microvascular injury in acute, experimental compartment syndrome. Clin Orthop Relat Res (339): 206-215.

Sheridan GW, Matsen FA (1975). An animal model of the compartmental syndrome. Clin Orthop Relat Res (113): 36-42.

Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray D, Harris KA, Parry N, Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol **296**(1): G9-G14.

Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R, Yard BA (2009). The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression. Br J Pharmacol **157**(5): 769-780.

Vadori M, Seveso M, Besenzon F, Bosio E, TOgnato E, Fante F, Boldrin M, Gavasso S, Ravarotto L, Mann BE, Simioni P, Ancona E, Motterlini R, Cozzi E (2009). In vitro and in vivo effects of the carbon monoxide-releasing molecule, CORM-3, in the xenogeneic pig-to-primate context. Xenotransplantation **16**(2): 99-114.

Whitesides TE, Haney TC, Morimoto K, Harada H (1975). Tissue pressure measurements as a determinant for the need of fasciotomy. Clin Orthop Relat Res (113): 43-51.

CHAPTER 4

SYSTEMIC ADMINISTRATION OF CARBON MONOXIDE RELEASING MOLECULE-3 (CORM-3) PROTECTS THE SKELETAL MUSCLE IN PORCINE MODEL OF COMPARTMENT SYNDROME.

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CHAPTER 4: SYSTEMIC ADMINISTRATION OF CARBON MONOXIDE RELEASING MOLECULE-3 (CORM-3) PROTECTS THE SKELETAL MUSCLE IN PORCINE MODEL OF COMPARTMENT SYNDROME.

4.1 INTRODUCTION

Compartment syndrome (CS) is a serious and devastating complication of musculoskeletal trauma. CS develops as a result of an increased pressure within a closed osseofascial compartment, resulting in muscle-threatening and limb-threatening ischemia (Matsen 1975, Matsen 1980, Matsen, Winquist et al. 1980, Hartsock, O'Farrell et al. 1998). Fasciotomy, to fully decompress all involved compartments, remains the only gold standard surgical treatment, but the surgery must be performed within a narrow window of 6-8 hours, before the damage to the involved tissues becomes permanent (Mubarak and Owen 1977, Mubarak, Owen et al. 1978, Hargens, Romine et al. 1979).

An elevation in intra-compartmental pressure (ICP) during CS compromises microcirculation (Hartsock, O'Farrell et al. 1998), restricting oxygen and nutrient delivery to the point where metabolic demands of the tissue cannot be met. This results in cellular anoxia and severe tissue necrosis; however, unlike complete ischemia, it occurs in the face of patent vessels (Whitesides, Haney et al. 1975). Reperfusion of previously ischemic tissue is known to trigger severe inflammation, both local and systemic (Blaisdell 2002, Gillani, Cao et al.

2011, Lawendy, Bihari et al. 2016, Bihari, Cepinskas et al. 2017). Since CS appears to be a form of reperfusion injury, the contribution of inflammation to the pathophysiology of CS is increasingly being recognized; studies from our group and others have demonstrated that leukocytes appear to play a primary role in producing both microvascular and parenchymal damage during CS (Sadasivan, Carden et al. 1997, Gute, Ishida et al. 1998, Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). The effect appears to be mediated by the release of pro-inflammatory cytokines, particularly TNF-α (Lawendy, Bihari et al. 2014, Donohoe 2015).

Apart from fasciotomy, few therapeutic options have been shown to be effective, primarily due to the lack of understanding the CS pathophysiology. Recently, exogenous application of carbon monoxide (CO), a normal byproduct of heme metabolism, has been shown to be protective to microvascular perfusion and provide anti-inflammatory benefits during various ischemic and inflammatory conditions (Otterbein, Bach et al. 2000, Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009). With the advent of development of water-soluble carbon monoxide releasing molecules (CO-RMs), particularly CORM-3, CO can be safely delivered to the tissue without significantly affecting the levels of carboxyhemoglobin (COHb) (Motterlini and Otterbein 2010, Lawendy, Bihari et al. 2014).

Previously, we have demonstrated that CORM-3 application at fasciotomy was able to diminish the CS-associated tissue injury and leukocyte activation, as well as block the systemic release of pro-inflammatory cytokine TNF-α in a rat model of CS (Lawendy, Bihari et al. 2014). Additionally, CORM-3 was capable of

blocking the formation of reactive oxygen species (ROS) within the human endothelial cells subjected to CS stimulus in the form of serum obtained from CS patients (Bihari, Cepinskas et al. 2014). While the results of these studies are promising, preclinical testing in a large, more human-like animal model of CS has not been undertaken yet; in addition, the effects and mechanisms of CORM-3 on modulation of CS-induced skeletal muscle injury and dysfunction remain to be elucidated.

The purpose of this study was to test the effect of CORM-3 on the severity of microvascular dysfunction, tissue injury and the activation of circulating leukocytes in a pre-clinical setting, using porcine model of CS. The ultimate goal is the development of a safe pharmacologic adjunctive treatment for compartment syndrome, which would reduce the morbidity and disability in patients.

4.2 MATERIALS AND METHODS

4.2.1 Animal Preparation

The experimental protocol was approved by the Animal Use Subcommittee of the Council on Animal Care at the University of Western Ontario. Male Yorkshire-Landrace pigs, body weight 49-60kg, were used for all experiments. Anesthesia was induced by TKX (0.5mg/kg telazol, 0.5mg/kg ketamine, 0.5mg/kg xylazine) injection. Animals were then intubated (ETT 6.5-8.0) and switched to mechanical ventilation (14-20 breaths/min) under

inhalational isoflurane (1.5-3% maintenance) in 100% oxygen for the remainder of the experiment. End-tidal CO_2, heart rate, oxygen saturation and arterial blood gases were continuously monitored.

Left femoral artery was cannulated percutaneously, using 6F introducer (Medtronic), for the invasive blood pressure monitoring and arterial blood sampling. A 24-gauge IV catheter (BD Insyte) was placed in the right auricular vein for continuous fluid replacement and intravenous drug administration. Body temperature was continuously monitored and kept at 38.0-38.5°C by BairHugger thermal blanket.

Compartment pressure monitoring probe (Synthes, Westchester PA) was inserted into the anterior compartment via gauge 16 angiocatheter (BD) of the pig right hind limb; a gauge 16 needle (BD) attached to an IV line was also inserted into the subfascial plane within the same compartment. The limb was then placed in a plaster cast. CS was induced by an infusion of isotonic saline enriched with 0.4g/L bovine serum albumin, leading to an elevation of intra-compartmental pressure (ICP) to 40-65 mmHg. Elevated ICP was maintained for 6 hours. A single-incision fasciotomy was performed to decompress the hind limb compartments; the muscles were allowed to reperfuse for 3 hours, followed by imaging of the microvascular perfusion of tibialis anterior (TA) and peroneus tertius (PT) muscles with a hand-held Cytoscan microscope equipped with orthogonal polarization spectroscopy (OPS) (Cytometrics).

Thirty minutes prior to OPS imaging, animals were injected intravenously with 50μg/kg each of the fluorescent vital dyes ethidium bromide (EB; exc.

482nm, em. 616nm), bisbenzimide (BB; exc. 343nm, em. 483nm) and carboxyfluorescein-labelled *in vivo* marker of apoptosis (FLIVO, ImmunoChemistry Technologies; exc. 492nm, em. 517nm). Following the OPS microscopy, TA and PT muscles were excised and fixed in 10% formalin for further analysis. Pigs were then euthanized by an overdose of anesthetic agent.

4.2.2 CORM-3

A water-soluble CORM-3 (tricarbonylchloro-glycinate-ruthenium(II), [$Ru(CO)_3Cl$-glycinate]; molecular weight 295$gmol^{-1}$), an equimolar CO donor, was synthesized by us (Mizuguchi, Stephen et al. 2009), in accordance with a previously-published method (Clark, Naughton et al. 2003). CORM-3 (10mg/ml stock solution) was prepared fresh by dissolving CORM-3 in isotonic saline just prior to injection. CO release from CORM-3 was confirmed spectrophotometrically, by conversion of deoxy-myoglobin to carbonmonoxymyoglobin. As a control, inactive CORM-3 (iCORM-3) was generated by dissolving CORM-3 in saline 72 hours prior to the experiment and allowing it to release all CO from the solution (lack of CO release was confirmed by myoglobin-binding assay) (Clark, Naughton et al. 2003, Lawendy, Bihari et al. 2014).

4.2.3 Experimental Design

Following induction of anesthesia, intubation and placement of all catheters, animals were randomly assigned into one of the three experimental

groups: Sham (n=4), CS+CORM-3 (n=4) and CS+iCORM-3 (n=4). CO-releasing molecule-3 (CORM-3), or its inactive form (iCORM-3), was administered to animals undergoing CS upon fasciotomy at the dose of 2mg/kg, IV.

Sham animals underwent all procedures, but the compartment pressure was maintained at the baseline level (0mmHg). In addition, the contralateral limb of each animal used in these experiments served as a control; it underwent all the procedures as the experimental limb, but did not receive saline infusion into the anterior compartment, and the ICP was maintained at the baseline level (0mmHg).

4.2.4 OPS Imaging

The hand-held objective of Cytoscan OPS microscope (Cytometrics, Devon, UK) was applied to the surface of the exposed TA and/or PT muscle, recording microvascular perfusion using epi-illumination with green polarized light at 10x objective, in five to ten adjacent fields of view. The microcirculation was recorded with a digital recorder (Sony) and stored on DV tapes for offline analysis.

4.2.4.1 Offline Video Analysis

Capillary perfusion was assessed by counting the number of continuously-perfused (CPC), intermittently-perfused (IPC) and non-perfused (NPC) capillaries that crossed three parallel lines drawn perpendicular to the capillary axis on the video monitor, and was expressed as % of total capillaries.

4.2.5 Tissue Leukocyte Infiltration and Necrosis

Following formalin fixation, TA and PT samples were processed and embedded in paraffin; 5μm sections were cut with a microtome. Sections were put on poly-L slides, cleared in xylene, rehydrated with graded alcohols and then subjected to a standard H&E staining protocol.

Levels of tissue leukocyte infiltration and tissue necrosis were assessed semi-quantitatively, by a blinded pathologist, Dr. Lee Ang of London Health Sciences Centre, in 5 adjacent fields of view in each muscle sample using conventional light microscopy. Histopathology grading scale ranged from 0 (no leukocyte infiltration, no necrosis, i.e. normal tissue) to 3 (severe leukocyte infiltration and/or severe tissue necrosis) (Table 4.1).

4.2.6 Tissue Injury and Apoptosis

Paraffin-embedded 5μm sections were cut with a microtome. Sections were put on poly-L slides, cleared in xylene, rehydrated with graded alcohols and mounted with Vectashield fluorescence mounting medium.

Levels of tissue injury (EB/BB) and apoptosis (FLIVO/BB) were quantified using conventional fluorescence microscopy. EB stains the nuclei of only those cells with damaged cell membrane, while BB stains the nuclei of all cells. FLIVO, a non-toxic marker, stains only the cells with apoptosis-associated caspases activation. Thus, EB/BB and FLIVO/BB ratios provided an index of tissue injury and apoptosis, respectively.

Table 4.1. Histopathology grading scale for skeletal muscle tissue leukocyte infiltration and necrosis in porcine model of CS. Four grades were assigned by a blinded pathologist (Dr Lee Ang at London Health Sciences Centre) to both leukocyte infiltration and tissue necrosis, with 0 representing normal tissue and 3 corresponding to severe fiber necrosis and/or leukocyte infiltration.

Grade	Muscle Leukocyte Infiltration	Muscle Necrosis
0	Normal, no infiltration	Normal, no necrosis
1	Mild	Mild
2	Moderate	Moderate
3	Severe	Severe

4.2.7 Systemic Leukocyte Isolation and Activation Assay

Systemic leukocyte activation was measured in arterial blood samples drawn at 16 time points: (1) baseline, (2) 1 hour into CS, (3) 2 hours into CS, (4) 3 hours into CS, (5) 4 hours into CS, (6) 5 hours into CS, (7) 6 hours into CS – just prior to fasciotomy and CORM-3 (or iCORM-3) injection, (8) 20 minutes post-fasciotomy, (9) 40 minutes post-fasciotomy, (10) 60 minutes post fasciotomy, (11) 75 minutes post-fasciotomy, (12) 90 minutes post-fasciotomy, (13) 105 minutes post-fasciotomy, (14) 2 hours post-fasciotomy, (15) 2.5 hours post-fasciotomy, (16) 3 hours post-fasciotomy – just before OPS imaging. Polymorphonuclear (PMN) leukocytes were isolated by density gradient centrifugation (Histopaque-1077, Sigma), as previously described by us (Mizuguchi, Stephen et al. 2009), followed by the lysing of red blood cells (ammonium chloride solution). Isolated leukocytes were reconstituted in 0.1M phosphate buffer saline, pH 7.4, adjusting the buffer volume to achieve the final cell count of $1x10^9$ leukocytes/ml. Leukocyte activation (i.e. superoxide (O_2^-) production) was assessed by L-012 assay, and expressed as relative luminescence units (RLU)/10^6 PMN.

4.2.8 Serum Tumor Necrosis Factor Alpha (TNF-α) Measurements

TNF-α levels in arterial blood samples were assessed using enzyme-linked immunosorbent assay (ELISA, Pierce Biotechnology, c/o Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The TNF-α detection limit was 5pg/mL.

4.2.9 Statistical Analysis

All parameters were expressed as mean ± SEM and analyzed using one-way ANOVA, repeated measures two-way ANOVA (systemic leukocyte activation, serum TNF-α), or Kruskal-Wallis test (histopathology scores), with Bonferroni or Dunn's post-hoc test as appropriate (GraphPad Prism 6.0 for Mac). Sample size calculation was performed using StatMate (GraphPad Software Inc., San Diego, CA), with power set at 85%. $p<0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Systemic Leukocyte Count and COHb

Elevation of ICP, coupled with subsequent fasciotomy and 3 hours of reperfusion led to a significant rise in leukocyte count; CORM-3 treatment was able to decrease the severity of the response (Table 4.2). Importantly, application of CORM-3 or iCORM-3 had no effect on COHb levels (Table 4.2).

4.3.2 Organ Function

4.3.2.1 Liver Enzymes

Elevation of ICP led to a significant increase in aspartate transaminase (AST), from 25.0±2.0U/L to 88.5±43.5U/L ($p<0.05$), and alkaline phosphatase (ALP) (208.5±27.5U/L versus 107.5±16.5U/L in sham), while having no effect on alanine transaminase (ALT) or bilirubin (Table 4.2). Systemic administration of CORM-3 resulted in a significant decrease in AST to 41.5±1.2U/L ($p<0.05$) and

Table 4.2. The effect of CORM-3 on hematological and biochemical parameters in porcine model of CS. CORM-3 at fasciotomy led to a significant decrease in systemic leukocytes and creatinine levels (*p<0.05 from sham and/or baseline, †p<0.05 from CS+iCORM-3)

	Sham	CS+iCORM-3	CS+CORM-3
LKC (Units x10^9/L)	12.9 ± 1.9	20.2 ± 5.4*	8.1 ± 0.5†
Hemoglobin (g/L)	98.5 ± 7.5	97.3 ± 2.8	100.3 ± 2.3
COHb (%)	2.7 ± 0.1	2.0 ± 0.7	2.1 ± 0.5
Urea (mM)	6.0 ± 0.4	17.4 ± 4.4*	8.6 ± 0.6†
Creatinine (mM)	109.0 ± 1.0	191.5 ± 5.5*	128.5 ± 2.0†
Bilirubin (mM)			
• Total	2.5 ± 0.1	3.7 ± 0.5	4.6 ± 0.9
• Direct	0.9 ± 0.1	2.1 ± 0.5	1.7 ± 0.3
ALT (U/L)	36.5 ± 0.5	32.5 ± 3.5	30.0 ± 2.9
AST (U/L)	25.0 ± 2.0	88.5 ± 43.5*	41.5 ± 1.2†
ALP (U/L)	107.5 ± 16.5	208.5 ± 27.5*	171.0 ± 13.5†
Lactate (mM)			
• Baseline	0.44 ± 0.04	0.43 ± 0.18	0.40 ± 0.08
• 6hr CS	0.50 ± 0.16	1.60 ± 0.08*	0.97 ± 0.23*†
• Final	0.54 ± 0.11	3.07 ± 1.26*	1.93 ± 0.36*†

ALP to 171.0±13.5U/L. No changes were observed in ALT or bilirubin due to CORM-3 administration.

4.3.2.2 Kidney

Elevation of ICP led to a significant increase in blood urea and creatinine levels, from 6.0±0.4mM and 109.0±1.0mM, respectively, in sham to 17.4±4.4mM and 191.5±5.5mM, respectively in CS ($p<0.05$). Systemic administration of CORM-3 at fasciotomy resulted in a significant decrease of both blood urea and creatinine, to 8.6±0.6mM and 128.5±2.0mM, respectively (Table 4.2).

4.3.2.3 Skeletal Muscle - Lactate

Elevation of ICP resulted in a significant increase in systemic lactate levels, from 0.43±0.18μmol/L at baseline, to 1.60±0.08μmol/L at 6hr CS ($p<0.05$). Following reperfusion, lactate levels rose to 3.07±1.26μmol/L, while in sham animals they remained unchanged from the baseline for the duration of the experiment.

Administration of CORM-3 at fasciotomy resulted in significant decrease of lactate levels measured at the conclusion of the experiment, to 1.93±0.36μmol/L ($p<0.05$) (Table 4.2).

4.3.3 Systemic Leukocyte Activation and TNF-α Levels

Elevation of ICP led to a progressive increase in systemic leukocyte activation, from the baseline of 1.0±0.0 RLU/10^6 PMNs to 1.4±2.0 RLU/10^6 PMNs,

4.1±2.1 RLU/10^6 PMNs, 6.2±2.9 RLU/10^6 PMNs, 13.5±6.3 RLU/10^6 PMNs, 16.5±7.9 RLU/10^6 PMNs and 14.7±5.7 RLU/10^6 PMNs at 1hr, 2hr, 3hr, 4hr, 5hr and 6hr, respectively (Figure 4.1A). Fasciotomy resulted in a transient decrease in leukocyte activation to 8.8±2.4 RLU/10^6 PMNs, followed by gradual progressive increase to 7.5±4.7 RLU/10^6 PMNs, 10.2±6.0 RLU/10^6 PMNs, 11.2±5.8 RLU/10^6 PMNs, 15.2±3.3 RLU/10^6 PMNs, 16.5±1.0 RLU/10^6 PMNs, 21.1±1.1 RLU/10^6 PMNs, 22.9±4.4 RLU/10^6 PMNs and 38.1±11.3 RLU/10^6 PMNs at 20min, 40min, 60min, 75min, 90min, 105min, 120min, 150min and 180min, respectively ($p<0.05$). Application of CORM-3 at fasciotomy decreased leukocyte activation to 5.5±2.5 RLU/10^6 PMNs, 6.3±2.9 RLU/10^6 PMNs, 6.0±3.4 RLU/10^6 PMNs, 6.3±3.5 RLU/10^6 PMNs, 6.2±3.1 RLU/10^6 PMNs, 6.9±3.8 RLU/10^6 PMNs, 7.4±3.2 RLU/10^6 PMNs, 10.4±5.5 RLU/10^6 PMNs and 11.1±5.8 RLU/10^6 PMNs at 20min, 40min, 60min, 75min, 90min, 105min, 120min, 150min and 180min, respectively ($p<0.05$ from CS control group) (Figure 4.1B).

