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Effect of Carbon Monoxide-Releasing Molecule-3 on the Severity of Endothelial Dysfunction Due to Elevation of Hydrostatic Pressure in an In Vitro Model of Compartment Syndrome

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

ABSTRACT

Compartment syndrome (CS) is a surgical emergency caused by elevated pressure within a closed osseofascial compartment. It leads to microvascular dysfunction, limiting oxygen and nutrient delivery, gas exchange, resulting in cellular anoxia, muscle necrosis and cell death.

Currently, the only effective treatment is surgical fasciotomy. Recently, carbon monoxide (CO) delivered via carbon monoxide releasing molecule-3 (CORM-3) has been shown to improve microvascular perfusion and convey anti-inflammatory benefits in animal models of CS.

The contribution of elevated hydrostatic pressure (EHP) to the pathophysiology of CS was examined in an *in vitro* model of CS. We found that EHP led to increased oxidative stress, apoptosis and structural changes within the human vascular endothelial cells; application of CORM-3 diminished the magnitude of these detrimental responses. The data suggest that CORM-3 provides beneficial effects by preventing endothelial activation while preserving endothelial integrity, making CORM-3 an excellent potential adjunct pharmacological therapeutic in CS.

Keywords: *compartment syndrome, elevated hydrostatic pressure, human vascular endothelial cells, carbon monoxide, CORM-3, endothelial integrity, oxidative stress, apoptosis, inflammation.*

COAUTHORSHIP

While each of the co-authors listed below made important contributions to this work, I am the principal author who designed the projects, performed the experimental data acquisition, collection, analysis and manuscript writing.

Abdel-Rahman Lawendy, MD, PhD, FRCSC, in his role as the supervisor, critically reviewed this work and provided leadership and guidance throughout the entire process. His clinical and basic sciences knowledge underlying the pathophysiology, clinical diagnosis and treatment of compartment syndrome is second to none.

Aurelia Bihari, PhD, was the driving force behind this study. She assisted in every facet of this project, from the conception, technical support, data collection, analysis, interpretation and manuscript editing. Her knowledge, effort and persistence are only surpassed by her patience.

Akira Chung, MD, tirelessly worked in the laboratory and dedicated his time to pushing this project along, contributing to the conception, experimentation and data gathering.

Gediminas Cepinskas, DVM, PhD, provided direction and guidance on data interpretation, resources, and the critical evaluation of the project.

DEDICATION

To my parents, Bryce and Marie-France, who gave me the opportunity to pursue a career in which I am truly happy. I hope one day to provide my family with as much love, devotion and understanding as they have been able to show me.

To my siblings, Marie-Noelle, Alexandra (Simon) and Jean-Francois, for their humour, understanding and patience, and who at times struggled to put up with a brother who could be, for lack of a better word, a jerk.

To Taylor Smith, although she may never read this, who has had more of a positive influence on me than she will ever know. The most unique person I have ever met; her humour, generosity and patience are only surpassed by her kindness.

To Daniel Peluso, David & Rebecca Gurau, Michael Secter, Michael Czerwinski, Kayvan Nateghi and Danny & Jessica Mendelsohn, for being great friends and raising the bar by simply being significantly more intelligent than me.

Last and definitely least: to myself, because it's nice to be mentioned on a dedication page.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AP-1, activator protein-1

ARF, acute renal failure

BB, bisbenzimide

BR, bilirubin

BV, biliverdin

BVR, biliverdin reductase

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

CS, compartment syndrome

DMSO, dimethyl sulfoxide

EB, ethidium bromide

EDL, extensor digitorum longus

EHP, elevated hydrostatic pressure

ELISA, enzyme-linked immunosorbent assay

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

HUVECs, 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

IP-10, interferon gamma-induced protein 10

I/R, 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

MIF, macrophage migration inhibitory factor

MIP-1β, macrophage inflammatory protein 1β

NAC, N-acetyl cysteine

NADPH, nicotinamide adenine dinucleotide phosphate

NFκB, nuclear factor kappa B

NO, nitric oxide

NOS, nitric oxide synthase

NPC, non-perfused capillaries

NSAIDs, non-steroidal anti-inflammatory drugs

PAF, platelet activating factor

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

PI, propidium iodide

PI3K, phosphatidylinositol 3-kinase

PMN, polymorphonuclear leukocytes

PSGL-1, P-selectin glycoprotein ligand-1

RFU, relative fluorescence units

RLU, relative luminescence units

ROS, reactive oxygen species

sGC, soluble guanylate cyclase

TEER, trans-endothelial electrical resistance

TNF-α, tumor necrosis factor alpha

TUF, tissue ultrafiltration

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling

VCAM-1, vascular cell adhesion molecule-1

VLA-4, very late antigen-4

CHAPTER 1. <u>INTRODUCTION AND HISTORICAL REVIEW</u>

1.1 COMPARTMENT SYNDROME

Compartment syndrome (CS) is a true medical and surgical emergency, with potential devastating consequences, caused by an elevated pressure within a closed osseofascial compartment (Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen, Winquist et al. 1980, Hartsock, O'Farrell et al. 1998). The intercompartmental fascia is unyielding and as such, individual compartments have limited ability to expand; this makes them vulnerable to small increases in intracompartmental pressure (ICP) or decreases in volume.

The increase in pressure within the compartment leads to microvascular dysfunction and compromise, thereby creating an ischemic environment within the compartment. This then limits oxygen and nutrient delivery, as well as gas exchange, resulting in cellular anoxia, muscle necrosis and eventual cell death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen, Winquist et al. 1980). Interestingly, the ischemic environment occurs in the presence of patent vasculature. Acute CS can result in severe functional impairment, permanent pain, disability, limb loss, and even death. CS may occur acutely, following both high- and low-energy trauma, but can also present as a chronic intermittent condition, such as exertional compartment syndrome, which is most commonly seen in the athletic or military populations.

Various types of injuries and medical conditions have been associated with the development of acute CS such as fractures, contusions, burns, tight casts & dressings, blast injuries, gunshot wounds, crush injuries, diabetes, bleeding disorders (Hope and McQueen 2004), statin medications (Chautems, Irmay et al. 1997, Jose, Viswanathan et al. 2004), various infections (Schnall, Holtom et al. 1994) and placing patients in prolonged lithotomy positions for surgical procedures (Goldsmith and McCallum 1996, Mathews, Perry et al. 2001). CS has been described in the arm, forearm, hand, buttock, thigh, lower leg, foot, abdomen, thorax and even the orbit (Greene and Louis 1983, Bonutti and Bell 1986, Brumback 1990, Kym and Worsing 1990, Frink, Hildebrand et al. 2010).

1.1.1 Brief Historical Review of CS

In 1881, the German surgeon Richard von Volkmann first described the clinical sequelae of CS following traumatic supracondylar distal humerus fractures. He attributed the devastating clinical outcome to the interruption of arterial blood supply but did not specify the cause (von Volkmann 1881). This observation by von Volkmann was further substantiated by Leser in 1884 who, by applying a tight bandage to the limbs of animals, noted time dependent necrotic changes in the muscle, as well as venous congestion and swelling (Leser 1884). In 1906, Hildebrand drew attention to the role of nerve involvement in the pathophysiology of ischemic contractures after replicating the experimental design of Leser, and coined the term 'Volkmann's contracture' to refer to the clinical sequelae following supracondylar distal humerus fractures (Hildebrand 1906). In 1914, Murphy changed the narrative, and instead of speaking of arterial injuries and

nerve involvement as underlying causes of ischemic contractures, he drew attention to venous obstruction as the driving force behind CS. Although we now know that venous obstruction is not a major underlying contributor of CS, Murphy importantly drew attention to the idea that 1. elevated ICP was a main driving force in the pathophysiology, that 2. arterial pulses were maintained during the process and finally that 3. by splitting the underlying deep fascia, the "obstruction" could be relieved (Murphy 1914). Until this point, treatments had been aimed towards the complications of CS and ischemia, such as fibrosis and contractures (Rowlands and Lond 1905).

In 1926, through a series of elegant ischemia-reperfusion experiments using the limbs of dogs, Jepson noted that elapsed time as well as increased pressure was a direct causal factor in the pathogenesis of ischemic contractures. More importantly, he also showed that by surgically decompressing the involved compartment, the function of the limb could be restored (Jepson 1926).

The next significant contribution was likely from the work of Griffiths (1940), and although he mistakenly argued (for the better part of two decades) that arterial spasm was the root cause of the resulting ischemic contracture, his research contributed significantly to our understanding and recognition of early clinical signs and symptoms of CS, such as pain out of proportion, pain with passive extension and 'puffiness', which are still widely taught to this day (Griffiths 1940).

The next main contribution to our understanding of CS came with the bombing raids known as the London Blitz in the early 1940s. Patients with crushed extremities would be taken to hospital, and a relatively stable clinical condition would quickly deteriorate into systemic decompensation, multi-organ failure and eventual death. The

condition became known as "crush syndrome" (Bywaters, Delory et al. 1941). This highlighted the importance of ischemia-reperfusion in the pathogenesis of CS, rather than strictly speaking of venous congestion and elevated ICP.