Elevation of ICP led to a progressive increase in systemic levels of pro-inflammatory cytokine TNF-α, from the baseline level of 8.9±5.8pg/ml to 14.6±7.1pg/ml, 20.8±8.2pg/ml, 30.9±7.8pg/ml, 48.7±5.0pg/ml, 72.5±11.9pg/ml and 99.2±22.1pg/ml at 1hr, 2hr, 3hr, 4hr, 5hr and 6hr, respectively (Figure 4.2A). Following fasciotomy, TNF-α levels continued to rise to 117.9±31.9pg/ml, 147.6±41.6pg/ml, 164.6±38.5pg/ml, 196.7±55.6pg/ml, 211.6±56.9pg/ml, 164.0±50.1pg/ml, 184.1±68.3pg/ml, 208.2±65.5pg/ml and 251.2±47.3pg/ml at 20min, 40min, 60min, 75min, 90min, 105min, 120min, 150min and 180min, respectively. Application of CORM-3 (but not iCORM-3) at fasciotomy completely

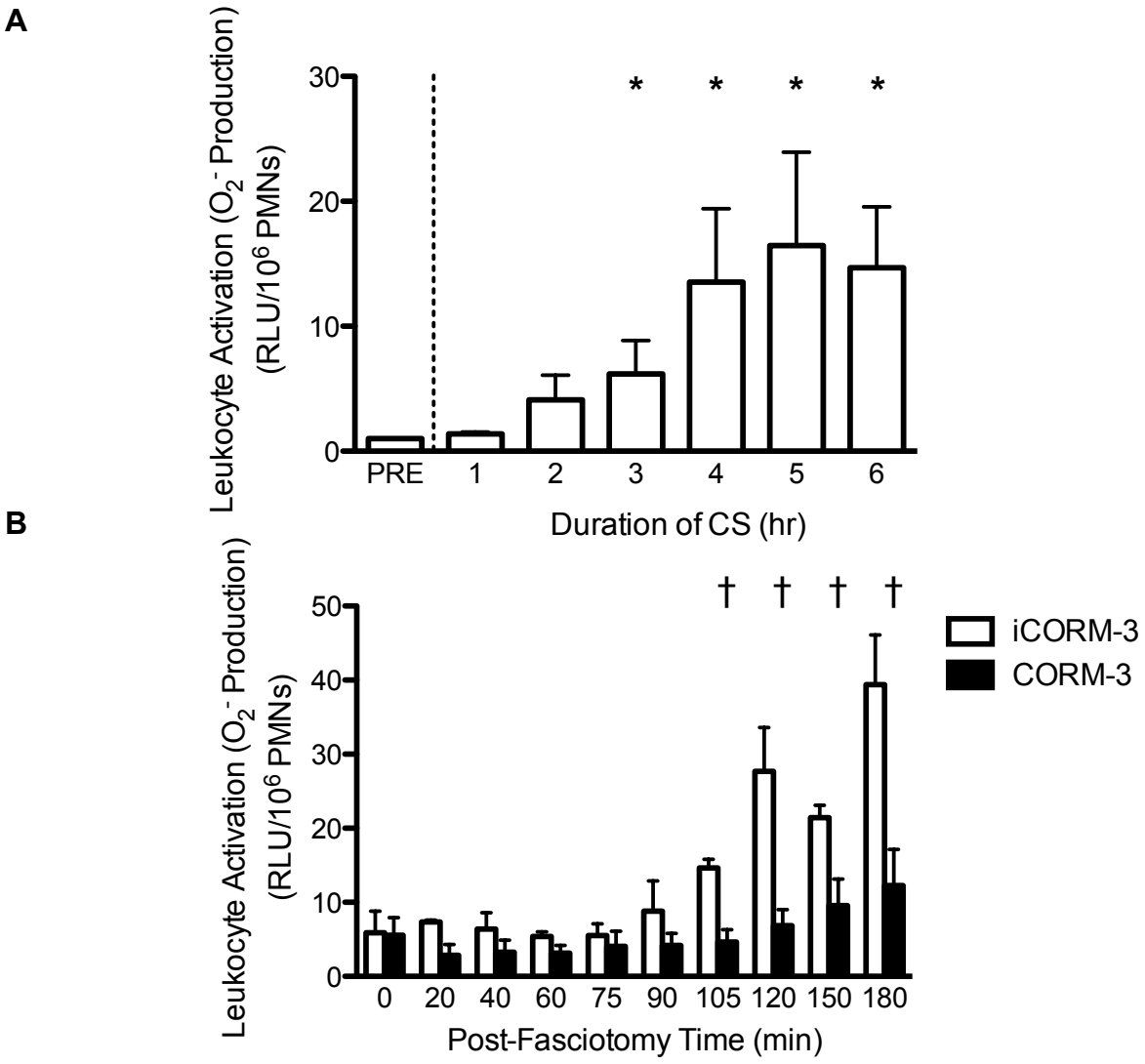

Figure 4.1. The effect of CORM-3 on systemic leukocyte activation in porcine model of CS. Elevated ICP resulted in (**A**) a progressive increase in leukocyte (PMN) activation, as assessed by O_2^- production; (**B**) fasciotomy resulted in a further circulating PMN activation, which was prevented by CORM-3 treatment; (repeated measures ANOVA; *p<0.001 from PRE, †p<0.05 from iCORM-3). *PRE*, pre-CS (i.e. baseline).

blocked the systemic TNF-α production by 20min reperfusion and for the remainder of the experiment ($p<0.05$) (Figure 4.2B).

4.3.4 Microvascular Perfusion

Six hours of elevated ICP, followed by fasciotomy and 3 hours of reperfusion resulted in 44±1% CPC versus 76±4% in sham ($p<0.001$) and 39±3% NPC in CS versus 13±2% in sham ($p<0.001$) (Figure 4.3). Application of CORM-3 (but not iCORM-3) at fasciotomy partially restored the microvascular perfusion to 68±3% CPC ($p<0.001$) and 25±3% NPC ($p<0.05$), while having no effect on the perfusion of the control limb (data not shown).

4.3.5 Tissue Injury and Apoptosis

Six hours of elevated ICP, coupled with fasciotomy and 3 hours of reperfusion resulted in EB/BB tissue injury index of 0.31±0.07 in CS versus 0.17±0.03 in sham ($p<0.05$) (Figure 4.4A), and FLIVO/BB apoptosis index of 0.26±0.06 in CS versus 0.13±0.03 in sham ($p<0.05$) (Figure 4.4B).

Systemic application of CORM-3 at fasciotomy resulted in EB/BB tissue injury index of 0.13±0.04 ($p<0.05$ from CS+iCORM-3) and FLIVO/BB apoptosis index of 0.12±0.03 ($p<0.05$ from CS+iCORM-3), while having no effect on the control limb (Figure 4.4).

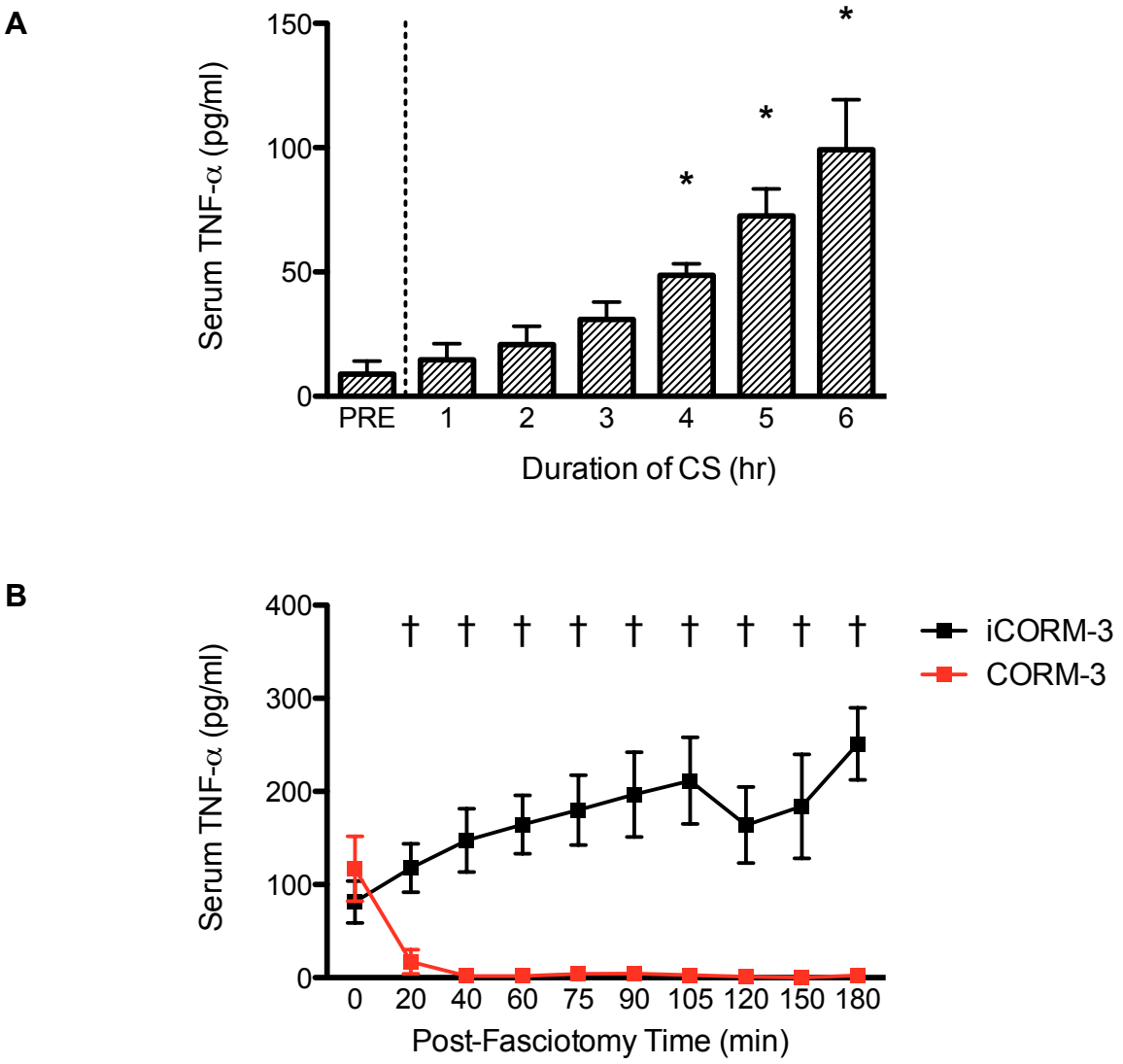

Figure 4.2. The effect of CORM-3 on serum TNF-α levels in porcine model of CS. (**A**) Elevated ICP resulted in a progressive increase in systemic TNF-α; (**B**) fasciotomy further upregulated circulating levels of TNF-α, which was effectively prevented by CORM-3 treatment (repeated measures ANOVA; *p<0.001 from PRE; †p<0.001 from CS+iCORM-3).

PRE, pre-CS (i.e. baseline).

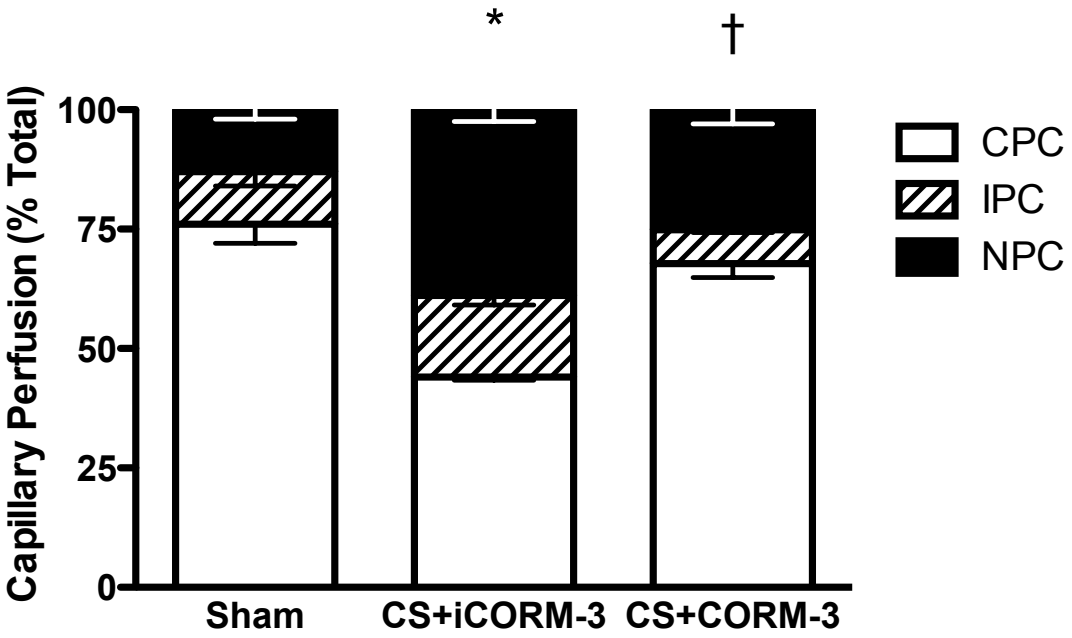

Figure 4.3. The effect of CORM-3 on skeletal muscle perfusion in porcine model of CS. Six hours of elevated ICP, coupled with fasciotomy and 3 hours of reperfusion resulted in severe microvascular perfusion deficit (indicated by a decreased number of CPC and an increased number of NPC); CORM-3 effectively reversed the microvascular perfusion deficit (one-way ANOVA; *$p<0.05$ from control, †$p<0.05$ from CS+iCORM-3; see the text for additional details).

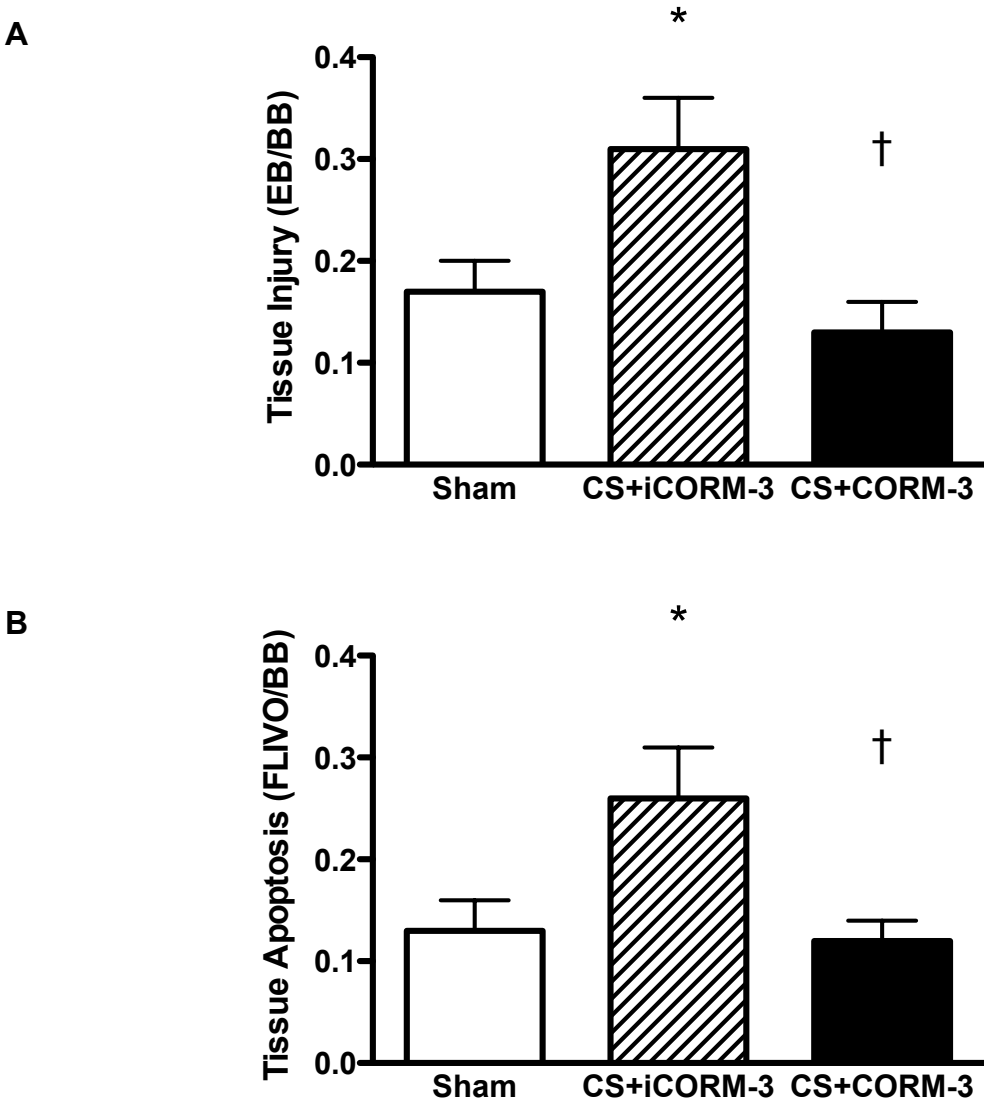

Figure 4.4. The effect of CORM-3 on skeletal muscle (A) tissue injury and (B) apoptosis in porcine model of CS. Six hours of elevated ICP, coupled with fasciotomy and 3 hours of reperfusion resulted in a significant increase in both (**A**) tissue injury and (**B**) apoptosis; the phenomenon was completely blocked by CORM-3 treatment (one-way ANOVA; *$p<0.05$ from control, †$p<0.05$ from CS+iCORM-3).

4.3.6 Tissue Leukocyte Infiltration and Necrosis

Elevation of ICP led to a histopathology leukocyte infiltration score of 2.5 (moderate to severe) versus 0 (none) in sham and control ($p<0.05$). Systemic administration of CORM-3 at fasciotomy resulted in a significant decrease of leukocyte infiltration to 1.5 (mild to moderate) within the skeletal muscle (Figure 4.5).

Elevation of ICP produced a histopathology tissue necrosis score of 2 (moderate) versus 0 (none) in contralateral limb and/or sham muscle ($p<0.05$). Systemic administration of CORM-3 at fasciotomy resulted in necrosis histological score of 1 (mild) within the skeletal muscle ($p<0.05$) (Figure 4.5).

4.4 DISCUSSION

Acute CS remains a challenging problem in the field of traumatology, not the least because the pathophysiology behind the CS injury is not well understood. Despite ongoing research, fasciotomy and supportive therapy to prevent other systemic sequelae during the recovery period remain the only gold-standard treatment choices of CS (McQueen, Gaston et al. 2000, Cascio, Pateder et al. 2005, Mithoefer, Lhowe et al. 2006, Ritenour, Dorlac et al. 2008, Farber, Tan et al. 2011, Dover, Memon et al. 2012).

In the current study, we demonstrated for the first time an improvement in skeletal muscle microvascular perfusion, inhibition of inflammation (both systemic and local) and attenuation of tissue injury, as well as apoptosis, by the systemic

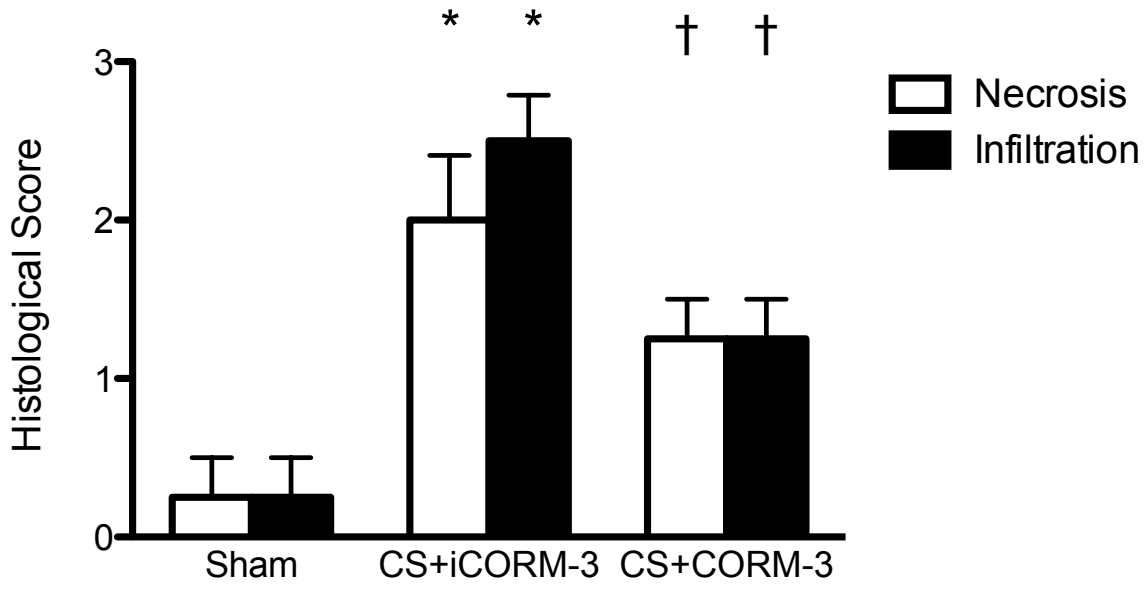

Figure 4.5. The effect of CORM-3 on skeletal muscle tissue necrosis and leukocyte infiltration in porcine model of CS. Six hours of elevated ICP, coupled with fasciotomy and 3 hours of reperfusion resulted in a significant increase in leukocyte infiltration (open bars) and tissue necrosis (solid bars) in skeletal muscle; CORM-3 treatment significantly reduced both leukocyte infiltration and tissue necrosis (Kruskall-Wallis ANOVA; *$p<0.01$ from sham, †$p<0.05$ from CS+iCORM-3).

administration of carbon monoxide (derived from a water-soluble CO donor, CORM-3) in a pre-clinical setting in a large animal model of CS.

CO, a gaseous signalling molecule produced endogenously, as a byproduct of heme degradation pathway (Ryter, Otterbein et al. 2002), has been shown to exhibit vasodilatory, anti-apoptotic and anti-inflammatory effects in various animal models (Motterlini and Otterbein 2010, Lawendy, Bihari et al. 2014). Nevertheless, therapeutic administration of CO via inhalation is hampered by the production of COHb, limiting its clinical applicability. It has been shown previously that, despite some beneficial effects, CO inhalation in humans (500ppm for 1 hour) increased COHb in the range of 1.2-7% (Mayr, Spiel et al. 2005). Similar findings were obtained from *in vivo* animal models, where COHb of 5-6% were obtained (Ott, Scott et al. 2005), some even reaching 15-25% (Fujita, Toda et al. 2001), depending on the experimental conditions.

Recently, transitional metal carbonyls, CO-RMs, have been developed as means of CO delivery in biological systems, with minimal or no effect on COHb formation (Clark, Naughton et al. 2003, Guo, Stein et al. 2004, Katada, Bihari et al. 2010, Lawendy, Bihari et al. 2014), making them potential candidates for therapeutic agents. Availability of water-soluble CO-RMs capable of liberating CO under physiological conditions makes their use even more attractive, due to the ease of application and no production of toxic levels of COHb (Lawendy, Bihari et al. 2014) (Table 4.1). In this regard, CO-RM-mediated protection against ischemia-reperfusion-induced injury to the heart (Clark, Naughton et al. 2003, Guo, Stein et al. 2004), kidney (Caumartin, Stephen et al. 2011), liver (Wei, Chen

et al. 2010) and intestine (Katada, Bihari et al. 2010), as well as the efficacy of CO-RMs to suppress severe systemic inflammatory conditions (e.g. sepsis) have been demonstrated (Mizuguchi, Stephen et al. 2009, Katada, Bihari et al. 2010).