In 1975, Matsen delivered his unified theory of CS by combining all the relevant data available to that point. The important aspects of his theory stated that CS was not restricted only to the upper extremity, that elevated pressure was a critical feature of the condition and finally that relieving the ICP via surgical fasciotomy was critical to avoid the devastating sequelae (Sheridan and Matsen 1975). The importance of Matsen's contribution cannot be overstated, as he shifted the discourse from understanding the underlying pathophysiology to better ways of diagnosing and treating acute CS.

1.1.2 Diagnosis of CS

The early identification, diagnosis and treatment of CS are critical in order to relieve ICP, prevent ongoing tissue anoxia, necrosis and optimize patient outcome, as well as prevent long-term disability. The diagnosis of CS is primarily a clinical one, which, in certain circumstances may be supplemented by direct ICP measurements. Understanding patient risk factors and the early identification of patient clinical signs and symptoms are paramount in the diagnosis and appropriate management of CS. Risk factors include male gender, age under 35, tibia fracture, high energy forearm fractures, high energy long bone fracture and comminuted fractures.

1.1.2.1 Clinical Diagnosis

There are several signs and symptoms that have traditionally been associated with acute CS. They appear in a stepwise fashion, although the timing can vary significantly from patient to patient and injury to injury (Myers 2000, Elliott and Johnstone 2003, Olson and Glasgow 2005, Shadgan, Menon et al. 2008). For this reason, the importance of serial and thorough clinical evaluations of all patients at risk of developing CS cannot be overstated; currently, this is considered the standard of care in the management of CS. The presence of symptoms should not only alert clinicians to the diagnosis of an acute CS, but also, unfortunately, likely suggests an advanced stage of disease.

The first signs and symptoms associated with CS are usually pain out of proportion to the apparent injury, and pain with passive stretch of the involved muscle compartment (Whitesides and Heckman 1996). The sensitivity and specificity of these clinical findings has been found to be between 13-19% and 97% respectively (Whitesides and Heckman 1996, Ulmer 2002). Other symptoms associated with CS include tense and painful muscle compartments, a persistent deep ache or burning pain, paresthesia or increasing analgesia requirement, with the last of these being an especially important finding in the pre-verbal pediatric population (Bae, Kadiyala et al. 2001).

1.1.2.2 Physical Examination

The first description of the 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 also added to the physical signs of CS (Cascio,

Wilckens et al. 2005). Unfortunately, some of these physical exam findings, such as paresthesia and paralysis, are considered late findings of *established* CS (McQueen, Christie et al. 1996), and often signify that irreversible vascular, muscular and neurological injury have likely already occurred (Matsen and Clawson 1975, Ulmer 2002). Although pulselessness was traditionally taught as one of the "5 Ps" in the clinical diagnosis of CS, the absence of a pulse is no longer considered a feature of CS (Manjoo, Sanders et al. 2010) and the presence of a pulse certainly does not rule out a diagnosis of CS. Failing to identify CS and obtain a timely diagnosis is the greatest cause of adverse clinical outcomes (Matsen and Clawson 1975, Rorabeck 1984, McQueen, Christie et al. 1996, Mars and Hadley 1998). In addition, missed CS is one of the most frequently argued cases in the field of medico-legal litigation, and is associated with frequent and significant judgements in the favour of the plaintiff (Bhattacharyya and Vrahas 2004).

1.1.2.3 Objective Compartment Pressure Monitoring

The direct measurement of ICP when attempting to diagnose CS, provided the proper technique is used, is a valuable tool in the clinician's armamentarium (Hargens and Ballard 1995). Various measurement techniques have been described, such as needle manometer, wick catheter, slit catheter and electronic transducer-tipped catheters (Hargens and Ballard 1995). In order to capture the peak ICP value, measurements should be taken at the level of the fracture, as well as additional sites up to 5 cm proximal and distal to the 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 CS is not missed. The electronic transducer-tipped systems have been found to be the most

accurate, as they do not rely on limb position or the height of the transducer. All direct compartment pressure measurement devices have their own specific technical steps, are user dependent, have their own advantages and disadvantages, and are not immune to false negatives (Lawendy and Sanders 2010). Although some authors have argued that all patients (especially young men) presenting with tibial diaphyseal fractures, high energy fractures of the tibial metaphysis, soft tissue injuries or in patients with an accompanied bleeding diathesis should undergo objective compartment pressure monitoring (McQueen, Gaston et al. 2000), this is currently not the usual practice. There are, however, several indications for using ICP monitors. These include unconscious patients, pediatric patients, pre-verbal and non-verbal patients, patients with equivocal signs and symptoms, patients with associated neurological injuries, or in a polytrauma scenario (Whitesides, Haney et al. 1975, Gelberman, Garfin et al. 1981, Hargens, Schmidt et al. 1981, Hargens, Akeson et al. 1989). In these cases, continuous compartment pressure monitoring may help confirm clinical findings, decrease the delay to fasciotomy and may, as a result, decrease the long-term complications of the disorder (McQueen, Christie et al. 1996).