Continuous perfusion is required in order to provide nutrients and oxygen to all tissues. CS has been shown to lead to a hypo-perfusion state within the affected compartment(s), to a point where the metabolic demands of the tissue cannot be met. This generates tissue ischemia, albeit a reduced-flow (rather than a complete) one (Blaisdell 2002, Lawendy, Sanders et al. 2011). Additionally, because not all microcirculation is shut down, a degree of reperfusion happens at the same time, producing a reperfusion injury concurrent with ischemia; this appears to be even more damaging than a complete ischemia itself (Better, Abassi et al. 1990, Gute, Ishida et al. 1998). Ischemia-reperfusion injury is comprised of a complex chain of events, leading to upregulation of the pro-inflammatory phenotype, and subsequent inflammatory interactions between the vascular endothelial cells, cells of the interstitium (e.g. fibroblasts, myofibroblasts, smooth muscle cells) and circulating cells (leukocytes). In turn, this leads to impaired vascular cell integrity, increased vascular permeability, and formation of edema (Sabido, Milazzo et al. 1994, Gute, Ishida et al. 1998, Hua, Al-Badawi et al. 2005). As a result, the ensuing increased interstitial pressure compresses adjacent capillaries, diminishing the continuous perfusion and increasing the degree of non-perfusion. The temporal changes in microvascular perfusion (i.e. the no-reflow phenomenon) appear to be caused not just by passive alterations in external pressure caused by edema, but also spastic changes in the

vasculature, leading to vasoconstriction (Nanobashvili, Neumayer et al. 2003). The non-perfused capillaries potentiate further damage by contributing to the endothelial cell swelling, capillary narrowing due to edema and increased intravascular blood viscosity from hemoconcentration (Tuma, Durian et al. 2008).

An increase of non-perfused capillaries from those being continuously perfused is a characteristic response of microvasculature to CS. In the current study, animals treated with CORM-3 had shown significant improvement in capillary perfusion, restoring the number of continuously-perfused capillaries, to a level well above that of the CS group (Figure 4.3). Thus, the exogenously applied CO, in the form of CORM-3, appeared to provide a significant protection in the maintenance of skeletal muscle blood flow. While it is unlikely that CORM-3-derived CO would have directly prevented ischemia-reperfusion-induced formation of edema, the potent vasodilatory properties of CO (mediated by soluble guanylate cyclase) (Foresti, Hammad et al. 2004) could explain the diminished spasticity of the microvasculature, resulting in the decreased severity of the no-reflow phenomenon and improved capillary flow of the affected tissue in the post-fasciotomy state (Motterlini and Otterbein 2010).

The parenchymal damage seen in CS appears to be associated, at least in part, with the direct microvascular dysfunction (resulting in severe hypoxia), and subsequent inflammation (recruitment of PMN and generation of oxidative stress) (Forbes, Carson et al. 1995, Gute, Ishida et al. 1998) that leads to the depletion of tissue ATP and initiation of tissue necrosis (Lindsay, Liauw et al. 1990). In our experiments, CS resulted in severe tissue injury, necrosis, tissue

and apoptosis within the muscle; all markers of tissue injury and dysfunction were significantly diminished in CORM-3-treated animals, suggesting a substantial protective role of exogenously applied CO (Figure 4.4 and Figure 4.5).

The tissue protective effects of CORM-3 may be attributed, at least in part, to its ability to interfere with the levels of oxidative stress in vascular endothelial cells (Mizuguchi, Capretta et al. 2010), cardiac myocytes (Lancel, Montaigne et al. 2012), and inflammatory cells (PMN, macrophages) (Sawle, Foresti et al. 2005, Mizuguchi, Stephen et al. 2009). While CO and CORM-3 (as well as other CO-RMs) may not act as free radical scavengers themselves, they may suppress the oxidative stress by interfering with the activity of heme-containing redox enzymes (e.g. cytochrome *c* oxidase, NADPH oxidase, etc.) (Kajimura, Fukuda et al. 2010). CORM-3 appears to be a potent inhibitor of myeloperoxidase activity (a PMN-derived enzyme responsible for the production of both hydrogen peroxide and hypochlorous acid – two of the most potent oxidizers in biological systems) (Patterson, Fraser et al. 2014), thus it may suppress/inhibit leukocyte (i.e. PMN)-induced oxidative damage/dysfunction to the vascular endothelium and interstitial cells.

Production of the pro-inflammatory cytokines within the tissue(s) and in the circulation, and a subsequent activation of leukocytes are considered key events contributing to induction/amplification of the inflammatory response under various conditions, including CS (Forbes, Harris et al. 1996, Sadasivan, Carden et al. 1997, Hua, Al-Badawi et al. 2005, Galasso, Schiekofer et al. 2014, Lawendy, Bihari et al. 2014, Lawendy, Bihari et al. 2016, Bihari, Cepinskas et al.

2017). It is well accepted that the production of acute-phase inflammation-relevant cytokines/chemokines (e.g. TNF-α, IL-1β, IL-6, IL-8) leads to PMN activation and recruitment into inflamed tissues (Gute, Ishida et al. 1998). Activated leukocytes, through the induction of oxidative and proteolytic stress, impair surrounding cell viability (Toyokuni 1999), and thus contribute to the tissue injury, dysfunction and a subsequent development of systemic inflammation and remote organ injury (Brock, Lawlor et al. 1999).

In our study, we observed a marked increase in the levels of circulating TNF-α (one of the most potent pro-inflammatory cytokines) in animals undergoing CS, particularly post-fasciotomy. This was associated with overwhelming systemic leukocyte activation, as well as leukocyte recruitment/infiltration within the CS-challenged muscle. Additionally, the effects could also be noticed in the diminished function of other organs, particularly the kidney and liver. Interestingly, the increase in tissue leukocyte infiltration was significantly diminished in animals treated with CORM-3, but not its inactive counterpart, iCORM-3. It is important to note that the decrease in leukocyte recruitment to CS-challenged muscle correlated with the CORM-3-dependent suppression of serum TNF-α and leukocyte activation in circulation (Figures 4.1 and 4.2). These findings are in line with our previous studies indicating that leukopenia diminished the degree of CS-associated tissue injury in a rodent model of CS (Lawendy, Bihari et al. 2015).

While the exact mechanism(s) of CORM-3-induced protective/anti-inflammatory effects are yet not completely understood, given the complex and

multi-component nature of CS pathophysiology (ischemic episode, oxidative stress, cytokine production, local and systemic responses), it is plausible to assume that the beneficial effects of the systemically administered CORM-3 may be related to the ability of CO to act simultaneously on multiple cellular and molecular targets (Otterbein, Bach et al. 2000, Ryter, Otterbein et al. 2002, Kajimura, Fukuda et al. 2010, Motterlini, Haas et al. 2012). Our current findings are in agreement with previous studies demonstrating CO-RM-dependent suppression of both vascular endothelial cell and leukocyte pro-adhesive phenotypes (e.g. reduced expression of adhesion molecules ICAM-1, VCAM-1, E-selectin, CD11b, L-selectin), and subsequent reduction/prevention of leukocyte-endothelium adhesive interaction (Urquhart, Rosignoli et al. 2007, Cepinskas, Katada et al. 2008, Mizuguchi, Stephen et al. 2009, Song, Bergstrasser et al. 2009, Vadori, Seveso et al. 2009, Mizuguchi, Capretta et al. 2010, Bergstraesser, Hoeger et al. 2012). As leukocytes are a significant contributor to tissue damage, any inhibition of leukocyte activation and/or reduced recruitment of these cells to the tissue(s) would reduce the magnitude of leukocyte-induced tissue injury and dysfunction (Patterson, Fraser et al. 2014). Whether CORM-3 preferentially interferes with the inflammatory activation of leukocyte or vascular endothelial cell (or both) in the model of limb CS used in this study remains to be determined.

While the results of our study are novel and look promising, there are some limitations that need to be addressed. First, we used only a single concentration of CORM-3 and a single route of administration (i.e. IV). Different

dosage, frequency, and alternative routes of CORM-3 administration (e.g. topical) may provide different dynamics of CORM-3-dependent protection. Second, despite the fact that CO-RMs (e.g. CORM-3) used at micromolar concentrations show no cytotoxic effects in cultured vascular endothelial cells and PMN (data not shown), the potential toxicity of various concentrations of CORM-3 *in vivo* is yet to be determined. Very little data exists on the safety of the systemic CORM-3 administration in large animals, although preclinical studies employing non-human primates suggest that there are few side effects associated with both single and repeated injections (Vadori, Seveso et al. 2009). As larger animals appear to be more sensitive to the effects of CORM-3 (Vadori, Seveso et al. 2009, Lawendy, Bihari et al. 2014) (for example, rats required 10 times the dose necessary to elicit a response in a pig), a lower therapeutic dose may be sufficient if this substance is to be used in humans, thus minimizing potential side effects. In support to the above, our previously published study using rodent model of CS (Lawendy, Bihari et al. 2014) and data presented in the current study indicate that, at concentrations that offer potent protective/anti-inflammatory effects, CORM-3 does not appear to be harmful with respect to hepatotoxicity and formation of toxic levels of COHb (Table 4.2).

In summary, this is the first study demonstrating the beneficial effects of CORM-3-derived CO as a therapeutic agent capable of mitigating the extent of acute CS-induced microvascular dysfunction in the skeletal muscle in a large animal model of CS. Our findings indicate that CORM-3 offers strong protective/anti-inflammatory effects by diminishing the post-fasciotomy tissue

injury to the skeletal muscle, and thus may potentially be used as a pharmacological adjunct therapy to fasciotomy.

4.5 REFERENCES

Bergstraesser C, Hoeger S, Song H, Ermantraut L, Hottenrot M, Czymal T, Schmidt M, Goebeler M, Ponelies N, Stich C, Loesel R, Molema G, Seelen M, van Son W, Yard BA, Rafat N (2012). Inhibition of VCAM-1 expression in endothelial cells by CORM-3: the role of the ubiquitin-proteasome system, p38, and mitochondrial respiration. Free Radic Biol Med **52**(4): 794-802.

Better OS, Abassi Z, Rubinstein I, Marom S, Winaver Y and Silberman M (1990). The mechanism of muscle injury in the crush syndrome: ischemic versus pressure-stretch myopathy. Miner Electrolyte Metab **16**(4): 181-184.

Bihari, A, Cepinskas G, Forbes TL, Potter RF and Lawendy AR (2017). Systemic application of carbon monoxide-releasing molecule-3 (CORM-3) protects skeletal muscle from ischemia-reperfusion injury. J Vasc Surg **65**: accepted Nov 30, 2016.

Bihari A, Cepinskas G, Sanders D and Lawendy AR (2014). Carbon monoxide releasing molecule-3 (CORM-3) diminishes the oxidative stress and leukocyte migration across human endothelium in an in vitro model of compartment syndrome. Orthopaedic Trauma Association Annual Meeting. Tampa, FL.

Blaisdell FW (2002). The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg **10**(6): 620-630.

Brock RW, Lawlor DK, Harris KA and Potter RF (1999). Initiation of remote hepatic injury in the rat: interactions between Kupffer cells, tumor necrosis factor-alpha, and microvascular perfusion. Hepatology **30**(1): 137-142.

Cascio BM, Pateder DB, Wilckens JH and Frassica FJ (2005). Compartment syndrome: time from diagnosis to fasciotomy. J Surg Orthop Adv **14**(3): 117-121; discussion 120-111.

Caumartin Y, Stephen J, Deng JP, Lian D, Lan Z, Liu W, Garcia B, Jevnikar AM, Wang H, Cepinskas G and Luke PP (2011). Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. Kidney Int **79**(10): 1080-1089.

Cepinskas G, Katada K, Bihari A and Potter RF (2008). Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates

inflammation in the liver of septic mice. Am J Physiol Gastrointest Liver Physiol **294**(1): G184-191.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R and Motterlini R (2003). Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ Res **93**(2): e2-8.

Donohoe ES (2015). Systemic Cytokines/Chemokines Contribute to Microvascular Dysfunction and Tissue Injury in Compartment Syndrome. MSc in Surgery, University of Western Ontario.

Dover M, Memon AR, Marafi H, Kelly G and Quinlan JF (2012). Factors associated with persistent sequelae after fasciotomy for acute compartment syndrome. J Orthop Surg (Hong Kong) **20**(3): 312-315.

Farber A, Tan TW, Hamburg NM, Kalish JA, Joglar F, Onigman T, Rybin D, Doros G and Eberhardt RT (2012). Early fasciotomy in patients with extremity vascular injury is associated with decreased risk of adverse limb outcomes: A review of the National Trauma Data Bank. Injury **43**(9): 1486-91.

Forbes TL, Carson M, Harris KA, DeRose G, Jamieson WG and Potter RF (1995). Skeletal muscle injury induced by ischemia-reperfusion. Can J Surg **38**(1): 56-63.

Forbes TL, Harris KA, Jamieson WG, DeRose G, Carson M and Potter RF (1996). Leukocyte activity and tissue injury following ischemia-reperfusion in skeletal muscle. Microvasc Res **51**(3): 275-287.

Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ and Motterlini R (2004). Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. Br J Pharmacol **142**(3): 453-460.

Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF and Pinsky DJ (2001). Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. Nat Med **7**(5): 598-604.

Galasso G, Schiekofer S, D'Anna C, Gioia GD, Piccolo R, Niglio T, Rosa RD, Strisciuglio T, Cirillo P, Piscione F and Trimarco B (2014). No-reflow phenomenon: pathophysiology, diagnosis, prevention, and treatment. A review of the current literature and future perspectives. Angiology **65**(3): 180-189.

Gillani S, Cao J, Suzuki T and Hak DJ (2012). The effect of ischemia reperfusion injury on skeletal muscle. Injury **43**(6): 670-5.

Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R, Bolli R (2004). Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. Am J Physiol Heart Circ Physiol **286**(5): H1649-1653.

Gute DC, Ishida T, Yarimizu K, Korthuis RJ (1998). Inflammatory responses to ischemia and reperfusion in skeletal muscle. Mol Cell Biochem **179**(1-2): 169-187.

Hargens AR, Romine JS, Sipe JC, Evans KL, Mubarak SJ and Akeson WH (1979). Peripheral nerve-conduction block by high muscle-compartment pressure. J Bone Joint Surg Am **61**(2): 192-200.

Hartsock LA, O'Farrell D, Seaber AV and Urbaniak JR (1998). Effect of increased compartment pressure on the microcirculation of skeletal muscle. Microsurgery **18**(2): 67-71.

Hua HT, Al-Badawi H, Entabi F, Stoner MC, Diamond RE, Bonheur JA, Houser S and Watkins MT (2005). CXC chemokine expression and synthesis in skeletal muscle during ischemia/reperfusion. J Vasc Surg **42**(2): 337-343.

Kajimura M, Fukuda R, Bateman RM, Yamamoto T and Suematsu M (2010). Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H2S gas biology. Antioxid Redox Signal **13**(2): 157-192.

Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF and Cepinskas G (2010). Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. Inflammation **33**(2): 92-100.

Lancel S, Montaigne D, Marechal X, Marciniak C, Hassoun SM, Decoster B, Ballot C, Blazejewski C, Corseaux D, Lescure B, Motterlini R and Neviere R (2012). Carbon monoxide improves cardiac function and mitochondrial population quality in a mouse model of metabolic syndrome. PLoS One **7**(8): e41836.

Lawendy AR, Bihari A, Sanders D, Badhwar A and Cepinskas G (2016). Compartment syndrome causes systemic inflammation in a rat. Bone Joint J **98-B**(8): 1132-7.

Lawendy AR, Bihari A, Sanders D, McGarr G, Badhwar A and Cepinskas G (2015). Contribution of inflammation to cellular injury in compartment syndrome in an experimental rodent model. Bone Joint J **97-B**(4): 539-543.

Lawendy AR, Bihari A, Sanders DW, Potter RF and Cepinskas G (2014). The severity of microvascular dysfunction due to compartment syndrome is diminished by the systemic application of CO-releasing molecule-3. J Orthop Trauma **28**(11): e263-268.

Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D and Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Lindsay TF, Liauw S, Romaschin AD and Walker PM (1990). The effect of ischemia/reperfusion on adenine nucleotide metabolism and xanthine oxidase production in skeletal muscle. J Vasc Surg **12**(1): 8-15.

Matsen FA, 3rd (1975). Compartmental syndrome. An unified concept. Clin Orthop Relat Res(113): 8-14.

Matsen FA, 3rd (1980). Compartmental syndromes. Hosp Pract **15**(2): 113-117.

Matsen FA, 3rd, Winquist RA and Krugmire RB, Jr (1980). Diagnosis and management of compartmental syndromes. J Bone Joint Surg Am **62**(2): 286-291.

Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, Wagner O and Jilma B (2005). Effects of carbon monoxide inhalation during experimental endotoxemia in humans. Am J Respir Crit Care Med **171**(4): 354-360.

McQueen MM, Gaston P and Court-Brown CM (2000). Acute compartment syndrome. Who is at risk? J Bone Joint Surg Br **82**(2): 200-203.

Mithoefer K, Lhowe DW, Vrahas MS, Altman DT, Erens V and Altman GT (2006). Functional outcome after acute compartment syndrome of the thigh. J Bone Joint Surg Am **88**(4): 729-737.

Mizuguchi S, Capretta A, Suehiro S, Nishiyama N, Luke P, Potter RF, Fraser DD, Cepinskas G (2010). Carbon monoxide-releasing molecule CORM-3 suppresses vascular endothelial cell SOD-1/SOD-2 activity while up-regulating the cell surface levels of SOD-3 in a heparin-dependent manner. Free Radic Biol Med **49**(10): 1534-1541.

Mizuguchi S, Stephen J, Bihari A, Markovic N, Suehiro S, Capretta A, Potter RF, Cepinskas G (2009). CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. Am J Physiol Heart Circ Physiol **297**(3): H920-929.

Motterlini R, Haas B and Foresti R (2012). Emerging concepts on the anti-inflammatory actions of carbon monoxide-releasing molecules (CO-RMs). Med Gas Res **2**(1): 28.

Motterlini R and Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nat Rev Drug Discov **9**(9): 728-743.

Mubarak SJ and Owen CA (1977). Double-incision fasciotomy of the leg for decompression in compartment syndromes. J Bone Joint Surg Am **59**(2): 184-187.

Mubarak SJ, Owen CA, Hargens AR, Garetto LP, Akeson WH (1978). Acute compartment syndromes: diagnosis and treatment with the aid of the wick catheter. J Bone Joint Surg Am **60**(8): 1091-1095.

Nanobashvili J, Neumayer C, Fuegl A, Blumer R, Prager M, Sporn E, Polterauer P, Malinski T and Huk I (2003). Development of 'no-reflow' phenomenon in ischemia/reperfusion injury: failure of active vasomotility and not simply passive vasoconstriction. Eur Surg Res **35**(5): 417-424.

Ott MC, Scott JR, Bihari, A, Badhwar A, Otterbein LE, Gray D, Harris KA, Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hind limb ischemia/reperfusion. FASEB J **19**(1): 106-108.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA and Choi AM (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med **6**(4): 422-428.

Patterson EK, Fraser DD, Capretta A, Potter RF and Cepinskas G (2014). Carbon monoxide-releasing molecule 3 inhibits myeloperoxidase (MPO) and protects against MPO-induced vascular endothelial cell activation/dysfunction. Free Radic Biol Med **70**: 167-173.

Ritenour AE, Dorlac WC, Fang R, Woods T, Jenkins DH, Flaherty SF, Wade CE and Holcomb JB (2008). Complications after fasciotomy revision and delayed compartment release in combat patients. J Trauma **64**(2 Suppl): S153-161; discussion S161-152.

Ryter SW, Otterbein LE, Morse D and Choi AM (2002). Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol Cell Biochem **234-235**(1-2): 249-263.

Sabido F, Milazzo VJ, Hobson RW, 2nd and Duran WN (1994). Skeletal muscle ischemia-reperfusion injury: a review of endothelial cell-leukocyte interactions. J Invest Surg **7**(1): 39-47.

Sadasivan KK, Carden DL, Moore MB, Korthuis RJ (1997). Neutrophil mediated microvascular injury in acute, experimental compartment syndrome. Clin Orthop Relat Res (339): 206-215.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ and Motterlini R (2005). Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. Br J Pharmacol **145**(6): 800-810.

Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray DK, Harris KA, Parry NG and Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol **296**(1): G9-G14.

Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R and Yard BA (2009). "The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression." Br J Pharmacol **157**(5): 769-780.

Toyokuni S (1999). Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int **49**(2): 91-102.

Tuma RF, Durian WN and Ley K (2008). Handbook of Physiology: Microcirculation. Oxford, UK, Academic Press (Elsevier).

Urquhart P, Rosignoli G, Cooper D, Motterlini R and Perretti M (2007). Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. J Pharmacol Exp Ther **321**(2): 656-662.

Vadori M, Seveso M, Besenzon F, Bosio E, Tognato E, Fante F, Boldrin M, Gavasso S, Ravarotto L, Mann BE, Simioni P, Ancona E, Motterlini R and Cozzi E (2009). In vitro and in vivo effects of the carbon monoxide-releasing molecule, CORM-3, in the xenogeneic pig-to-primate context. Xenotransplantation **16**(2): 99-114.

Wei Y, Chen P, de Bruyn M, Zhang W, Bremer E and Helfrich W (2010). Carbon monoxide-releasing molecule-2 (CORM-2) attenuates acute hepatic ischemia reperfusion injury in rats. BMC Gastroenterol **10**: 42.