1.1.3 Consequences of Missed CS

The early identification and diagnosis of acute CS is critical to its successful management, and will maximize the chances of a positive clinical outcome. The inability to obtain a timely diagnosis is the most common cause of adverse clinical outcomes (Matsen and Clawson 1975, Rorabeck 1984, McQueen, Christie et al. 1996, Mars and Hadley 1998). A missed or late diagnosis of acute CS can lead to serious complications,

such as muscle infarction, muscle and joint contractures, secondary joint and soft tissue deformities, limb weakness and neurologic dysfunction (Whitesides and Heckman 1996). Less common, but nonetheless important complications of missed CS include infection, gram-negative sepsis, amputation and end-organ involvement (Whitesides and Heckman 1996). The end result of missed CS is often irreversible myoneural ischemia leading to various degrees of permanent neuromuscular deficits and dysfunction.

The severity of the clinical outcome and dysfunction depends on the amount of tissue affected, and can range from mild weakness and sensory changes to severe ischemic contractures and limb dysfunction. When a sufficiently large amount of muscle tissue is involved (often combined with a weakened and compromised immune system), CS can lead to severe systemic complications such as crush syndrome, rhabdomyolysis, renal failure (secondary to myoglobinuria) and systemic shock (Sanghavi, Aneman et al. 2006, West 2007).

A missed or late diagnosis can be the result of clinical inexperience, a lack of suspicion, or a confusing clinical presentation (McQueen, Christie et al. 1996). These situations can occur when patients present with altered pain perception, altered level of consciousness, regional anesthesia, patient-controlled analgesia and nerve injury; all are known risk factors for late diagnosis (Mubarak and Wilton 1997, Harrington, Bunola et al. 2000).

1.2 THERAPEUTIC APPROACHES TO CS

The therapeutic goals of treating acute CS are to minimize chronic, long lasting injury and dysfunction of the involved limb, by accurately and efficiently diagnosing the condition and restoring the compartments microcirculatory environment. This is done in order to avoid the devastating consequences of myonecrosis, ischemic contracture and limb dysfunction. Non-operative techniques and adjuncts have been studied in various animal models and human case series with limited success. These nonoperative treatments remain unproven, making surgical decompression through fasciotomy of all involved compartments the only gold standard therapy for acute *established* CS, provided it is carried out within 6-8 hours of CS onset (Eaton and Green 1972, Matsen, Winquist et al. 1980, Rorabeck 1984, McQueen, Hajducka et al. 1996, Lawendy and Sanders 2010).

1.2.1 Fasciotomy

Fasciotomy, as a technique for the surgical treatment of patients with impeding Volkmann's contracture, was first described in 1911 by Bardenheuer (Bardenheuer 1911) although at the time, he used the term 'aponeurectomy'. Eventually, Murphy in 1914 suggested early surgical fasciotomy for the treatment of increased pressure within a fascial-enclosed space due to hemorrhage and edema in order to prevent paralysis and contractures (Murphy 1914). The concept of surgical fasciotomy, as described by Murphy, is the mainstay for the treatment of CS today. The first detailed record of the actual operative technique was provided by Benjamin (Benjamin 1957), describing the surgical approach to the forearm. Fasciotomy is urgently performed to normalize compartment pressures, in the hope of restoring normal perfusion to the affected tissues,

halting the inflammatory process and ultimately preventing the devastating clinical sequelae. After 6-8 hours, the risk of permanent tissue damage increases exponentially. Once muscle and tissue necrosis has occurred, surgical fasciotomy is contraindicated as it increases the risk of infection significantly. Adding to the complexity of this clinical presentation, one often does not know the exact time of injury or the subsequent events that have led to the patient's current state, making decision making difficult. Therefore, frequent serial clinical examinations and reassessment are extremely important.

Fasciotomy releases the involved compartment(s), allowing soft tissues to swell and expand, thus allowing for an increased compartmental volume while decreasing the ICP. Following decompression, surgical wounds are usually left open for 48-72 hours prior to skin closure, which is often accompanied by split thickness skin grafting (Lawendy and Sanders 2010).

1.2.1.1 Threshold for Decompression

Considering the significant cost of missing a CS, some authors have expanded the indications of using ICP monitoring to include all traumas and fractures with a high risk of CS (McQueen, Gaston et al. 2000). To complicate matters further, various ICP thresholds have been proposed, at which fasciotomy should be performed, although there is currently no clear consensus. Protocols have included absolute values of 30 mmHg, 40 mmHg and 45 mmHg (Mubarak, Owen et al. 1978, Matsen, Winquist et al. 1980, Schwartz, Brumback et al. 1989) while others, rather than considering the absolute ICP of a compartment, have used the difference between a compartment's ICP and the patient's diastolic pressure (ΔP), with 20 to 30 mmHg being considered an indication for

fasciotomy (Whitesides, Haney et al. 1975). Most trauma surgeons prefer using this ΔP as a cut-off measure to perform fasciotomy rather than using an absolute ICP threshold, as this becomes more useful in hypotensive trauma patients leading to a lower overall fasciotomy rate when compared to an absolute pressure threshold (Matsen, Winquist et al. 1980, McQueen, Hajducka et al. 1996). A recent study by Whitney et al (2014) looked at false positive rates of CS diagnosis based on one-time ICP measurements alone. When using a ΔP threshold of 30 and 20 mmHg they reported a false positive rate of 35% and 24% respectively (Whitney, O'Toole et al. 2014).

1.2.1.2 Fasciotomy Techniques

Surgical fasciotomy techniques have been well described for the upper and lower extremities as well as the trunk (McQueen, Gaston et al. 2000). Fasciotomies for CS of the lower leg (80% of all cases), forearm and hand are among the most commonly performed.

1.2.1.2.1 Fasciotomy in the Lower Leg

The lower leg is divided into 4 osseofascial compartments: anterior, lateral, posterior superficial and posterior deep (Gray 2000) (Figure 1.1). The anterior compartment is the most commonly affected in CS (Rorabeck and Macnab 1976). There are two commonly described techniques for the surgical decompression of the lower leg: the two-incision and single-incision, four-compartment fasciotomy.
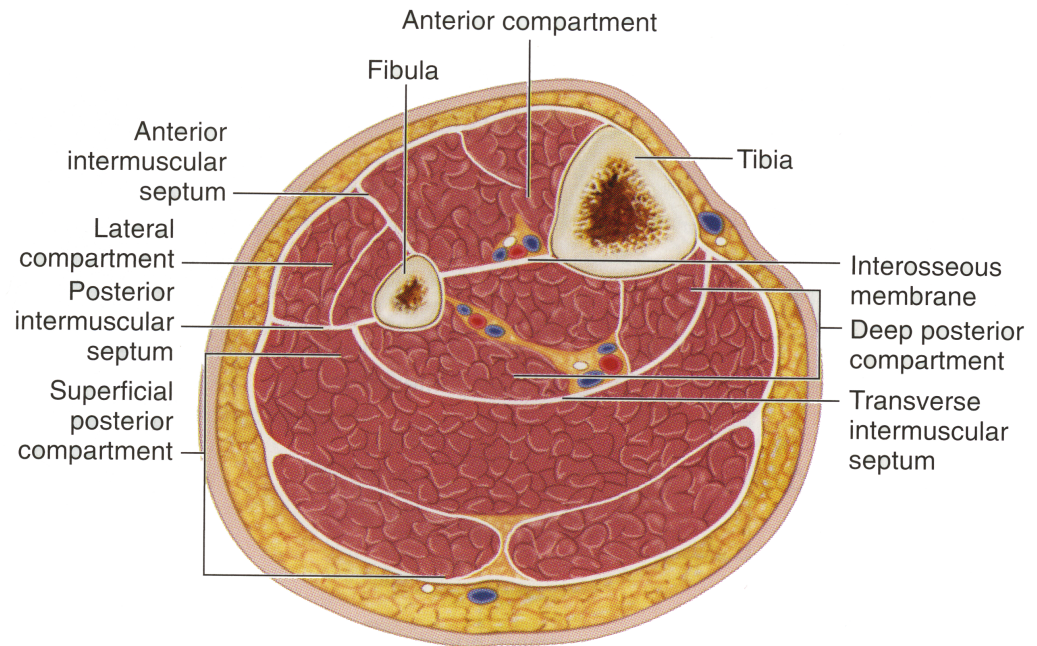
Figure 1.1.   **Anatomy and compartments of the lower leg**. The lower leg is comprised of the tibia and fibula, with interconnecting fascial planes separating the various muscles into anterior, lateral, superficial posterior and deep posterior compartments.