Whitesides TE, Haney TC, Morimoto K, Harada H (1975). Tissue pressure measurements as a determinant for the need of fasciotomy. Clin Orthop Relat Res(113): 43-51.

CHAPTER 5

CARBON MONOXIDE-RELEASING MOLECULE-3 (CORM-3) OFFERS PROTECTION IN AN *IN VITRO* MODEL OF COMPARTMENT SYNDROME.

A version of this chapter will be submitted for publication to Microcirculation (2017).

CHAPTER 5: CARBON MONOXIDE-RELEASING MOLECULE-3 (CORM-3) OFFERS PROTECTION IN AN *IN VITRO* MODEL OF COMPARTMENT SYNDROME.

5.1 INTRODUCTION

Compartment syndrome (CS) is a devastating complication of musculoskeletal trauma. CS develops when the pressure within the closed osseofascial compartment rises, producing muscle-threatening and limb-threatening ischemia (Whitesides, Haney et al. 1975, Matsen 1980, Mubarak and Hargens 1983, McQueen, Christie et al. 1996). Fasciotomy, to fully decompress all affected compartments, remains the only gold-standard surgical therapy (Eaton and Green 1972, Rorabeck 1984), but the procedure must be carried out within 6-8 hours of CS onset, before the damage to the affected limb becomes permanent.

An increase in extremity compartmental pressure during CS compromises microcirculatory perfusion, thus restricting oxygen and nutrient delivery to vital tissues, resulting in cellular anoxia and severe tissue necrosis (Whitesides, Haney et al. 1975, Hargens, Schmidt et al. 1981, Matsen and Rorabeck 1989). Unlike complete ischemia, CS causes myonecrosis in the face of patent vessels. As such, the pathologic contribution of inflammation to the pathophysiology of CS is being increasingly recognized; studies from our group (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015) and others (Sadasivan, Carden et al. 1997,

Kalns, Cox et al. 2011) have broadly implicated leukocytes as playing a primary role in both microvascular and parenchymal injury during CS.

Few therapeutic options have been shown to be effective. Recently, carbon monoxide (CO), a byproduct of heme oxygenase (HO) activity, has been shown to offer both protection to microvascular perfusion, and anti-inflammatory benefits during systemic inflammation. While exogenous administration of CO via inhalation results in increased carboxyhemoglobin (COHb) levels (presenting a potential threat to the host), transitional metal carbonyls, CO-releasing molecules (CO-RMs) can be used to deliver CO in a controlled manner without significantly altering COHb (Motterlini, Clark et al. 2002, Clark, Naughton et al. 2003, Motterlini 2007). While most CO-RMs are only soluble in organic solvents, carbon monoxide releasing molecule-3 (CORM-3) is water soluble (Motterlini and Otterbein 2010), making it well suited to clinical applications.

It has been demonstrated that application of CORM-3 at fasciotomy was able to diminish the CS-associated tissue injury and leukocyte activation, as well as block the systemic release of pro-inflammatory cytokine TNF-α in a rodent and porcine models of CS (Lawendy, Bihari et al. 2014, Bihari, Cepinskas et al. 2015). While the results of the animal studies look promising, CORM-3 would have to be thoroughly tested before being used as a therapeutic agent in human patients. Moreover, the actual effects and cellular mechanisms of CORM-3-derived CO protection still remain to be elucidated.

The purpose of this study was to lay the foundation for the translation of the animal studies into human subjects: to test the effect of CORM-3-derived CO

on human endothelial cells. We undertook the task of developing an *in vitro* model of CS*,* in order to investigate the mechanisms of CORM-3-derived CO protection on human vascular endothelial cells. The ultimate goal is the development of a safe pharmacologic adjunctive treatment for compartment syndrome, which would reduce the morbidity and disability in patients.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Medium-199 (M199), fetal bovine serum, penicillin, streptomycin and Dulbecco's PBS (DPBS) (pH 7.4) were purchased from Invitrogen Canada (Life Technologies Inc., Burlington, ON). Dihydrorhodamine (DHR)-123 was obtained from Molecular Probes Inc. (Eugene, OR), and L-012 was purchased from Wako Pure Chemical (Osaka, Japan). Sera of CS patients (acquired at fasciotomy) (Appendix IV) (N=12) and healthy volunteers (N=6) were obtained from venous blood by whole blood coagulation and subsequent centrifugation at 1,500xg for 20min, as per standard operating procedures (Gillio-Meina, Cepinskas et al. 2012), and stored at -80°C until use. A water-soluble CORM-3 (tricarbonylchloro-glycinate-ruthenium(II), [$Ru(CO)_3Cl$-glycinate]; molecular weight 295 $gmol^{-1}$) was synthesized by us (Mizuguchi, Stephen et al. 2009), in accordance with the previously-published method (Motterlini, Clark et al. 2002). CORM-3 (100μM stock solution) was always prepared fresh by dissolving CORM-3 in M199 just prior to use. Inactive CORM-3 (iCORM-3) was generated by leaving CORM-3

solution for 72hrs at room temperature, to liberate all CO from the molecule, as previously described (Clark, Naughton et al. 2003).

5.2.2 Cells

Human vascular endothelial cells (HUVECs), isolated from human umbilical veins by collagenase treatment (Cepinskas, Savickiene et al. 2003), were grown to confluence on fibronectin-coated cellware (12-well plates, transwell inserts with 3μm and/or 1μm diameter pores, 96-well plates and parallel-flow perfusion microslides). HUVECs at passages 1-3 were used for all of the experiments.

Human neutrophils (PMNs) were isolated from the venous blood of healthy adults by 1% Dextran (Sigma, Mississauga, ON) sedimentation and gradient separation on Histopaque-1077 (Sigma, Mississauga, ON), as previously described (Kuhns, Long Priel et al. 2015). PMN viability was confirmed by Trypan blue dye exclusion test.

5.2.3 *In vitro* Model of CS

HUVECs were stimulated for 3 or 6 hours with human serum (40% v/v, diluted in isotonic saline) isolated from CS patients (N=12) (Appendix IV). Sera of healthy human volunteers (N=6) were used as a time-matched control. All experiments were performed in triplicate, in the presence of CORM-3 (100μM), or its inactive form, iCORM-3.

5.2.5 Reactive Oxygen Species (ROS) Production

The production of ROS in HUVECs was measured by intracellular oxidation of DHR-123, a pan-oxidant-sensitive fluorochrome, as previously described (Mizuguchi, Stephen et al. 2009). HUVECs ($1x10^6$ cells), grown to confluence in 12-well fibronectin-coated plates, were loaded with DHR-123 (10μM) for 45min, and then stimulated for 3 hours with CS serum or serum obtained from healthy volunteers, in the presence of CORM-3, or its inactive counterpart, iCORM-3. After stimulation, cells were washed with PBS, lysed in 0.5% CHAPS buffer and analysed spectrofluorometrically (FR-1501 spectrofluorometer, Shimadzu) at excitation/emission wavelengths of 495/523nm. Protein concentration in the cell lysate was assessed by DC protein assay (BioRad, Mississauga, ON). ROS production was expressed as DHR-123 fluorescence intensity (FI) per mg protein.

In parallel, levels of extracellular ROS production by PMNs were assessed using L-012, an O_2^--sensitive chemiluminescence probe, as previously described (Mizuguchi, Stephen et al. 2009). Briefly, freshly isolated PMNs ($1x10^6$ cells) were resuspended in 120μl DPBS containing 5.5mM glucose and 100μM L-012. Cells were placed in Lumitrac 96-well plates (Greiner Bio-One); subsequently, PMNs were stimulated in the presence of CORM-3 (or iCORM-3) by the CS serum or serum of healthy volunteers, continuously recording chemiluminescence intensity over 30 minutes at 37°C in a Victor-3 Multilabel plate reader (Perkin-Elmer). Superoxide production was expressed as relative luminescence units (RLU)/10^6 PMN.

5.2.5 Measurement of the Endothelial Monolayer Integrity

HUVECs were grown to confluence on fibronectin-coated transwell inserts (1μm diameter pores) (BD Falcon). Cells were stimulated with CS serum or serum obtained from healthy volunteers in the presence of CORM-3 (or iCORM-3). The integrity of the endothelial layer was assessed by measuring the trans-endothelial electrical resistance (TEER) using EndOhm chamber method (EndOhm-6, World Precision Instruments) following 1hr, 3hr and 6hr serum exposure, and expressed as Ωcm^2. Changes in TEER (ΔTEER) from the baseline were evaluated at each time point.

5.2.6 Quantification of Apoptosis

HUVECs grown on black fibronectin-coated 96-well plates with clear bottom (Greiner Bio-One) were stimulated with CS serum or serum obtained from healthy volunteers for 6 hours, in the presence of CORM-3 (or iCORM-3). Levels of the activation of active caspases were assessed by FAM-FLICA poly caspase apoptosis kit (Immunochemistry Technologies, LLC), as per manufacturer's instructions. Briefly, cells were incubated with FAM-FLICA poly caspase reagent for one hour at 37°C, washed and immediately assessed for fluorescence using Victor-3 plate reader (Perkin-Elmer), at excitation/emission wavelengths of 480nm/530nm. Levels of apoptosis were expressed as relative fluorescence units (RFU) (i.e. fluorescence intensity/10^4).

5.2.7 PMN Rolling/Adhesion Assay

HUVECs grown on the parallel-flow perfusion microslides (μ-slide $VI^{0.4}$; ibidi, Madison, WI) were stimulated with CS serum or serum of healthy volunteers for 6 hours in the presence of CORM-3 or iCORM-3. Following this, microslides with HUVECs were placed into an air-heated chamber (37°C) attached to an inverted phase-contrast microscope (Diaphot 300, Nikon). Following the 10min wash with M199 in the presence of CORM-3 or iCORM-3 at a shear stress of 1dyn/cm^2 using syringe pump (Harvard Apparatus, St. Laurent, QC), PMNs ($1x10^6$/ml) isolated from healthy adults were added to the perfusion medium and the perfusion was continued for 15 minutes at the same shear stress. PMN-HUVECs adhesive interactions (i.e. rolling, adhesion) were captured in six random fields of view (10s/field) with a digital CCD camera (Sony Corp., Japan) connected to a computer, and analyzed offline. PMNs with velocity less than 100μm/s were considered “rolling”. Adhesion was defined as PMNs that remained stationary for at least 10s. PMN rolling/adhesion was expressed as a number of PMN/mm^2.

5.2.8 Transendothelial PMN Migration Assay

HUVECs were grown to confluence on the apical aspect of fibronectin-coated transwell inserts (3μm diameter pores) (BD Falcon), and stimulated with CS serum or serum of healthy volunteers placed in the basal compartment of the inserts (mimicking the interstitial aspect of the blood vessel) for 6 hours, in the presence of CORM-3 or iCORM-3.

For the PMN migration assay, $5x10^7$ PMN/ml, isolated from healthy volunteers, were radiolabelled with 50μCi $Na^{51}CrO_4$ in PBS for 60 minutes at 37°C (Cepinskas, Katada et al. 2008). Radiolabelled PMNs ($5x10^5$ cells/insert) were added to HUVEC monolayer; PMN transendothelial migration was assessed 60min later. PMN migration was quantified as follows: %migration = basal fluid (cpm)/[wash fluid (cpm)+membrane lysate (cpm)+basal fluid (cpm)]. The amount of radioactivity in the samples was assessed by a γ-counter (Wallac 1480 Wizard, Turku, Finland).

5.2.9 Statistical Analysis

All parameters were expressed as means ± standard error of the mean (SEM), and analyzed using two-way analysis of variance (ANOVA) (GraphPad Prism, v. 5.0, San Diego, CA), with Bonferroni post-hoc test as needed. $p<0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 ROS Production

Incubation of HUVECs with 40% human CS serum induced a significant increase in the production of ROS within the endothelial cells, as shown in Figure 5.1. DHR-123 fluorescence intensity increased from 644.8±114.5 FI/mg protein in cells treated with serum of healthy volunteers to 1059.6±56.3 FI/mg protein in CS serum-treated endothelial cells ($p<0.01$). CORM-3 treatment completely

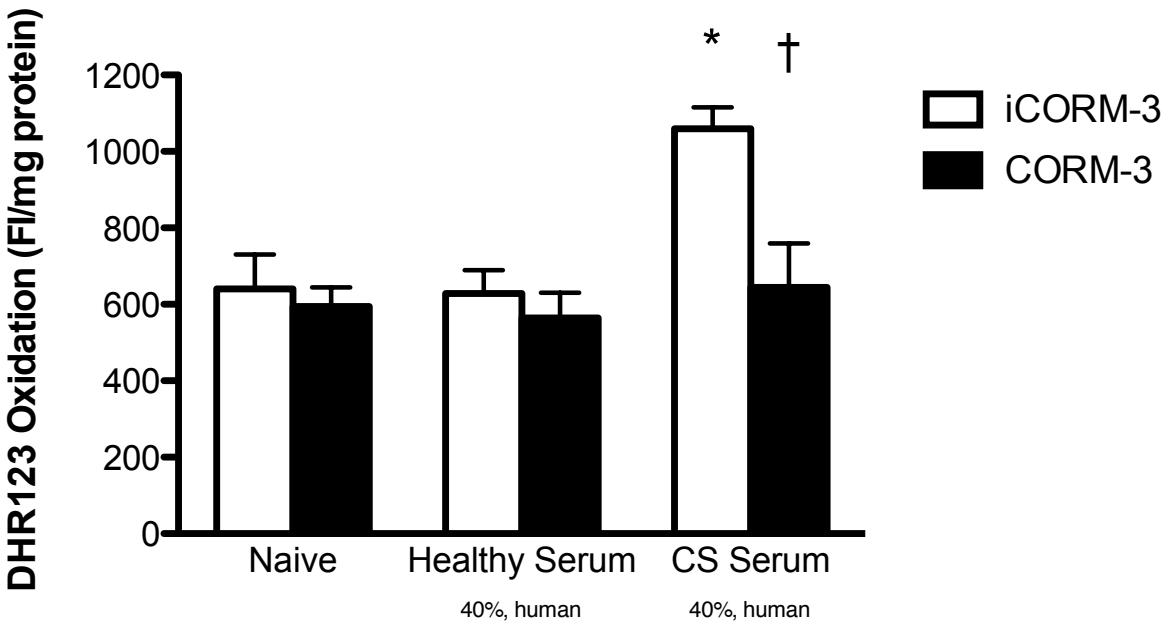

Figure 5.1. The effect of CORM-3 on the oxidative stress response in human vascular endothelial cells, elicited by stimulation with human CS serum. HUVECs were stimulated by patients' CS serum for 3 hours in the presence of CORM-3 (or its inactive form, iCORM-3), and the levels of ROS production were assessed by DHR-123 assay. *CS serum-induced ROS production was reversed by CORM-3 application* (two-way ANOVA $p<0.05$; *$p<0.001$ from control; †$p<0.001$ from CS serum+iCORM-3; N=6 per group). Serum of healthy patients served as a control.

prevented CS serum-induced ROS production (640.6±89.7 FI/mg protein versus 1061.1±53.6 FI/mg protein in iCORM-3 group, $p<0.01$), while it had no effect on control or un-stimulated endothelial cells (Figure 5.1).

Incubation of naïve PMNs with CS serum led to an increase in extracellular ROS production, as assessed by the oxidation of superoxide-sensitive probe, L-012. Superoxide production by the CS serum-challenged PMNs persisted for up to 24 minutes, with the most profound increase observed at 2-8 minute time points after the addition of sera to PMNs (Figure 5.2). Superoxide production increased from 13.4±2.4 RLU/10^6 PMN in healthy serum-stimulated PMNs to 36.1±5.9 RLU/10^6 PMN in CS-serum stimulated PMNs ($p<0.001$) (Figure 5.2). The presence of CORM-3 resulted in a significant decrease of superoxide production in CS-stimulated PMNs (10.1±4.6 RLU/10^6 PMN, $p<0.001$), while having no appreciable significant effect on PMNs treated with the serum of healthy patients (9.0±2.7 RLU/10^6 PMN) (Figure 5.2).

5.3.2 Transendothelial Electrical Resistance (TEER)

Incubation of HUVECs with CS serum produced a gradual decrease in TEER, from the baseline of 55.3±3.3Ωcm^2 to 26.2±3.2Ωcm^2 20.0±4.7Ωcm^2 and 12.2±3.2Ωcm^2 at 1hr, 3hr and 6hr stimulation, respectively (Figure 5.3), resulting in ΔTEER of 28.0±7.0Ωcm^2, 34.3±7.5Ωcm^2 and 41.8±6.47.5Ωcm^2, respectively, when compared to baseline. TEER remained virtually unchanged at 53.7±3.2Ωcm^2 in the endothelial cells treated with the serum of healthy volunteers for the duration of the experiment. CORM-3 treatment significantly

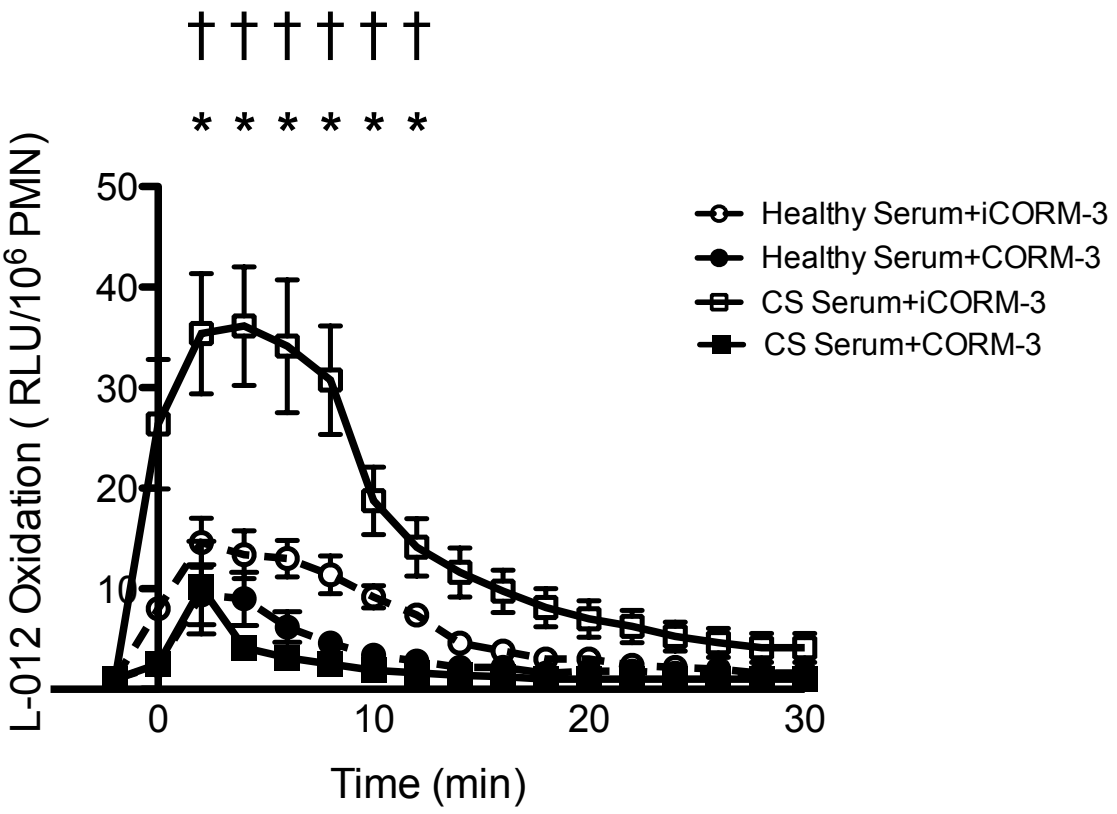

Figure 5.2. The effect of CORM-3 on leukocyte activation (quantified by the production of superoxide by PMNs), in response to stimulation with human CS serum. Naïve PMNs were stimulated with patients' CS sera in the presence of CORM-3 (or its inactive form, iCORM-3), and the levels of superoxide production were assessed by L-012 assay. *CS serum-associated PMN superoxide production was reversed by CORM-3 application* (two-way repeated measures ANOVA $p<0.05$; *$p<0.001$ from healthy serum; †$p<0.001$ from CS serum+iCORM-3; N=10 per group). Serum of healthy patients served as a control.