Reproduced with permission from Lawendy and Sanders (2010).

Some surgeons will routinely employ a two-incision (medial and anterolateral) technique, while others perform a single anterolateral approach in patients with CS in order to decompress both the anterior and lateral compartments, and then reassess the remaining compartments with ICP monitors before releasing the posterior superficial and posterior deep compartments (Tornetta, Puskas et al. 2016). Better visualization of tissue planes, neurovascular structures and ability to assess the conditions of soft tissues have been described as reasons to preferentially perform a two-incision approach (Lawendy and Sanders 2010). However, both single-incision and two-incision approaches have been shown to adequately decompress all 4 lower leg compartments (Neal, Henebry et al. 2016).

1.2.1.2.2 Fasciotomy in the Forearm

The forearm is made up of the volar compartment, the extensor compartment and the mobile wad. The flexor compartment is divided into superficial, middle and deep muscle layers. The dorsal extensor compartment contains superficial and deep layers (Gray 2000).

A curvilinear incision is made, extending from proximal and medial aspect to the elbow flexion crease, which then crosses the flexor surface of the elbow at an oblique angle. This is then followed by moving lateral to the midline, allowing for an extensile approach and the ability to release the carpal tunnel if needed. Medial and lateral flaps are elevated; the lateral antebrachial cutaneous nerve is found and protected. The lacertus fibrosis, the most proximal tether, is also released. The fascia overlying the superficial volar compartment is then incised, as well as the mobile wad laterally. The interval

between the flexor carpi ulnaris and the flexor digitorum superficialis is exploited to reveal the deep volar compartment which can then also be released (Gray 2000).

1.2.1.2.3 Fasciotomy in the Hand

The hand is divided into ten compartments; these include the thenar, hypothenar, adductor pollicis, four dorsal interossei and three volar interossei compartments (Gray 2000).

Two longitudinal incisions are centered over the index and ring fingers on the dorsum of the hand. Soft tissues are bluntly dissected on either side of the metacarpals, incising through the dorsal interossei muscle fascia. If the thenar and hypothenar compartments need to be released, two separate incisions are made on the volar radial aspect of the thumb and the volar ulnar aspect of the 5th digit, respectively. The carpal tunnel can be released through a 4cm longitudinal incision, in line with the ring finger, with the proximal extent being the flexor crease of the wrist. The transverse carpal ligament is then released under direct visualization (Kalyani, Fisher et al. 2011).

1.2.1.3 Complications of Fasciotomy

Surgical fasciotomy, although being the gold standard treatment of CS, it is not without its risks and complications. A high percentage of patients report postoperative neurologic symptoms and chronic pain associated with their surgical wounds (Fitzgerald, Gaston et al. 2000). Other complications include dry skin, pruritus, wound discolouration, swelling, tendon tethering, hypertrophic scarring, ulceration and muscle herniation (Johnson, Weaver et al. 1992, Heemskerk and Kitslaar 2003, Schmidt 2007). The risk of

infection is also not insignificant, and can create potentially devastating complications; this is directly related to the timing of the surgical intervention.

Fasciotomies which are delayed for greater than 12 hours have a 28% rate of infection, while those performed early have an infection rate of 7.3% (Williams, Luchette et al. 1997). In a retrospective study looking at a trauma patient population, Dover et al (2011) found an early post-operative complication rate of 20%. Of these, 80% experienced clinical symptoms which they rated as severe. On long-term follow-up, 70% of patients experienced persistent symptoms, which severely limited them from either an occupational or social point of view (Dover, Marafi et al. 2011, Dover, Memon et al. 2012).

Fitzgerald et al (2000) retrospectively assessed complications of fasciotomy in both upper and lower extremities over an 8-year period (Fitzgerald, Gaston et al. 2000). They found that one in every ten patients had chronic pain associated with their fasciotomy wounds and more than 20% of patients covered their scars due to the aesthetic appearance of the wound. They also found complications to be detrimental both socially and occupationally, with 28% of patients changing their hobbies and 12% having to change their occupation, secondary to the complications of their fasciotomy (Fitzgerald, Gaston et al. 2000). Another post-operative complication of surgical fasciotomies *is* CS, which has been found to occur in 3 to 20% of cases (Barr 2008), and is believed to be caused by excessive post-operative scar tissue formation and/or inadequate release of compartmental fascia (Schmidt 2007).

1.2.2 Non-Surgical Interventions

Currently, non-operative treatment modalities for CS are utilized in cases where surgical fasciotomy is contraindicated: when the affected limb is nonviable due to severe ischemia, or missed CS (Schmidt 2007). Before 1911, non-operative treatment options mainly consisted of limb mobilization and muscle stretching in order to prevent or treat ischemic contractures. Today, the most common non operative treatment is the removal of a cast or occlusive splints in a patient who presents with symptoms suggestive of CS. In these cases, if symptoms persist, fasciotomy is indicated.