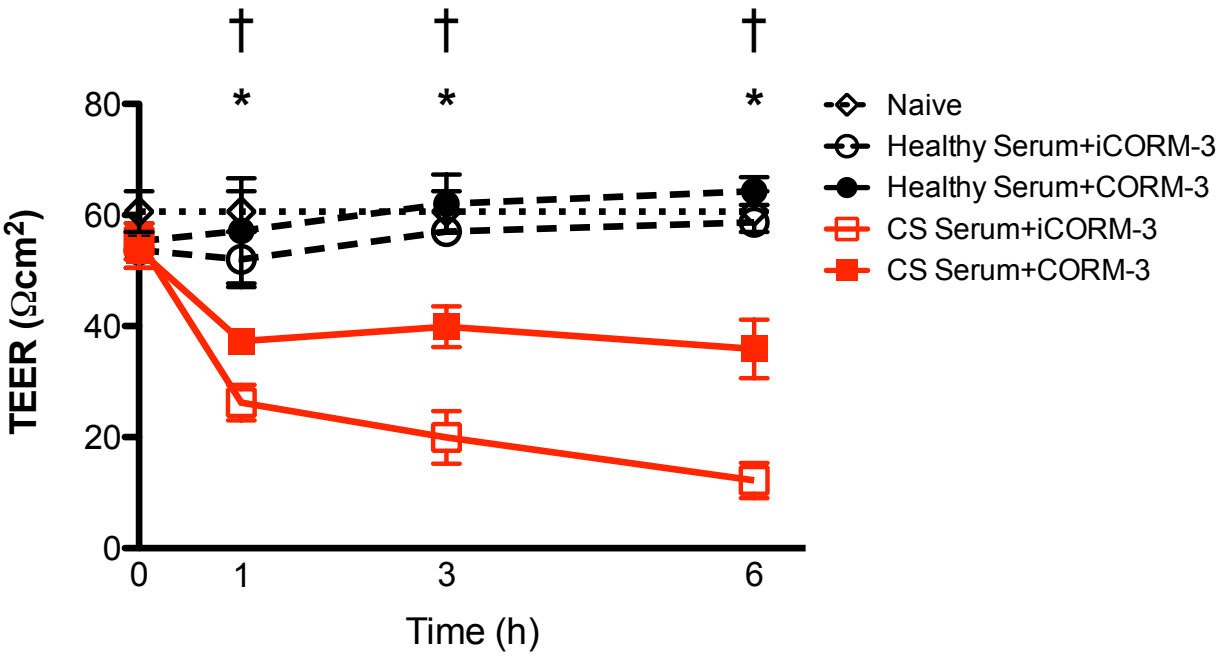

Figure 5.3. The effect of CORM-3 on the integrity of human vascular endothelial cell monolayer following stimulation with CS serum. HUVECs were stimulated by patients' CS sera for up to 6 hours in the presence of CORM-3 (or its inactive form, iCORM-3), measuring transendothelial electrical resistance (TEER). *CS serum-associated decrease in TEER was diminished by CORM-3 application* (two-way repeated measures ANOVA $p<0.05$; *$p<0.01$ from control; †$p<0.01$ from CS serum+iCORM-3; N=6 per group). Serum of healthy patients served as a control.

diminished the magnitude of changes in TEER to 37.3±2.0Ωcm^2, 39.9±3.7Ωcm^2 and 35.9±5.3Ωcm^2 (ΔTEER of 6.2±2.3Ωcm^2, 7.2±3.1Ωcm^2 and 12.9±2.8Ωcm^2 from the baseline, respectively) at 1hr, 3hr and 6hr, respectively ($p<0.01$ from CS serum+iCORM-3), while having no effect on cells incubated with serum of healthy volunteers (Figure 5.3).

5.3.3 Apoptosis

Incubation of HUVECs with human CS serum led to a significant increase in the activation of caspases, as shown in Figure 5.4 (10.3±1.0 RFU versus 2.4±0.7 RFU in control, $p<0.001$). CORM-3 treatment resulted in a significant decrease in the caspases activation to 4.0±0.4 RFU ($p<0.001$), while having no effect on control or untreated endothelial cells (Figure 5.4) Treatment of HUVECs with serum of healthy volunteers produced no changes in the activity of caspases in both CORM-3 and iCORM-3 groups.

5.3.4 PMN Rolling/Adhesion

Incubation of HUVECs with CS serum resulted in a marked increase in PMN activation behaviour, from 0.5±0.1 rolling PMNs/0.1mm^2 and 4.7±0.7 adherent PMNs/0.1mm^2 in control to 2.3±0.3 rolling PMNs/0.1mm^2 and 17.0±1.8 adherent PMNs/0.1mm^2 in CS serum-treated HUVECs ($p<0.001$) (Figure 5.5). While iCORM-3 treatment had no effect, CORM-3 treatment of HUVECs resulted in a significant decrease in PMN rolling and adhesion in CS serum-treated cells

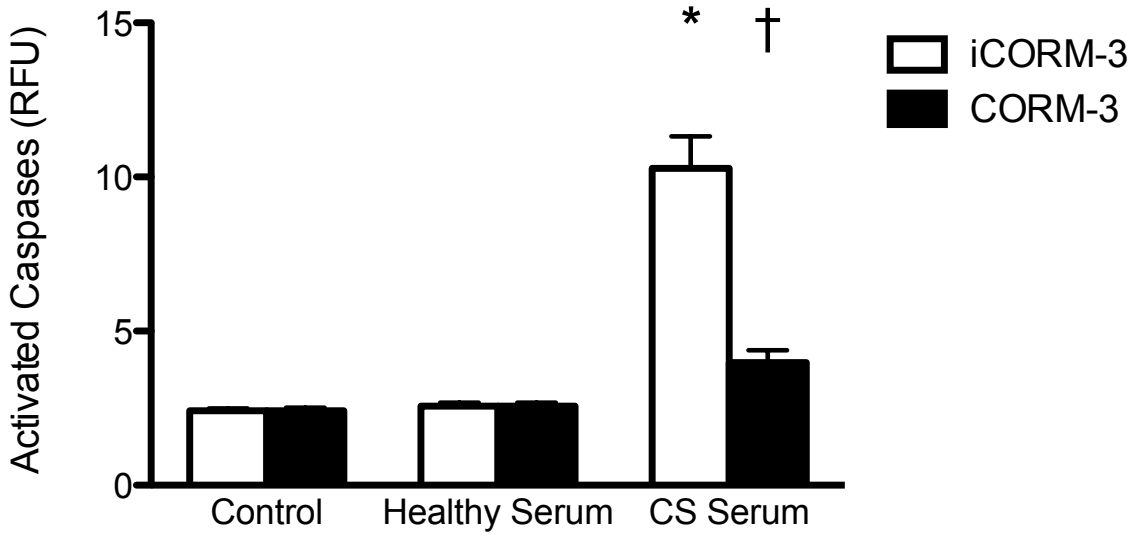

Figure 5.4. The effect of CORM-3 on the level of apoptosis in human vascular endothelial cells, elicited by stimulation with human CS serum. HUVECs were stimulated by patients' CS sera for 6 hours in the presence of CORM-3 (or its inactive form, iCORM-3), and the levels of active caspases were assessed by FAM-FLICA polycaspase assay. *CS serum-induced activation of caspases (apoptosis) was inhibited by CORM-3 application* (two-way ANOVA $p<0.05$; *$p<0.001$ from control; †$p<0.001$ from CS serum+iCORM-3; N=6 per group). Serum of healthy patients served as a control.

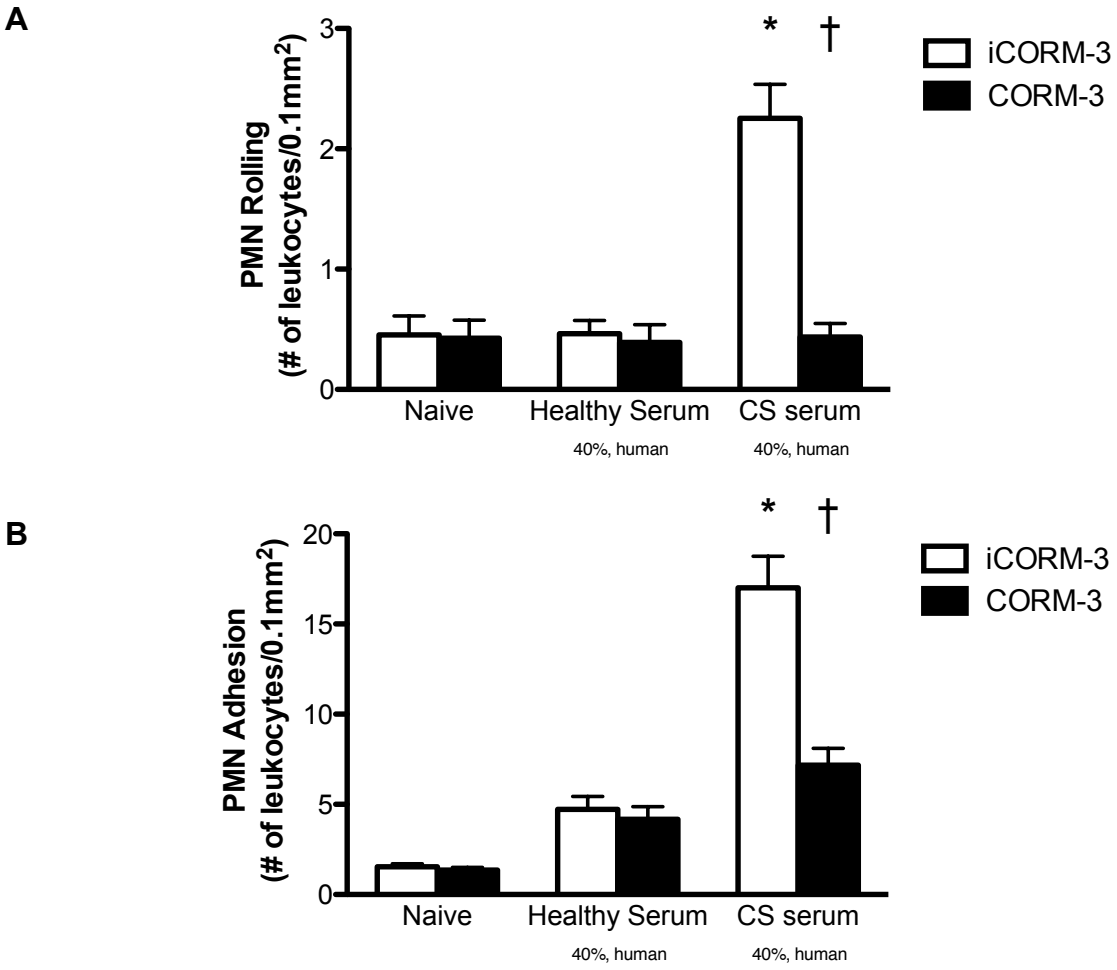

Figure 5.5. The effect of CORM-3 on leukocyte (A) rolling and (B) adhesion in response to stimulation of the human vascular endothelial cells by CS serum. HUVECs were stimulated with CS patients' sera for 6 hours, followed by application of healthy PMNs, while being superfused at a constant rate of 1dyn/cm^2 with M199, in the presence of CORM-3 or iCORM-3. *CS serum-induced PMN rolling and adhesion were significantly diminished by CORM-3 application* (two-way ANOVA $p<0.05$; *$p<0.001$ from control and healthy serum; †$p<0.001$ from CS serum+iCORM-3, N=10 per group). *PMN*, polymorphonuclear cells.

(0.4±0.1 rolling PMN/0.1mm^2 and 7.2±0.9 adherent PMN/0.1mm^2, $p<0.001$) (Figure 5.5). Inactive CORM-3 treatment had no effect on PMN rolling/adhesion.

5.3.5 PMN Migration

In order to mimic changes occurring at the vascular-interstitial interface during the clinical conditions of compartment syndrome (i.e. impaired vascular function resulting in accumulation of serum in the interstitium and formation of severe edema (Blaisdell 2002)), HUVECs were grown on the apical aspect of the permeable supports (transwell inserts) and stimulated for 6 hours with the CS serum or serum obtained from healthy volunteers placed in to the basal compartment of the inserts, in the presence of CORM-3 or iCORM-3 (100μM). Subsequently, ^{51}Cr-labeled PMNs were added to the apical aspect of HUVEC monolayer and allowed to migrate across the HUVECs for 60 minutes.

Stimulation of HUVECs with CS serum in the basal compartment (representing interstitial aspect) led to an increased PMN migration across HUVECs, from 10.0±2.0% in control to 43±0.1% in CS serum-treated cells ($p<0.05$), as shown in Figure 5.6. The presence of CORM-3/iCORM-3 in the absence of serum had no effect on PMN migration; however, CORM-3 treatment completely prevented CS serum-induced PMN migration across the endothelial cells (18.5±0.5% versus 43.5±0.5% in iCORM-3 group, $p<0.05$). Stimulation of HUVECs with serum of healthy subjects had no significant effect on trans-endothelial PMN migration in both CORM-3 and iCORM-3 groups (11.0±1.8%, n.s.).

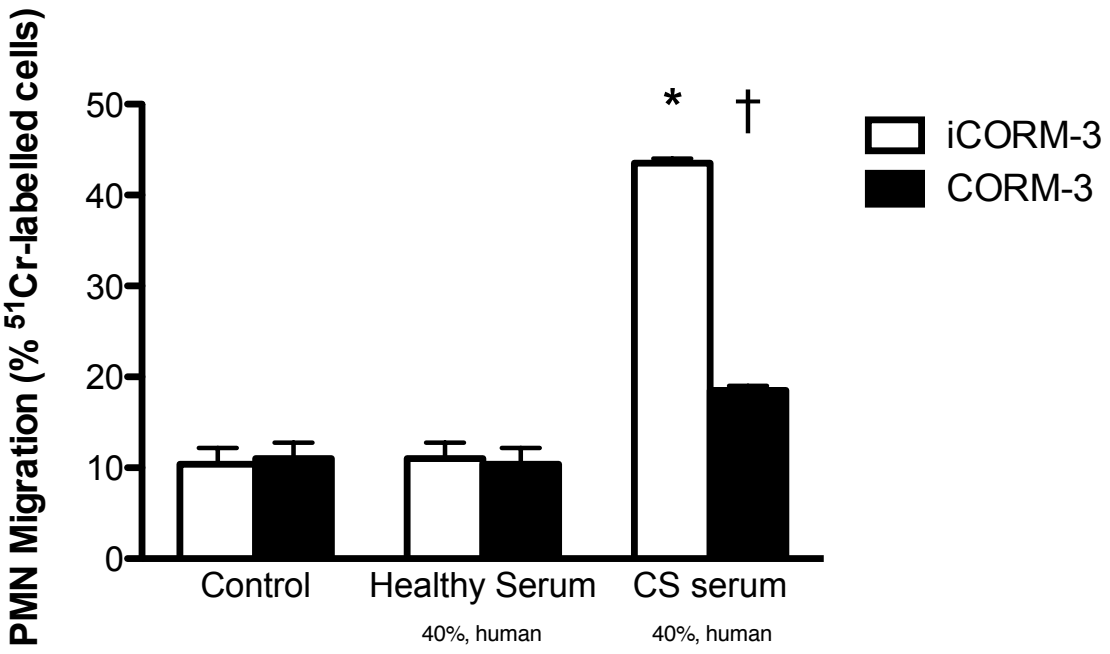

Figure 5.6. The effect of CORM-3 on transendothelial leukocyte migration in response to stimulation of human vascular endothelial cells with CS serum. $Na^{51}CrO_4$-labelled human PMNs ($5x10^5$ cells) were added to the top of stimulated HUVECs (grown on apical surface) and allowed to migrate for 60min in response to CS serum (basal compartment), in the presence of CORM-3 (or iCORM-3). The amount of ^{51}Cr radioactivity was assessed and percentage of PMN migration was calculated. *CS stimulus-associated PMN migration was significantly diminished by CORM-3 application* (two-way ANOVA $p<0.05$; *$p<0.05$ from control; †$p<0.05$ from CS+iCORM-3, N=3 per group). *PMN*, polymorphonuclear cells.

5.4 DISCUSSION

CS poses a challenging clinical problem associated with significant patient disability (Giannoudis, Nicolopoulos et al. 2002). Few treatment modalities exist, apart from the gold standard of fasciotomy (Olson and Glasgow 2005). While some supportive therapies have shown limited potential in animal models (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2014), their use is hindered by the lack of understanding of the originating mechanism(s) of CS injury.

CS is comprised of a complex chain of events, leading to upregulation of pro-inflammatory phenotype and subsequent inflammatory interactions between the vascular endothelial cells and leukocytes. As a result, impaired vascular cell integrity, increased vascular permeability and edema ensue (Sabido, Milazzo et al. 1994). One of the key features of CS appears to be an overwhelming production of pro-inflammatory mediators, and an accumulation of leukocytes in the affected limb. Leukocyte recruitment to the site of injury is an entirely normal host response (to remove pathogens or dead cells), but the extensive accumulation of PMNs and subsequent production of cytotoxic ROS, followed by the release of proteolytic enzymes contribute significantly to the extensive parenchymal damage seen in CS. Activated leukocytes impair surrounding cell viability (Toyokuni 1999), thus contributing to the tissue injury, dysfunction and subsequent development of systemic inflammation.

Recent findings indicate that CO, either produced by the inducible form of HO (i.e. HO-1) or administered by inhalation (100-250ppm) offers potent anti-inflammatory effects (Motterlini and Otterbein 2010). Nevertheless, the clinical

applicability of inhaled CO is limited due to a rapid formation of toxic levels (higher than 10%) of COHb (Ryter, Alam et al. 2006, De Backer, Elinck et al. 2009). To overcome this limitation, transitional metal carbonyls, CO-releasing molecules (CO-RMs), have been developed that allow delivery of CO in a controlled manner without significant effects on COHb formation (<3-5%COHb) (Motterlini, Mann et al. 2005, Motterlini 2007, De Backer, Elinck et al. 2009) The major advantage of using CO-RMs (e.g. the water soluble CORM-3) over the inhaled CO gas is the ability to control CO delivery and choice of various routes (IV, IP, SC or tissue superfusion) of CO administration to target specific organs/tissues. It has been demonstrated that CORM-derived CO offers cytoprotective/anti-inflammatory effects in animal models of inflammation and injury such as ischemia/reperfusion (Guo, Stein et al. 2004, Katada, Bihari et al. 2010), pulmonary hypertension (Zuckerbraun, Chin et al. 2006), transplantation (Song, Kubo et al. 2003, Caumartin, Stephen et al. 2011) and sepsis (Cepinskas, Katada et al. 2008, Mizuguchi, Stephen et al. 2009). Research aimed at understanding the mechanisms of CO-mediated protection suggests the role of soluble guanylate cyclase (Ndisang, Tabien et al. 2004, Failli, Vannacci et al. 2012), mitogen-activated protein kinases (MAPK) (Otterbein, Bach et al. 2000), phosphatidylinositol 3-kinase (PI3K), and NF-κB signaling pathways (Cepinskas, Katada et al. 2008). Hence, the actions mediated by CO appear to invoke unique stimulus-dependent signaling mechanism(s) in various cell types and organs.

The studies addressing the role of CO-RMs in modulation of inflammatory response in compartment syndrome are extremely poorly investigated. Recently,

we have developed an experimental small animal model of CS (Lawendy, Sanders et al. 2011) and demonstrated potent protective effects of CORM-3 through the suppression of CS-induced inflammation, tissue injury and microvascular perfusion deficits (Lawendy, Bihari et al. 2014). The cellular/molecular aspects of CS-induced tissue injury and dysfunction are also poorly understood. Moreover, no previous studies have used human material to assess vascular endothelial cell and/or leukocyte inflammatory activation in response to stimulation with CS-relevant stimulus.

In the present study, we attempted to mimic the CS conditions *in vitro*, employing *human* vascular endothelial cells, *human* leukocytes and serum obtained from CS patients (as a CS-relevant stimulus) in order to assess the pro-inflammatory potential of CS, as well as mechanisms of CORM-3 protection via its effect on modulation of cellular responses. To our knowledge, this is the first study to model CS in cell culture; not only does it permit the exploration of the mechanistic aspects of CS, but it allows for interventions currently not possible (or unethical) in humans.

Incubation of endothelial cells (HUVECs) with serum obtained from CS patients resulted in a significant increase in intracellular production of ROS (Figure 5.1). It can be surmised that increased ROS production in response to CS is due, at least in part, to the upregulation of pro-inflammatory cytokines found in the CS serum (Donohoe 2015). Cytokines are known to induce oxidative stress, leading to cellular membrane compromise, changes in internal protein structure and downstream effects on enzymes (Sprague and Khalil 2009).

Similarly, incubation of naïve PMNs with CS serum resulted in the same type of response – significant PMN activation, as evidenced by an increase in the superoxide production (Figure 5.2).

Application of CORM-3, but not of iCORM-3, was able to effectively diminish the CS serum-induced ROS production in both the endothelial cells and PMNs. The latter may be the result of CORM-3-dependent suppression of ROS-generating pathways, such as inhibition of heme-containing redox enzymes (e.g. cytochrome *c* oxidase, NADPH oxidase (Babior, Lambeth et al. 2002), and/or myeloperoxidase (Patterson, Fraser et al. 2014). It is important to note that CORM-3 also interferes with PMN proteolytic potential by reducing the levels of cell surface-bound elastase in an inflammatory stimulus-activated PMN, thus reducing/preventing PMN tissue infiltration and proteolytic injury (Mizuguchi, Stephen et al. 2009).

Under normal circumstances, intact endothelium provides a semi-selective barrier between the vessel lumen and surrounding tissue, controlling the passage of materials and the transit of leukocytes between the blood and interstitial space. *In vivo*, excessive increase in permeability of the endothelial monolayer may lead to tissue edema, creating non-perfused segments within the capillary system, thus further contributing to ischemia and the microvascular dysfunction (Sabido, Milazzo et al. 1994). In this regard, exposure of human endothelial cells to CS serum led to a significant, progressive breakdown of endothelial barrier, as evidenced by a decrease in trans-endothelial electrical resistance (Figure 5.3), suggesting the presence of potent injurious substances present in the circulation

of CS patients. Unlike plasma obtained from patients with pronounced systemic response (e.g. diabetic ketoacidosis), which had failed to impair the integrity of HUVECs (Omatsu, Cepinskas et al. 2014), CS serum appears to contain strong injurious and pro-inflammatory compounds that produce detrimental effect on cellular integrity.

On the other hand, it is important to note that CS serum-induced impairment of endothelial cell integrity was coupled with endothelial cell apoptosis, as evidenced by activation of multiple caspases (marker of apoptosis) (Figure 5.4). Application of CORM-3 (but not iCORM-3) prevented both CS serum-induced decrease in HUVEC monolayer integrity and induction of cell apoptosis.