The consequences of a missed CS or of delaying fasciotomy are significant, as a result, non-operative treatments have been limited to an adjunctive role to fasciotomy. It would be beneficial to develop non-surgical modalities that could prolong the treatment window between the onset of CS and the time where irreversible neurological, vascular or muscular changes occur. Potential medical treatments have been described in both animal models and human case series. These include mannitol (Better, Zinman et al. 1991), hyperbaric oxygen (Wattel, Mathieu et al. 1998), tissue ultrafiltration (Odland, Schmidt et al. 2005), anti-inflammatories (Manjoo, Sanders et al. 2010) and anti-oxidants (Kearns, Daly et al. 2004).

1.2.2.1 Mannitol

Mannitol is an osmotic diuretic, volume expander and free radical scavenger. It is commonly used to acutely reduce intracranial pressure, prevent or treat acute kidney failure secondary to crush injuries as well as treat raised intraorbital pressure. Crush

injuries associated with CS can lead to rhabdomyolysis, acidosis, acute renal failure (ARF) and even death (Bywaters, Delory et al. 1941, Better and Stein 1990).

The severity of rhabdomyolysis can be confirmed and clinically followed by measuring serum creatine kinase (CK) levels. One of the most severe complications of rhabdomyolysis is ARF, which has a mortality rate of 3-50% (Slater and Mullins 1998, Malinoski, Slater et al. 2004). One of the tenets in the management of the crush syndrome and rhabdomyolysis is aggressive fluid resuscitation, in an attempt to prevent both systemic and renal complications (Odeh 1991, Malinoski, Slater et al. 2004).

Mannitol has been shown to decrease extracellular fluid volume by promoting water and sodium excretion. It has been shown to reduce ICP in a canine model of CS (Better, Zinman et al. 1991). Daniels et al. (1998) described the case of a healthy 19–years old male presenting with heat stroke, who subsequently developed a lower leg CS, and who was treated only with mannitol. The patient was discharged 10 days after his admission to the hospital, with only "mild residual weakness" in the involved leg (Daniels, Reichman et al. 1998).

1.2.2.2 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy involves the medical use of oxygen at levels higher than the atmospheric content of 21%. Hyperbaric oxygen therapy creates a 3-fold increase in the oxygen diffusion into the tissues (Wattel, Mathieu et al. 1998). This allows continued delivery of oxygen even in the presence of ischemia. Hyperbaric oxygen has been described as either the main treatment, or as an adjunct for various medical conditions, such as decompression sickness, arterial gas embolism, smoke

inhalation, severe carbon monoxide (CO) poisoning, osteoradionecrosis, skin flap healing, clostridial myonecrosis and CS (Leach, Rees et al. 1998).

With respect to CS, hyperbaric oxygen treatment is believed to exert its beneficial effects on intracompartmental bleeding, swelling and edema by causing oxygen-induced vasoconstriction and allowing oxygen perfusion at lower perfusion pressures (as are seen in situations of CS) (Nylander, Nordstrom et al. 1987). As the interstitial edema is decreased, flow through the microcirculation is restored, or at least improved. The benefit of hyperbaric oxygen therapy has been reported in several ischemia-related clinical scenarios including traumatic ischemic lesions, ulcerations, infections and open fractures (Smith, Stevens et al. 1961, Hanson, Slack et al. 1966, Szekely, Szanto et al. 1973). Published case studies have reported success in averting fasciotomy in patients presenting with CS (Strauss, Hargens et al. 1983, Wattel, Mathieu et al. 1998, Gold, Barish et al. 2003); a recent case report by Karam et al. (2010) described the case of an NCAA football player with acute paraspinal CS following weight-lifting: he was successfully treated with forced diuresis and hyperbaric oxygen chamber treatment (Karam, Amendola et al. 2010).

However, due to the lack of definitive evidence and the need for costly and specialized equipment, hyperbaric oxygen is infrequently used and currently seen only as an adjunct to, and not a substitute for, surgical fasciotomy.