Increased oxidative stress through ROS-based signalling (Toyokuni 1999) can modify endothelial cell cytoskeleton (e.g. F-actin assembly) and associated adherens junction protein function (e.g. VE-cadherin, β-catenin) (Corada, Liao et al. 2001, Giannotta, Trani et al. 2013), contributing to the breakdown of endothelial barrier. ROS also play a critical role in activation of the pro-inflammatory pathways, as well as activation of caspases (e.g. caspase-3) responsible for the induction of cell apoptosis (Elmore 2007). Given that CS produces a strong pro-oxidant and pro-inflammatory environment (characterized by the presence of ROS and inflammatory cytokines, particularly TNF-α (Lawendy, Bihari et al. 2014, Lawendy, Bihari et al. 2015)), it is plausible to assume that both intrinsic (mitochondria/cytochrome *c*-mediated) and extrinsic (TNF-α receptor-mediated) apoptotic pathways would be activated (Elmore 2007).

Therefore, the protective effects of CORM-3 associated with endothelial cell barrier function and/or cell apoptosis may be, at least in part, due to CORM-3-dependent suppression of oxidant-generating redox system(s). It remains to be determined which specific redox system in the endothelial cells and/or PMN is affected by CORM-3.

In our study, the application of CS serum to human endothelial cells led to a significant increase in leukocyte rolling and adhesion (Figure 5.5), a key feature of leukocyte and/or vascular endothelial cell inflammatory activation (Butcher 1991, Ley, Laudanna et al. 2007). These results support our recent findings demonstrating significant accumulation of leukocytes in the microcirculation of the skeletal muscle of CS animals (Lawendy, Sanders et al. 2011, Bihari, Cepinskas et al. 2015). The potency of CS serum in the induction of PMN adhesion to endothelial cells *in vitro* was greater in comparison to that observed in sepsis-relevant stimulus (lipopolysaccharide (LPS)-challenged human cerebrovascular endothelial cells) (Serizawa, Patterson et al. 2015) or endothelial cells stimulated with plasma of diabetic ketoacidosis patients (Omatsu, Cepinskas et al. 2014).

PMN recruitment to the inflamed tissues involves a series of complex, yet well coordinated PMN-endothelial cell adhesive interactions (PMN rolling, firm adhesion and migration across the endothelial barrier (Ley, Laudanna et al. 2007)). It is well accepted that adhesion molecules expressed on the vascular endothelium (e.g. P-selectin, E-selectin, ICAM-1, VCAM-1) and their ligands on

PMNs (e.g. L-selectin, sialyl-Lewis^X, β2 integrins) play a critical role in leukocyte recruitment.

In the current study, treatment of human endothelial cells with CORM-3 effectively reduced PMN rolling and adhesion to CS serum-stimulated HUVECs (Figure 5.5), replicating our *in vivo* animal data (Lawendy, Bihari et al. 2014, Bihari, Cepinskas et al. 2015). The exact mechanism(s) of CORM-3-induced suppression of the vascular endothelial cell pro-adhesive phenotype in an *in vitro* model of CS is not clear, yet it warrants further investigation. While previous studies in the field indicate that CO-RMs can modulate activation or expression of adhesion molecules on both the PMN and vascular endothelial cells, the experimental data appear to be rather controversial. It has been demonstrated that CORM-2 (DMSO-soluble CO-RM) suppressed tissue levels of intercellular adhesion molecule-1 (ICAM-1) in the liver of septic mice, as well as subsequent PMN adhesion to LPS-stimulated HUVECs (Cepinskas, Katada et al. 2008). In addition, CORM-2 effectively reduced tissue levels of E-selectin and ICAM-1 in the small intestine of mice undergoing ischemia-reperfusion (Katada, Bihari et al. 2010). Studies *in vitro* indicate that CORM-2 reduces high glucose-induced expression of ICAM-1 in HUVECs (Nizamutdinova, Kim et al. 2009). CORM-3-dependent inhibition of E-selectin and VCAM-1 expression in TNF-α-stimulated HUVECs has also been demonstrated (Song, Bergstrasser et al. 2009). On the other hand, and contrary to the above, CORM-3 failed to suppress ICAM-1 and E-selectin expression in TNF-α-stimulated HUVECs (Urquhart, Rosignoli et al. 2007) or LPS-stimulated human cerebrovascular endothelial cells, while showing

high effectiveness in reducing expression of VCAM-1 (Serizawa, Patterson et al. 2015). Finally, it has been found that CORM-3 suppressed PMN-HUVEC adhesive interaction by reducing CD11b ($a_M\beta_2$-integrin) surface levels in platelet activating factor (PAF)-stimulated PMN (Urquhart, Rosignoli et al. 2007).

Following firm leukocyte adhesion to the vascular endothelium, PMN migration into the interstitium is driven primarily by chemokines (e.g. CXCL1, CXCL8) and other chemotactic substances (e.g. PAF, leukotriene B_4); these are produced by vascular endothelial cells and/or interstitial cells, such as macrophages, pericytes and fibroblasts. PMN migration across the vascular barrier is controlled by PECAM-1 (CD31), CD99 and JAM-A/B/C adhesion molecules, expressed on both leukocytes and the endothelial cells (Ley, Laudanna et al. 2007). It has been demonstrated that excessive migration of leukocytes (particularly neutrophils) across the vascular endothelium compromises microvessel integrity; this is associated with the disruption of the endothelial cell adherens/tight junctions and degradation of basement membrane (Cepinskas, Sandig et al. 1999, Cepinskas, Savickiene et al. 2003, Granger and Senchekova 2010). As a result, fluid (i.e. plasma) is allowed to pass into the parenchyma, contributing to edema formation (Giannotta, Trani et al. 2013).

In our current study, basolateral stimulation of endothelial cells with the serum of CS patients (to mimic vascular-interstitial interface) resulted in significant increase in PMN transendothelial migration, the phenomenon that was effectively prevented by CORM-3 treatment (Figure 5.6). These findings suggest that CS serum may upregulate expression or activation of adhesion molecules

involved in PMN migration (e.g. CD31, JAM-C). Alternatively, CS serum may induce production of chemokines by the endothelial cells, or it itself contains sufficient amount of chemotactic molecules promoting PMN transendothelial migration. In fact, the pro-inflammatory potential of plasma obtained from patients with severe systemic inflammatory disorders was demonstrated, showing that the levels of pro-inflammatory cytokines IL-6 and CCL2 (MCP-1), as well as chemokines CXCL8 (IL-8), CCL4 (MIP-1β), and CXCL1 (GRO-α) are markedly upregulated in the circulation of human patients with severe sepsis. In addition, the circulating levels of IL-6, CXCL1 (GRO-α), CXCL8 (IL-8), and CXCL10 (IP-10) chemokines have also been detected in the plasma of diabetic ketoacidosis patients (Omatsu, Cepinskas et al. 2014). While we have previously demonstrated, in a rat model, that CS resulted in upregulation of pro-inflammatory cytokines and chemokines (Donohoe 2015), it remains to be determined whether pro-inflammatory cytokines/chemokines can be found in the serum of human CS patients.

In summary, this is the first study demonstrating the beneficial effects of carbon monoxide, delivered by CORM-3, in an *in vitro* human model of compartment syndrome. It represents the first step in an attempt to translate the promising results obtained from animal studies into human patients. While the exact mechanisms of CORM-3 protective action remain to be determined, the obtained data strongly indicate a potential therapeutic application of CORM-3 to patients at risk of developing CS.

5.5 REFERENCES

Babior BM, Lambeth JD and Nauseef W (2002). The neutrophil NADPH oxidase. Arch Biochem Biophys **397**(2): 342-344.

Bihari A, Cepinskas G, Sanders D and Lawendy A (2015). Systemic administration of carbon monoxide-releasing molecule-3 (CORM-3) protects the skeletal muscle in porcine model of compartment syndrome. Thirty-First Orthopaedic Trauma Association Annual Meeting, San Diego, CA.

Blaisdell FW (2002). The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg **10**(6): 620-630.

Butcher EC (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell **67**(6): 1033-1036.

Caumartin Y Stephen J, Deng P, Lian D, Lan Z, Liu W, Garcia B, Jevnikar AM, Wang H, Cepinskas G and Luke PP (2011). Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. Kidney Int **79**(10): 1080-1089.

Cepinskas G, Sandig M and Kvietys PR (1999). PAF-induced elastase-dependent neutrophil transendothelial migration is associated with the mobilization of elastase to the neutrophil surface and localization to the migrating front. J Cell Sci **112 (Pt 12)**: 1937-1945.

Cepinskas G, Savickiene J, Ionescu CV and Kvietys PR (2003). PMN transendothelial migration decreases nuclear NFkappaB in IL-1beta-activated endothelial cells: role of PECAM-1. J Cell Biol **161**(3): 641-651.

Cepinskas G, Katada K, Bihari A and Potter RF (2008). Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. Am J Physiol Gastrointest Liver Physiol **294**(1): G184-191.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R and Motterlini R (2003). Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ Res **93**(2): e2-8.

Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, Muller WA, Hicklin DJ, Bohlen P and Dejana E (2001). Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood **97**(6): 1679-1684.

De Backer O, Elinck E, Blanckaert B, Leybaert L, Motterlini R and Lefebvre RA (2009). Water-soluble CO-releasing molecules reduce the development of postoperative ileus via modulation of MAPK/HO-1 signalling and reduction of oxidative stress. Gut **58**(3): 347-356.

Donohoe ES (2015). Systemic Cytokines/Chemokines Contribute to Microvascular Dysfunction and Tissue Injury in Compartment Syndrome. MSc in Surgery, University of Western Ontario.

Eaton RG and Green WT (1972). Epimysiotomy and fasciotomy in the treatment of Volkmann's ischemic contracture. Orthop Clin North Am **3**(1): 175-186.

Elmore S (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol **35**(4): 495-516.

Failli P, Vannacci A, Di Cesare Mannelli L, Motterlini R and Masini E (2012). Relaxant Effect of a Water Soluble Carbon Monoxide-Releasing Molecule (CORM-3) on Spontaneously Hypertensive Rat Aortas. Cardiovasc Drugs Ther **26**(4): 285-292

Giannotta M, Trani M and Dejana E (2013). VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. Dev Cell **26**(5): 441-454.

Giannoudis PV, Nicolopoulos C, Dinopoulos H, Ng A, Adedapo S and Kind P (2002). The impact of lower leg compartment syndrome on health related quality of life. Injury **33**(2): 117-121.

Gillio-Meina C, Cepinskas G, Cecchini EL, Fraser DD (2013). Translational research in pediatrics II: blood collection, processing, shipping and storage. Pediatrics **131**(4): 754-766.

Granger DN and Senchekova E (2010). Inflammation and the Microcirculation. San Rafael, CA, Morgan & Claypool Life Sciences.

Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li, QH, Dawn B, Motterlini R and Bolli R (2004). Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. Am J Physiol Heart Circ Physiol **286**(5): H1649-1653.

Hargens AR, Schmidt DA, Evans KL, Gonsalves MR, Cologne JB, Garfin SR, Mubarak SJ, Hagan PL and Akeson WH (1981). Quantitation of skeletal-muscle necrosis in a model compartment syndrome. J Bone Joint Surg Am **63**(4): 631-636.

Kalns J, Cox J, Baskin J, Santos A, Odland R and Fecura S, Jr. (2011). Threshold model for extremity compartment syndrome in swine. J Surg Res **167**(1): e13-19.

Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF and Cepinskas G (2010). Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. Inflammation **33**(2): 92-100.

Kuhns DB, Long Priel DA, Chu J and Zarember KA (2015). Isolation and Functional Analysis of Human Neutrophils. Curr Protoc Immunol **111**: 7 23 21-16.

Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D and Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Lawendy AR, Bihari A, Sanders DW, Potter RF and Cepinskas G (2014). The Severity of Microvascular Dysfunction Due to Compartment Syndrome Is Diminished by the Systemic Application of CO-Releasing Molecule (CORM-3). J Orthop Trauma **28**(11): e263-e268.

Lawendy AR, Bihari A, Sanders D, McGarr G, Badhwar A and Cepinskas G (2015). Contribution of inflammation to cellular injury in compartment syndrome in an experimental rodent model. Bone Joint J **97-B**(4): 539-543.

Ley K (2005). Adhesion molecules and the recruitment of leukocytes in postcapillary venules. Microvascular Research: Biology and Pathology. Shepro D, Elsevier**:** 317-322.

Manjoo A, Sanders D, Lawendy A, Gladwell M, Gray D, Parry N and Badhwar A (2010). Indomethacin reduces cell damage: shedding new light on compartment syndrome. J Orthop Trauma **24**(9): 526-529.

Matsen FA 3rd (1980). Compartmental syndromes. Hosp Pract **15**(2): 113-117.

Matsen FA 3rd and Rorabeck CH (1989). Compartment syndromes. AAOS Instr Course Lect **38**: 463-472.

McQueen MM, Christie J and Court-Brown CM (1996). Acute compartment syndrome in tibial diaphyseal fractures. J Bone Joint Surg Br **78**(1): 95-98.

Mizuguchi S, Stephen J, Bihari A, Markovic N, Suehiro S, Capretta A, Potter RF and Cepinskas G (2009). CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. Am J Physiol Heart Circ Physiol **297**(3): H920-929.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE and Green CJ (2002). Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res **90**(2): E17-24.

Motterlini R, Mann BE and Foresti R (2005). Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin Investig Drugs **14**(11): 1305-1318
Motterlini R (2007). Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem Soc Trans **35**(Pt 5): 1142-1146.

Motterlini R and Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nat Rev Drug Discov **9**(9): 728-743.

Mubarak SJ and Hargens AR (1983). Acute compartment syndromes. Surg Clin North Am **63**(3): 539-565.

Ndisang JF, Tabien HE and Wang R (2004). Carbon monoxide and hypertension. J Hypertens **22**(6): 1057-1074.

Nizamutdinova IT, Kim YM, Kim HJ, Seo HG, Lee JH and Chang KC (2009). Carbon monoxide (from CORM-2) inhibits high glucose-induced ICAM-1 expression via AMP-activated protein kinase and PPAR-gamma activations in endothelial cells. Atherosclerosis **207**(2): 405-411.

Olson SA and Glasgow RR (2005). Acute compartment syndrome in lower extremity musculoskeletal trauma. J Am Acad Orthop Surg **13**(7): 436-444.

Omatsu T, Cepinskas G, Clarson C, Patterson EK, Alharfi IM, Summers K, Couraud PO, Romero IA, Weksler B, Fraser DD and Canadian Critical Care Translational Biology Group (2014). CXCL1/CXCL8 (GROalpha/IL-8) in human diabetic ketoacidosis plasma facilitates leukocyte recruitment to cerebrovascular endothelium in vitro. Am J Physiol Endocrinol Metab **306**(9): E1077-1084.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA and Choi AM (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med **6**(4): 422-428.

Patterson EK, Fraser DD, Capretta A, Potter RF and Cepinskas G (2014). Carbon monoxide-releasing molecule 3 inhibits myeloperoxidase (MPO) and protects against MPO-induced vascular endothelial cell activation/dysfunction. Free Radic Biol Med **70**: 167-173.

Rorabeck CH (1984). The treatment of compartment syndromes of the leg. J Bone Joint Surg Br **66**(1): 93-97.

Ryter SW, Alam J and Choi AM (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev **86**(2): 583-650.

Sabido F, Milazzo VJ, Hobson RW, 2nd and Duran WN (1994). Skeletal muscle ischemia-reperfusion injury: a review of endothelial cell-leukocyte interactions. J Invest Surg **7**(1): 39-47.

Sadasivan KK, Carden DL, Moore MB and Korthuis RJ (1997). Neutrophil mediated microvascular injury in acute, experimental compartment syndrome. Clin Orthop Relat Res(339): 206-215.

Serizawa F, Patterson E, Potter RF, Fraser DD and Cepinskas G (2015). Pretreatment of human cerebrovascular endothelial cells with CO-releasing molecule-3 interferes with JNK/AP-1 signaling and suppresses LPS-induced proadhesive phenotype. Microcirculation **22**(1): 28-36.

Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R and Yard BA (2009). The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression. Br J Pharmacol **157**(5): 769-780.

Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry RK and Choi AM (2003). Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. Am J Pathol **163**(1): 231-242.

Sprague AH and Khalil RA (2009). Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol **78**(6): 539-552.

Toyokuni S (1999). Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int **49**(2): 91-102.

Urquhart P, Rosignoli G, Cooper D, Motterlini R and Perretti M (2007). Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. J Pharmacol Exp Ther **321**(2): 656-662.

Whitesides TE, Haney TC, Morimoto K and Harada H (1975). Tissue pressure measurements as a determinant for the need of fasciotomy. Clin Orthop Relat Res(113): 43-51.

Whitesides TE Jr, Haney TC, Harada H, Holmes HE and Morimoto K (1975). A simple method for tissue pressure determination. Arch Surg **110**(11): 1311-1313.

Zuckerbraun BS, Chin BY, Wegiel B, Billiar TR, Czsimadia E, Rao J, Shimoda L, Ifedigbo E, Kanno S and Otterbein LE (2006). Carbon monoxide reverses established pulmonary hypertension. J Exp Med **203**(9): 2109-211

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS.

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6.1 OVERVIEW OF RESULTS

6.1.1 Pathophysiology of CS and Current Therapeutic Considerations

Ever since the first description of limb ischemic contracture by Richard von Volkmann (von Volkmann 1881), CS has been recognized as one of the most devastating complications of musculoskeletal trauma, resulting in severe acute myonecrosis, with significant patient disability as an outcome. An attempt to highlight the major conceptual contributions to the current state of knowledge of CS and current therapeutic approaches was undertaken in Chapter 1. While the pathophysiology of the condition has not been completely elucidated, CS appears to occur as a consequence of ischemia-reperfusion injury secondary to an elevation of ICP. An increase in intra-compartmental pressure creates hypo-perfused state, where oxygen and nutrient delivery to the tissue is limited, resulting in cellular anoxia and tissue necrosis (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Sheridan, Matsen et al. 1977, Rorabeck and Clarke 1978). As a consequence, severe inflammatory response occurs, further contributing to the ischemia-induced microvascular dysfunction (Sadasivan, Carden et al. 1997, Lawendy, Sanders et al. 2011). Unlike complete ischemia, however, CS occurs in the face of patent vessels, since distal pulse is often present (Seddon 1966); the low-flow ischemic state appears to be even more injurious than complete ischemia, with severe acidosis and metabolic stress

rendering a more severe degree of the muscle deterioration (Heppenstall, Scott et al. 1986).

CS constitutes surgical emergency, with fasciotomy as the only current gold-standard treatment. Fasciotomy, to decompress all tissues in the involved compartments must be carried out in a timely manner (within a surgical window of 6-8 hours), before the damage becomes permanent. Due to the uncertainty of the actual onset of CS (the condition does not develop instantaneously) and the lack of objective diagnostic test (CS diagnosis is based on a subjective clinical exam), targeting the window may not be easy – hence the need for prolongation of surgical window.

6.1.2 Carbon Monoxide and CS

It has been known for some time that ischemia results in an upregulation of protective stress-induced proteins, particularly HO (Otterbein, Lee et al. 1999, Petrache, Otterbein et al. 2000, Sato, Balla et al. 2001). While the global upregulation of HO may not be feasible clinically, exogenous application of its endproducts, particularly CO, can be accomplished with relative ease.

The effect of inhalational CO on the extent of CS-induced microvascular dysfunction in a rat model of CS is described in Chapter 2. Since CS poses great challenge to study in humans, various animal models have been developed to investigate the condition (Jepson 1926, Sheridan and Matsen 1975, Sadasivan, Carden et al. 1997, Vollmar, Westermann et al. 1999, Kalns, Cox et al. 2011, Lawendy, Sanders et al. 2011). The rat model (Lawendy, Sanders et al. 2011)

was chosen due to its relative ease of preparation, excellent reproducibility and low cost. Most importantly, rat model of CS allowed for the use of IVVM as a real-time analysis of microcirculation.

Direct imaging of the microcirculation by IVVM validated significant perfusion derangements, marked tissue injury and extensive leukocyte activation in response to 2 hours of elevated ICP; these effects were suppressed by administration of CO or CORM-3 at fasciotomy. Two hours were chosen based on previous studies, demonstrating that 1 hour of ischemia in a rodent approximates 4 hours of ischemia in human (Sheridan, Matsen et al. 1977, Hulbert, Pamplona et al. 2007). The CO dose of 250ppm was chosen on the basis of previous research demonstrating its effectiveness in suppressing various pathological inflammatory conditions (Otterbein, Mantell et al. 1999, Otterbein, Bach et al. 2000, Moore, Otterbein et al. 2003, Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009).

In our study, exogenous application of CO resulted in a significant improvement in microvascular perfusion, complete inhibition of leukocyte activation and tissue injury in CS-challenged animals; however, inhalation of CO significantly elevated COHb.

6.1.2.1 CORM-3 as a Source of CO

With the synthesis of a novel class of transition metal carbonyls, CO-releasing molecules (CO-RMs) (Motterlini, Mann et al. 2005), as a pharmacological means of CO delivery, a new avenue of research opened up,

allowing application of CO directly to the tissues (i.e. bypassing the inhalational component). The effect of exogenous application of CO in the form of injectable CORM-3 on the severity of microvascular dysfunction due to CS is described in Chapter 3. CORM-3 was selected because of its excellent water solubility and rapid release of CO under physiological conditions, upon administration. *Ex vivo* studies indicate that CORM-3 is an equimolar CO donor (Motterlini, Clark et al. 2002); the CORM-3 dose of 10mg/kg was chosen based on our inhalational CO studies (250ppm), to deliver the equivalent amount of CO.

In our study, systemic application of CORM-3 at fasciotomy resulted in a significant improvement in muscle perfusion, 4-fold decrease in tissue injury, 8-fold decrease in leukocyte activation, coupled with abolishment of CS-induced TNF-α release, all without any changes in blood levels of COHb.

6.1.3 Preclinical Testing of CORM-3 in Porcine CS

While the results of rat CS experiments show promise, it would be quite a leap to apply these to human subjects. Hence, the effect of CORM-3 was tested in a pre-clinical setting, using a large animal model – an animal more akin to human (i.e. pig), described in Chapter 4. Rodents respond differently to CO than humans (previous inhalational CO studies in humans using bacterial sepsis failed to demonstrate any significant effects of inhaled CO on the LPS-induced systemic response) (Mayr, Spiel et al. 2005). The purpose of the study described in this thesis was to evaluate the effect of CORM-3 on the severity of microvascular dysfunction in pigs undergoing CS.

We found that six hours of elevated compartment pressure in pigs resulted in severe microvascular perfusion deficit, progressive release of TNF-α coupled with significant systemic leukocyte activation, as well as a large increase in tissue necrosis, apoptosis and leukocyte infiltration (tissue inflammation). In addition, some end-organ damage was also observed (particularly in the kidney). Systemic administration of CORM-3 led to a marked decrease in tissue inflammation coupled with some improvement in microvascular dysfunction, lessening of tissue necrosis and a large decrease in tissue apoptosis. Systemically, CORM-3 blocked CS-induced increase in circulating TNF-α levels, coupled with complete abolishment of systemic leukocyte activation. Additionally, CORM-3 also attenuated the severity of end organ dysfunction (i.e. kidney).

6.1.4 Human *in vitro* Model

In order to translate the results of animal experimentation, human CS was modelled *in vitro*, as described in Chapter 5. This study was designed to test the effect of CORM-3 on the cultured primary human vascular endothelial cells stimulated with CS-like conditions, attempting to provide a broad mechanistic insight into CS in terms of oxidative stress and leukocyte activation. The model did not involve elevation of hydrostatic pressure; rather, the CS-like conditions were mimicked by the use of serum obtained from CS patients, covering only the inflammatory component of CS pathophysiology. As such, the model may not accurately and completely mimic conditions that CS patients experience.

In our study, we found that CS challenge of the endothelial cells, in the form of serum isolated from CS patients, resulted in an increase in oxidative stress in the endothelial cells (as evidenced by increased ROS production), breakdown of endothelial layer integrity (changes in trans-endothelial electrical resistance), and significant upregulation of pro-adhesive phenotype, leading to changes in leukocyte-endothelial interactions (increased leukocyte adhesion and rolling, leukocyte transmigration). Application of CORM-3 was able to diminish the severity of both the oxidative stress and endothelial activation necessary for leukocyte recruitment within the endothelial cells.

Our findings suggest that CORM-3 has potent anti-oxidant and anti-inflammatory properties. While not a free-radical scavenger, it may have the ability to suppress the oxidative stress indirectly (e.g. by modulating the activity of redox enzymes), minimizing the extent of endothelial injury.

6.2 LIMITATIONS AND FUTURE DIRECTIONS

The significance of the results described this thesis lies, in part, with the demonstration of the protective effects of CORM-3 on the severity of outcomes in multiple models of CS: that of a rat, pig and human. While the data looks promising, the studies were not without limitations. First, we used only a single concentration of CORM-3 and a single route of administration in each species (i.e. IP in rats, IV in pigs, direct exposure in human endothelial cells). Different

dosage, frequency, and alternative routes of CORM-3 administration may provide different dynamics of CORM-3-dependent protection.

Second, despite the fact that the use of CORM-3 at micromolar concentrations shows no cytotoxic effects in cultured vascular endothelial cells and leukocytes, the potential toxicity of various concentrations of CORM-3 *in vivo* is yet to be determined. Very little data exists on the safety of the systemic CORM-3 administration in large animals, although they appear to be more sensitive to the effects of CORM-3. The data presented in our studies indicate that, at concentrations that offer potent anti-inflammatory effects, CORM-3 does not appear to be harmful with respect to hepatotoxicity and formation of toxic levels of COHb. However, CORM-3 is a ruthenium-based molecule; ruthenium is a transition metal that does not naturally occur in human body (Emsley 2003). Although ruthenium is relatively inert to other chemicals, heavy metal toxicity and effects on the DNA (Bergamo and Sava 2011) are still of major concern.

Finally, the studies described here did not examine the effects of CORM-3 on pathophysiology of CS in a very detailed mechanistic manner, primarily due to the lack of availability of genetically modified animals. The main purpose was to test the potential therapeutic applicability of CORM-3 in a rather crude approach: does it work or not?

In the future, the evaluation of the effect of CORM-3 on the magnitude of functional deficits following CS would be of immense benefit. The studies that evaluate gait could be accomplished in rats with relative ease using automated gait analysis (e.g. the CatWalk^{TM} system), currently available to us.

Another avenue of research could explore the role of CORM-3 in tissue repair and muscle regeneration. The concepts of developmental biology could be applied to tissue engineering and stem cell production. Successful muscle regeneration requires careful regulation of inflammation to clear up the debris, and the initiation of activation of muscle satellite cells (the normally quiescent muscle precursors) (Tidball 2011). Given that different classes of macrophages fulfill different function (M1 type furthers inflammation, while M2 type promotes tissue repair) the role of CORM-3 on the differential expression (induction or modulation) of M1 and M2 phenotypes could be explored.

Finally, the first transition into an actual patient could be undertaken, by evaluating the efficacy of topical application of CORM-3 following fasciotomy.

6.3 CONCLUSIONS

The data in this thesis indicates that CORM-3 has strong anti-inflammatory properties, and may be capable of diminishing the post-fasciotomy tissue injury. While the exact mechanisms of its protective action remain to be determined, CORM-3 may have an enormous potential as a pharmacological adjunct to fasciotomy, and might, at least, be capable of prolongation of surgical window and improvement of muscle salvage after fasciotomy.

6.4 REFERENCES

Bergamo A and Sava G (2011). Ruthenium anticancer compounds: myths and realities of the emerging metal-based drugs. Dalton Trans **40**(31): 7817-7823.

Emsley J (2003). Ruthenium. Nature's Building Blocks: An A to Z Guide to the Elements. Oxford, England, UK, Oxford University Press**:** 368-370.

Heppenstall RB, Scott R, Sapega A, Park YS and Chance B (1986). A comparative study of the tolerance of skeletal muscle to ischemia. Tourniquet application compared with acute compartment syndrome. J Bone Joint Surg Am **68**(6): 820-828.

Hulbert AJ, Pamplona R, Buffenstein R and Buttemer WA (2007). Life and death: metabolic rate, membrane composition, and life span of animals. Physiol Rev **87**(4): 1175-1213.

Jepson PN (1926). Ischaemic Contracture: Experimental Study. Ann Surg **84**(6): 785-795.

Kalns J, Cox J, Baskin J, Santos A, Odland R and Fecura S, Jr. (2011). Threshold model for extremity compartment syndrome in swine. J Surg Res **167**(1): e13-19.

Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D and Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg **54**(3): 194-200.

Mayr FB, Spiel A, Leitner J, Marsik C, Germann P, Ullrich R, Wagner O and Jilma B (2005). Effects of carbon monoxide inhalation during experimental endotoxemia in humans. Am J Respir Crit Care Med **171**(4): 354-360.

Moore BA, Otterbein LE, Turler A, Choi AM and Bauer AJ (2003). Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. Gastroenterology **124**(2): 377-391.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE and Green CJ (2002). Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ Res **90**(2): E17-24.

Motterlini R, Mann BE and Foresti R (2005). Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin Investig Drugs **14**(11): 1305-1318.

Ott MC, Scott JR, Bihari A, Badhwar A, Otterbein LE, Gray DK, Harris KA and Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hind limb ischemia/reperfusion. FASEB J **19**(1): 106-108.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA and Choi AM (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med **6**(4): 422-428.

Otterbein LE, Lee PJ, Chin BY, Petrache I, Camhi SL, Alam J and Choi AM (1999). Protective effects of heme oxygenase-1 in acute lung injury. Chest **116**(1 Suppl): 61S-63S.

Otterbein LE, Mantell LL and Choi AM (1999). Carbon monoxide provides protection against hyperoxic lung injury. Am J Physiol **276**(4 Pt 1): L688-694.

Petrache I, Otterbein LE, Alam J, Wiegand GW and Choi AM (2000). Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. Am J Physiol Lung Cell Mol Physiol **278**(2): L312-319.

Rorabeck CH, and Clarke KM (1978). The pathophysiology of the anterior tibial compartment syndrome: an experimental investigation. J Trauma **18**(5): 299-304.

Sadasivan KK, Carden DL, Moore MB and Korthuis RJ (1997). Neutrophil mediated microvascular injury in acute, experimental compartment syndrome. Clin Orthop Relat Res(339): 206-215.

Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH and Soares MP (2001). Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol **166**(6): 4185-4194.

Scott JR, Cukiernik MA, Ott MC, Bihari A, Badhwar A, Gray DK, Harris KA, Parry NG and Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol **296**(1): G9-G14.

Seddon HJ (1966). Volkmann's ischaemia in the lower limb. J Bone Joint Surg Br **48**(4): 627-636.

Sheridan GW and Matsen FA (1975). An animal model of the compartmental syndrome. Clin Orthop Relat Res(113): 36-42.

Sheridan GW, Matsen FA, 3rd and Krugmire RB, Jr. (1977). Further investigations on the pathophysiology of the compartmental syndrome. Clin Orthop Relat Res(123): 266-270.

Tidball JG (2011). Mechanisms of muscle injury, repair, and regeneration. Compr Physiol **1**(4): 2029-2062.

Vollmar B, Westermann S and Menger MD (1999). Microvascular response to compartment syndrome-like external pressure elevation: an in vivo fluorescence microscopic study in the hamster striated muscle. J Trauma **46**(1): 91-96.

von Volkmann, R (1881). Die Ischaemischen Muskellahmungen und Kontrakturen. Zentralbl Chir **8**: 801-803.

Whitesides TE, Jr., Haney TC, Harada H, Holmes HE and Morimoto K (1975). A simple method for tissue pressure determination. Arch Surg **110**(11): 1311-1313.

APPENDICES

APPENDIX I

SURGICAL APPROACHES TO LIMB COMPARTMENT SYNDROME

APPENDIX I: SURGICAL APPROACHES TO LIMB COMPARTMENT SYNDROME

There is only one gold standard surgical option for the treatment of compartment syndrome: fasciotomy. The ultimate goal of the procedure is salvage of a functional extremity; this must be kept in mind by the surgeon when deciding on the fasciotomy technique to be employed.

The surgical techniques for complete limb fascial release have been well studied in both the leg and the forearm. In the leg, three techniques are most commonly described: two-incision fasciotomy, single incision perifibular fasciotomy, and fibulectomy. In the forearm, two techniques are most commonly described: dorsal approach and volar approach.

I.1 FASCIOTOMY IN THE LEG

Most surgeons prefer the double incision technique, which allows for adequate visualization of all compartments, assessment of muscle viability, and sufficient surgical control to avoid neurovascular structures. The single incision four-compartment fasciotomy without fibulectomy is safe and can be useful in cases where soft tissue trauma or contamination is of concern, including situations in which only a single vessel perfuses the leg, or when flap coverage may be necessary.

Kelly and Whitesides (1967) described a four-compartment release with fibulectomy performed through one lateral incision (Kelly and Whitesides 1967). The technique takes advantage of the fascial anatomy, since all the fascial membranes insert onto the fibula. However, this method is technically challenging, may place the peroneal vessels at risk, and sacrifices the fibula (which is usually unnecessary). Both the double and single incision techniques are sufficiently effective at decreasing intracompartmental pressure (ICP) (Mubarak and Owen 1977, Vitale, Richardson et al. 1988).

Subcutaneous fasciotomy is a technique in which the fascia is incised blindly with dissecting scissors through a small skin incision (Hutchinson and Ireland 1994). Advantages include technical ease and cosmesis; however, access is limited to the deep posterior compartment and the neurovascular bundle. In addition, it is now recognized that intact skin may not allow for complete release of ICP.

In acute compartment syndrome, the skin is an important boundary of all compartments that must be released to achieve the greatest decrease in ICP. While small incision fasciotomy and endoscopically-assisted fasciotomy may have a role in chronic exertional compartment syndrome, these techniques should not be used in *acute* compartment syndrome: the recurrence of limb-threatening ischemia may occur despite fascial release when the skin is left intact (Illig, Ouriel et al. 1998, Leversedge, Casey et al. 2002, Hutchinson, Bederka et al. 2003, Apaydin, Basarir et al. 2008).

I.1.1 Surgical Technique: Single-Incision Fasciotomy

Single-incision fasciotomy was described in detail by Davey et al (Davey, Rorabeck et al. 1984). The operative technique starts with the patient positioned supine with a bump under the hip, tourniquet applied but not insufflated. The limb is prepped and draped free. The surgery begins with a single longitudinal, lateral incision in line with the fibula (Figure I.1). The incision extends from the fibular head to 3-4 cm proximal to the lateral malleolus, taking care to minimize the risk of injuring the superficial peroneal nerve toward the distal aspect of the incision. Skin flaps are developed anterior, and a longitudinal fasciotomy of the anterior and lateral compartments is performed with dissecting scissors.

Next, a posterior flap is developed and a fasciotomy of the superficial posterior compartment is performed. The surgeon should identify the interval between and superficial and lateral compartments distally, and develop this interval proximally by detaching the soleus from the fibula. Flexor hallucis longus should be dissected subperiosteally from the fibula; the muscle and the peroneal vessels are retracted posteriorly. At this point, all four compartments would have been decompressed. However, on occasion tibialis posterior exists within a self-contained fascial envelope and, therefore, it is beneficial to continue the deep dissection until tibialis posterior is decompressed. The muscle and the peroneal vessels should be retracted posteriorly; the fascial attachment of the tibialis posterior muscle to the fibula should be identified and this fascia then incised longitudinally. At the conclusion of the surgery, wounds are packed open, or the skin may be loosely closed over suction drains.

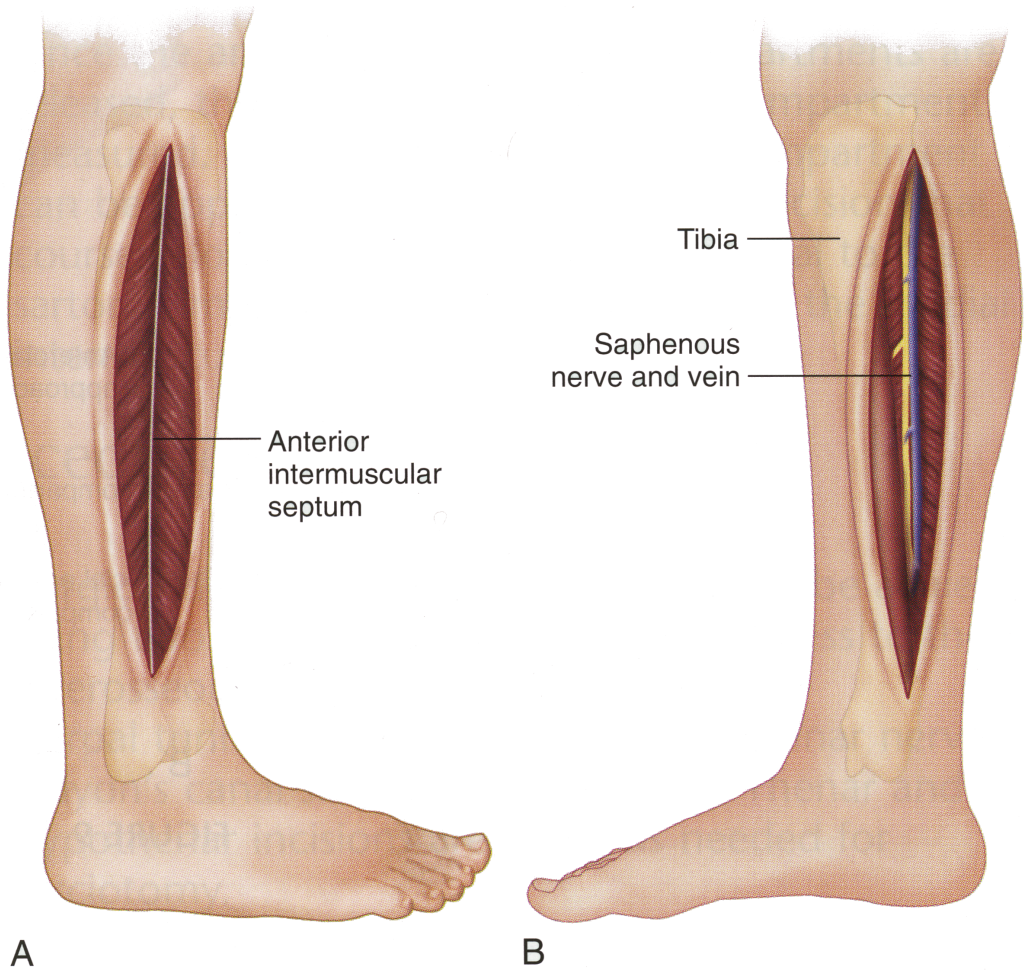

Figure I.1. **Single-incision fasciotomy**. (**A**) Lateral aspect and (**B**) medial aspect of the leg. The incision extends from the fibular head to 3 cm proximal to the lateral malleolus. Skin flaps are developed anterior, and a longitudinal fasciotomy of the anterior and lateral compartments is performed with dissecting scissors. Posterior flap is developed and a fasciotomy of the superficial posterior compartment is performed. Wounds are packed open or the skin may be loosely closed over suction drains.

Reproduced with permission from Lawendy and Sanders (2010).

I.1.2. Surgical Technique: Two-Incision Fasciotomy

Two-incision fasciotomy was described in detail by Mubarak and Hargens (Mubarak and Hargens 1981). Patient is positioned supine, tourniquet applied and not insufflated. The limb is prepped and draped free. The procedure begins with a 20-25cm incision in the anterior compartment, centered halfway between the fibular shaft and the crest of the tibia (Figure I.2), utilizing subcutaneous dissection for wide exposure of the fascial compartment. Transverse incision is made to expose the lateral intermuscular septum, and the superficial peroneal nerve lying posterior to the septum is identified. Using dissecting scissors, the anterior compartment is released proximally and distally in line with the tibialis anterior. The lateral compartment is accessed, and a fasciotomy of the lateral compartment is performed proximally and distally, in line with the fibular shaft.

A second longitudinal incision 2 cm posterior to the posterior margin of the tibia is made, using wide subcutaneous dissection to allow identification of the fascial planes. Skin flaps are elevated and the saphenous vein and nerve are identified and protected. The septum between the deep and superficial posterior compartments is then identified, and the fascia over the gastrocnemius-soleus complex is released over its entire length. Another fascial incision is made over the flexor digitorum longus muscle and the entire deep posterior compartment is released. As the dissection is carried proximally, if the soleus bridge extends more than halfway down the tibia, this extended origin must also be released. After release of the posterior compartment, the tibialis posterior muscle compartment is identified and released over the extent of the muscle belly if

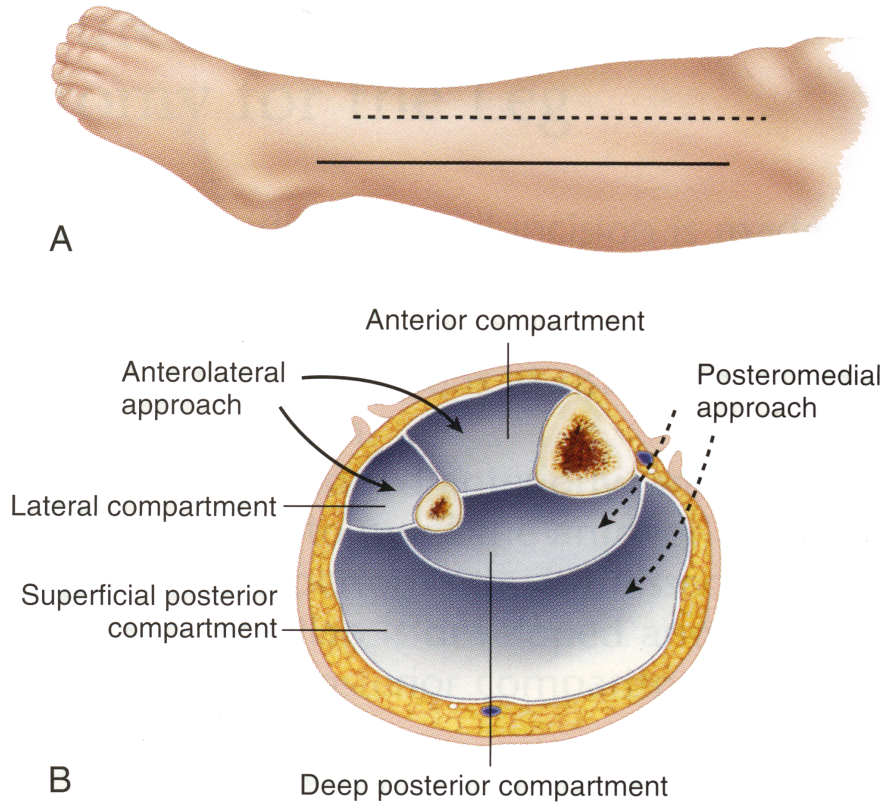

Figure I.2. **Two-incision fasciotomy**. An incision in the anterior compartment is centered halfway between the fibular shaft and the crest of the tibia. Lateral intermuscular septum is exposed. The anterior compartment is released proximally and distally in line with the tibialis anterior. Fasciotomy of the lateral compartment proximally and distally in line with the fibular shaft is performed. A second longitudinal incision 2cm posterior to the posterior margin of the tibia is made; fascia over the gastroc-soleus complex is released over its entire length. Then, the deep posterior compartment is released via fascial incision over the flexor digitorum longus.

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increased tension is evident in this compartment. The wound is packed open and a posterior plaster splint is applied, with the foot plantigrade

I.2 FASCIOTOMY IN THE FOREARM

Decompression fasciotomy of the forearm is performed through a volar approach, a dorsal approach, or both. Unlike the fascial compartments of the leg, the volar compartment, dorsal compartment, and mobile wad compartment (containing the brachioradialis and radial wrist extensors) are interconnected; thus, fasciotomies of all 3 compartments may be unnecessary.

Superficial fasciotomy is usually adequate to decompress the entire forearm (Lawendy and Sanders 2010). The flexor digitorum profundus and flexor pollicis longus muscles (deep volar compartment) are among the most severely affected muscles, due to their deep location adjacent to the radius and ulna. Pre-fasciotomy and post-fasciotomy pressures often are obtained from all compartments of the volar forearm, and if deep compartment pressures remain high after superficial fasciotomy, an additional release is indicated.

Forearm fasciotomy requires decompression from the wrist to mid arm, including the lacertus fibrosus fascia, the fascial compartments over the flexor carpi ulnaris, and the edge of the flexor superficialis muscles. With median nerve involvement, in addition to carpal tunnel release, the surgeon must explore the nerve in the proximal forearm.

The median nerve is decompressed throughout its course, including high-risk areas that are deep to the bicipital aponeurosis (lacertus fibrosus), between the humeral and ulnar heads of the pronator teres, the proximal arch, and deep fascial surface of the flexor digitorum superficialis, and the carpal tunnel. Preoperative prophylactic antibiotics against *Staphylococcus aureus* are generally recommended.

I.2.1 Surgical Technique: Volar Approach

Volar approach to the superficial and deep flexors of the forearm was first described in detail by Henry (Henry 1927). Standard surgical preparation and draping are performed, but no tourniquet should be used. A volar curvilinear incision medial to the biceps tendon, crossing the elbow flexion crease at an angle is first made (Figure I.3). The incision is carried distally into the palm to allow for a carpal tunnel release (similar to the McConnell's combined exposure of the median and ulnar nerves), avoiding crossing the wrist flexion crease at a right angle.

Lacertus fibrosus is divided proximally, evacuating any hematoma. The brachial artery is exposed to determine whether there is a normal blood flow. In case of the unsatisfactory flow, the adventitia is removed to expose any underlying clot, spasm or intimal tear, resecting the adventitia if necessary, and anastomosing or grafting the artery. The superficial volar compartment is then released throughout its length with open scissors under direct vision, freeing the fascia over the superficial compartment muscles. The flexor carpi ulnaris is

identified and retracted with its underlying ulnar neurovascular bundle medially. The flexor digitorum superificalis and median nerve are retracted laterally to expose the flexor digitorum profundus in the deep compartment. If its overlying fascia is tight, it is incised longitudinally.

The dissection is continued distally, by incising the transverse carpal ligament along the ulnar border of the palmaris longus tendon and median nerve. The median nerve is then inspected and examined, to ensure that it is not injured or entrapped between the ulnar and humeral head of the pronator teres (if it is, a partial pronator tenotomy will be necessary). In the distal forearm, if the median nerve is exposed, the distal radial-based forearm skin flap is loosely sutured over the nerve, leaving the rest of the incision open. In case that an associated fracture is present, the fracture is reduced and stabilized to obtain hemostasis.

At this point, the dorsal compartments are checked clinically, or repeated pressure measurements are made. Usually, the volar fasciotomy decompresses the dorsal musculature sufficiently; however, if the involvement of the dorsal compartments is still suspected, they are also released. A sterile moist dressing and a long arm splint are applied, taking care not to leave the elbow flexed beyond 90°.

The volar ulnar approach (similar to the Henry approach) is used to release the flexor carpi ulnaris and flexor digitorum superficialis. The proximal edge of the flexor digitorum superficialis is carefully identified, and the ulnar nerve at the wrist is decompressed.

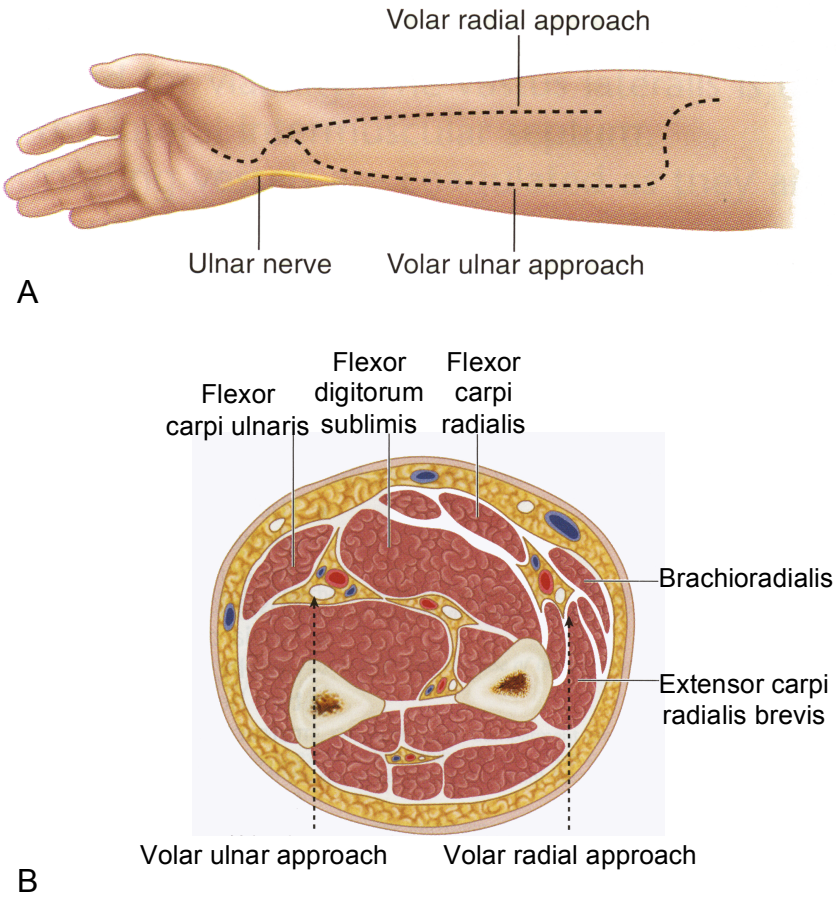

Figure I.3. **Fasciotomy of the forearm**. A volar curvilinear incision is made, releasing superficial volar compartment. The flexor digitorum superificalis and median nerve are retracted to expose the flexor digitorum profundus in the deep compartment, incising the fascia longitudinally. The dissection is continued by incising the transverse carpal ligament along the ulnar border of the palmaris longus tendon. The volar ulnar approach is used to release the flexor carpi ulnaris and flexor digitorum superficialis, decompressing the ulnar nerve at the wrist.

Reproduced with permission from Lawendy and Sanders (2010).

I.2.2 Surgical Technique: Dorsal Approach

The technique for the dorsal approach has been described by Thompson (Thompson 1918). The arm is pronated, and the incision distal to the lateral epicondyle is made between the extensor digitorum communis and extensor carpi radialis brevis, extending approximately 10 cm distally toward the midline of the wrist. The subcutaneous tissue is gently undermined, and the fascia overlying the mobile wad of Henry and the extensor retinaculum is released.

The skin is not closed at this time, anticipating secondary closure later. A sterile moist dressing and a long arm splint are applied, taking care not to leave the elbow flexed beyond 90°.

I.3. REFERENCES

Apaydin N, Basarir K, Loukas M, Tubbs RS, Uz A and Kinik H (2008). Compartmental anatomy of the superficial fibular nerve with an emphasis on fascial release operations of the leg. Surg Radiol Anat **30**(1): 47-52.

Davey JR, Rorabeck CH and Fowler PJ (1984). The tibialis posterior muscle compartment. An unrecognized cause of exertional compartment syndrome. Am J Sports Med **12**(5): 391-397.

Henry AK (1927). Complete exposure of the radius. Exposures of Long Bones and Other Surgical Methods. Bristol, England, John Wright & Sons, Ltd.**:** 9-12.

Hutchinson MR, Bederka B and Kopplin M (2003). Anatomic structures at risk during minimal-incision endoscopically assisted fascial compartment releases in the leg. Am J Sports Med **31**(5): 764-769.

Hutchinson MR and Ireland ML (1994). Common compartment syndromes in athletes. Treatment and rehabilitation. Sports Med **17**(3): 200-208.

Illig KA, Ouriel K, DeWeese JA, Shortell CK and Green RM (1998). A condemnation of subcutaneous fasciotomy. Mil Med **163**(11): 794-796.

Kelly RP, Whitesides TE (1967). Transfibular route for fasciotomy of the leg. J Bone Joint Surg Am **49A**: 1022-1024.

Lawendy AR, and Sanders D (2010). Compartment Syndrome: Evidence based surgical approaches. In Operative Techniques: Orthopaedic Trauma Surgery, Schemitsch E, ed. Elsevier/Saunders, Philadelphia, PA; pp. 679-702.

Leversedge FJ, Casey PJ, Seiler JG, 3rd and Xerogeanes JW (2002). Endoscopically assisted fasciotomy: description of technique and in vitro assessment of lower-leg compartment decompression. Am J Sports Med **30**(2): 272-278.

Mubarak SJ and Hargens AR (1981). Compartment syndromes and Volkmann's ischemic contracture. In Monographs in Clinical Orthopedics, vol 3, WB Saunders, Philadelphia.

Mubarak SJ and Owen CA (1977). Double-incision fasciotomy of the leg for decompression in compartment syndromes. J Bone Joint Surg Am **59**(2): 184-187.

Thompson, J. E. (1918). Anatomical Methods of Approach in Operations on the Long Bones of the Extremities. Ann Surg **68**(3): 309-329.

Vitale GC, Richardson JD, George SM, Jr. and Miller FB (1988). Fasciotomy for severe, blunt and penetrating trauma of the extremity. Surg Gynecol Obstet **166**(5): 397-401.

APPENDIX II

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II.1 Journal of Orthopaedic Trauma 2014; 28(11): e263-8.

II.2 Operative Techniques: Orthopaedic Trauma Surgery 2010;

Compartment Syndrome: Evidence-Based Approaches, Elsevier, pp.679-702

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s.orenigordillo@elsevier.com

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Institute/company: University of Western Ontario and London Health Sciences Centre
Address: Rm A6-152, VRL, 800 Commissioners Rd East
Post/Zip Code: N6A 4G5
City: London
State/Territory: Ontario
Country: Canada
Telephone: 519-685-8300, x. 55468

Type of Publication: Book

Book Title:Operative Techniques: Orthopaedic Trauma Surgery
Book ISBN: 9781416049357
Book Author: Lawendy AR, Sanders D
Book Year: 2010
Book Pages: 679 to 702
Book Chapter number: Section II, Procedure 39
Book Chapter title: Compartment syndrome: evidence based surgical approaches

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APPENDIX III

ANIMAL PROTOCOL APPROVAL

APPENDIX III: ANIMAL PROTOCOL APPROVAL LETTER

11.01.13
This is the original approval for this protocol
A full protocol submission will be required in 2018

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 03.31.18** 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

APPENDIX IV

HUMAN RESEARCH ETHICS BOARD APPROVAL

APPENDIX IV: HUMAN RESEARCH ETHICS BOARD APPROVAL LETTER

Use of Human Participants – Ethics Approval Notice

Principal Investigator: Dr. Abdel-Rahman Lawendy
Review Number: 17889E
Review Level: Delegated
Approved Local Adult Participants: 100
Approved Local Minor Participants: 0
Protocol Title: Identification of Serum Inflammatory Markers in Compartment Syndrome
Department & Institution: Surgery, London Health Sciences Centre
Sponsor:
Ethics Approval Date: April 29, 2011 **Expiry Date**: March 31, 2017
Documents Review & Approved &Documents Received for Information:

Document Name	Comments	Version Date
UWO Protocol		
Letter of Information & Consent		2011/01/20

This is to notify you that the University of Western Ontario Research Ethics Board for Health Sciences Research

Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic request for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[redacted]
Signature

Ethics Officer to Contact for Further Information

_____ Janice Sutherland (jsutherl@uwo.ca)	_____ Elizabeth Wambolt (ewambolt@uwo.ca)	__✓___ Grace Kelly (grace.kelly@uwo.ca)

VITA

Name: Aurelia Bihari

Post-secondary Education and Degrees:

University of Western Ontario
London, Ontario, Canada
1989 – 1992 BSc (Physiology)

University of Western Ontario
London, Ontario, Canada
1993 – 1995 Honours Diploma (Biology)

University of Western Ontario
London, Ontario, Canada
1996 – 1997 MSc (Anatomy and Cell Biology)

University of Western Ontario
London, Ontario, Canada
2013 – 2017 PhD (Medical Biophysics)

Honours and Awards:

Province of Ontario Graduate Scholarship
2015 – 2016

Queen Elizabeth II Graduate Scholarship in Science and Technology
2016 – 2017

Related Work Experience:

Research Associate
London Health Sciences Centre
1998 – 2013

Publications:

Bihari A, Cepinskas G, Forbes T, Potter R, Lawendy A (2017). Systemic application of carbon monoxide (CO), liberated from CO-releasing molecule-3 (CORM-3), protects skeletal muscle from ischemia-reperfusion injury. J Vasc Surg. pii: S0741-5214(17)30074-5. doi: 10.1016/j.jvs.2016.11.065.

Lawendy AR, **Bihari A**, Sanders D, Badhwar A, Cepinskas G (2016). Compartment syndrome causes systemic inflammation in a rat. Bone Joint J 98-B(8): 1132-7.

Bihari A, Cepinskas G, Sanders D, Lawendy A (2015). Carbon monoxide releasing molecule-3 (CORM-3) protects the skeletal muscle in porcine model of compartment syndrome. Orthopaedic Trauma Association Annual Meeting, San Diego, CA, p.172

Lawendy A, **Bihari A**, Sanders D, McGarr G, Badhwar A, Cepinskas G (2015). Contribution of inflammation to cellular injury in compartment syndrome in an experimental rodent model. Bone Joint J 97-B(4): 539-43.

Chadi S, Abdo H, **Bihari A**, Parry N, Lawendy A (2015). Hepatic microvascular changes in rat abdominal compartment syndrome. J Surg Res 197(2): 398 – 404.

Lawendy A, **Bihari A**, Sanders D, Potter R, Cepinskas G (2014). Microvascular dysfunction due to compartment syndrome is diminished by the systemic application of CO-releasing molecule-3 (CORM-3). J Orthop Trauma 28(11): e263-8.

Bihari A, Cepinskas G, Sanders D, Lawendy A (2014). CORM-3 diminishes oxidative stress and leukocyte migration in an in vitro model of compartment syndrome. Orthopaedic Trauma Association Annual Meeting, Tampa, FL, p. 172

Bihari A, Cepinskas G, Forbes T, Potter R, Lawendy A (2014). Ischemia-reperfusion injury in skeletal muscle is diminished by systemic application of CO-releasing molecule-3 (CORM-3). Society for Free Radical Research International, Kyoto, Japan, p.

Zhu JX, Kalblfeisch M, Yang YX, **Bihari A**, Lobb I, Davison M, Mok A, Cepinskas G, Lawendy AR, Sener A (2012). Detrimental effects of warm renal ischemia-reperfusion injury are abrogated by supplemental hydrogen sulphide: an analysis using real-time intravital microscopy and polymerase chain reaction. BJU Int 110(11 Pt. C): E1218-27.

Hundt H, Fleming JC, Phillips JT, Lawendy AR, Gurr K, Bailey SI, Sanders D, **Bihari A**, Gray D, Parry N, Bailey CS, Badhwar A (2011). Assessment of hepatic inflammation after spinal cord injury using intravital video microscopy. Injury 42(7): 691-6.

Lawendy A, **Bihari A**, Sanders D, Parry N, Gray D, Badhwar A (2011). Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Can J Surg 54(3): 194-200.

Katada K, **Bihari A**, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF, Cepinskas G (2010). Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. Inflammation 33(2): 92-100.

Mizuguchi S, Sephen J, **Bihari A**, Markovic N, Suehiro S, Capretta A, Potter RF, Cepinskas G (2009). CORM-3 derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. Am J Physiol Heart Circ Physiol 297(3): H920-9.

Katada K, **Bihari A**, Badhwar A, Yoshida N, Yoshikawa T, Potter RF, Cepinskas G (2009). Hindlimb ischemia/reperfusion-induced remote injury to the small intestine: role of inducible nitric oxide synthase-derived nitric oxide. J Pharmacol Exp Ther 329(3): 919-27.

Scott JR, Cukiernik MA, Ott MC, **Bihari A**, Badhwar A, Gray DK, Harris KA, Parry NG, Potter RF (2009). Low-dose inhaled carbon monoxide attenuates the remote intestinal inflammatory response elicited by hindlimb ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 296(1): G9-G14.

Cepinskas G, Katada K, **Bihari A**, Potter RF (2008). Carbon monoxide (CO) liberated from CO-releasing molecule (CORM-2) attenuates inflammation in the liver of septic mice. Am J Physiol Gastrointest Liver Physiol 294(1): G184-91.

Dungey AA, Badhwar A, **Bihari A**, Kvietys PR, Harris KA, Forbes TL, Potter RF (2006). Role of heme oxygenase in the protection afforded skeletal muscle during ischemic tolerance. Microcirculation 13(2): 71-9.

Scott JR, Gray DK, **Bihari A**, Badhwar A, Zhang X, Shan PY, Lee PJ, Chakrabarti S, Harris KA, Potter RF (2005). Heme oxygenase modulates small intestine leukocyte adhesion following hindlimb ischemia/reperfusion by regulating the expression of ICAM-1. Crit Care Med 33(11): 2563-70.

Ott MC, Scott JR, **Bihari A**, Otterbein LE, Gray DK, Harris KA, Potter RF (2005). Inhalation of carbon monoxide prevents liver injury and inflammation following hindlimb ischemia/reperfusion. FASEB J. 2005 Jan; 19(1): 106-108.

McCarter SD, Badhwar A, Scott JR, Akyea TG, **Bihari A**, Dungey AA, Harris KA, Potter RF (2004). Remote liver injury is attenuated by adenovirus-mediated gene transfer of heme oxygenase-1 during the systemic inflammatory response syndrome. Microcirculation 11(7): 587-95.

McCarter SD, Akyea TG, Lu X, **Bihari A**, Scott JR, Badhwar A, Dungey AA, Harris KA, Feng Q, Potter RF (2004). Endogenous heme oxygenase induction is a critical mechanism attenuating apoptosis and restoring microvascular perfusion following limb ischemia/reperfusion. Surgery 136(1): 67-75.

Wunder C, Scott JR, Lush CW, Brock RW, **Bihari A**, Harris K, Eichelbrönner O, Potter RF (2004). Heme oxygenase modulates hepatic leukocyte sequestration via changes in sinusoidal tone in systemic inflammation in mice. Microvasc Res 68(1): 20-9.

Badhwar A, **Bihari A**, Dungey AA, Scott JR, Albion CD, Forbes TL, Harris KA, Potter RF (2004). Protective mechanisms during ischemic tolerance in skeletal muscle. Free Radic Biol Med 36(3): 371-9.

McCarter SD, Scott JR, Lee PJ, Zhang X, Choi A, McLean CA, Badhwar A, Dungey AA, **Bihari A**, Harris KA, Potter RF (2003). Co-transfection of heme oxygenase-1 prevents the acute inflammation elicited by a second adenovirus. Gene Ther 10(19): 1629 - 35.

Bihari A, Hrycyshyn AW, Brudzynski SM (2003). Role of the mesolimbic cholinergic projection to the septum in the production of 22kHz alarm calls in rats. Brain Res Bull 60(3): 263-74.

Mitchell D, **Bihari A**, Sandig M, Tyml K. Endothelin-A receptor in rat skeletal muscle microvasculature. Microvasc Res. 2002 Jul; 64(1): 179-85.

Scott JA, Mehta S, Duggan M, **Bihari A**, McCormack DG (2002). Functional inhibition of constitutive nitric oxide synthase in a rat model of sepsis. Am J Respir Crit Care Med 165(10): 1426-32.

Wunder C, Brock RW, McCarter SD, **Bihari A**, Harris K, Eichelbrönner O, Potter RF (2002). Inhibition of haem oxygenase activity increases leukocyte accumulation in the liver following limb ischaemia-reperfusion in mice. J Physiol 540(Pt 3): 1013-21.

Nie RG, McCarter SD, Harris KA, Lee PJ, Zhang X, **Bihari A**, Gray D, Wunder C, Brock RW, Potter RF (2002). The role of endogenous heme oxygenase in the initiation of liver injury following limb ischemia/reperfusion. J Hepatol 36(5): 624-30.

Yu J, **Bihari A**, Lidington D, Tyml K (2000). Gap junction uncouplers attenuate arteriolar response to distal capillary stimuli. Microvasc Res 59(1): 162-8