1.2.2.3 Tissue Ultrafiltration

Tissue ultrafiltration (TUF) was first described as a method of analyzing the contents of the interstitial space (Linhares and Kissinger 1992). TUF involves the

insertion of small-diameter semi-permeable hollow fibers into the tissue compartment of interest. The catheter is connected to suction, in order to filter interstitial fluid. This enables researchers to not only decompress the tissues, but also analyze the extracted fluid for biomarkers (Odland, Schmidt et al. 2005). The use of ultrafiltration in CS has been shown to lower the intramuscular pressure while maintaining perfusion pressure (Odland, Schmidt et al. 2005). In their porcine model of CS, using bovine serum albumin-enriched saline infusion into the anterior compartment of the hind limb, Odland et al. (2005) measured serum and filtrate creatinine kinase (CK) and lactate dehydrogenase (LDH) levels over a 10 hour period. They found that the biomarker levels were 80 times higher in the ultrafiltrate compared to the serum (Odland, Schmidt et al. 2005). Significantly lower pressures were recorded in experimental limbs connected to negative pressure, coupled with a markedly lessened cellular injury. The authors undertook a small human clinical trial, to test the safety and efficacy of ultrafiltration. They examined ten patients with tibial fractures treated with intramedullary nailing with and without tissue ultrafiltration, and found no difference in ICP between the two groups; however, 2 patients in the control group developed CS, while none in the ultrafiltration treatment group (Odland and Schmidt 2011).

1.2.2.4 Anti-Inflammatories

There is a significant body of evidence describing an increase in ICP as the underlying cause of microcirculatory dysfunction. However, the significant impact of inflammation and leukocyte activation in the pathophysiology of CS is increasingly being recognized (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). Activated

leukocytes directly impair perfusion, increase intravascular protein leakage, thus contributing to tissue edema, as well as causing direct parenchymal injury (Kurose, Anderson et al. 1994, Forbes, Carson et al. 1995, Forbes, Harris et al. 1996, Harris and Skalak 1996).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of medication that interfere with arachidonic acid metabolism, via inhibition of the 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). Manjoo (2010), looked at the effects of indomethacin, a selective COX-2 inhibitor, on capillary perfusion, cell damage and inflammatory activation in a rat model of CS. They found that indomethacin improved tissue perfusion and viability, decreased the number of non-perfused capillaries and significantly lowered tissue injury, lending support to the suggestion that anti-inflammatory treatments have the potential to reduce the damage in the presence of elevated ICP (Manjoo, Sanders et al. 2010).

1.2.2.5 Anti-Oxidants

Ischemia-reperfusion is known to lead to a significant release of reactive oxygen species (ROS) – extremely damaging free radicals, both locally within the tissue, as well as from the release of activated neutrophils. Thus, the resulting tissue injury is not only seen at the local level in skeletal muscle, but also in distant organ systems, such as the lungs and kidneys (Xiao, Eppihimer et al. 1997, Kearns, Kelly et al. 1999). In a study by Perler et al. (1990), the authors concluded that the most important component of CS

appeared to be the free radical-mediated reperfusion injury: an increase in free radicals (such as H_2O_2) causes direct injury to the endothelium. Furthermore, by interacting with lymphocytes, ROS further stimulate a pro-inflammatory state by increasing cytokines levels (e.g. TNF-α and IL-8) (Perler, Tohmeh et al. 1990). These, in turn, lead to the activation of neutrophils and these activated neutrophils then release ROS, which further contributes to the endothelial injury.

Due to expanding knowledge regarding the contribution of oxidative damage in CS and ischemia-reperfusion injury, various anti-oxidant therapies have been attempted, to prevent both the local and systemic injuries. These include N-acetylcysteine (NAC), taurine and vitamin C. NAC is a free radical scavenger that also restores the host cellular anti-oxidant defenses by upregulating glutathione levels in the cell (Sjodin, Nilsson et al. 1989). The primary clinical use of NAC is in acetaminophen overdose, to reduce injury to hepatocytes (Flanagan and Meredith 1991). It is also used for its nephroprotective effects in patients with kidney failure prior to administering IV contrast (Tepel, van der Giet et al. 2000), as well as to protect against oxidative injury in lung parenchyma (Bernard 1991). A study by Kearns et al. (1999) examining the effects of NAC in a rat model of CS found that CS led to decreased muscle contractility and increased tissue myeloperoxidase activity and treatment with NAC attenuated neutrophil activation and preserved muscle contractility (Kearns, Kelly et al. 1999).

Taurine (2-aminoethane sulfonic acid) is a sulphur-containing amino acid, derived from the metabolism of methionine. The major source of taurine is from a person's diet. Taurine has been implicated in the inhibition of lipid peroxidation, cell membrane stabilization, osmoregulation, as well as modulation of calcium levels (Kingston, Kelly et

al. 2004). Studies have found that exogenous administration of taurine can have protective effects against ischemia-reperfusion tissue injury in the kidney, heart, liver and skeletal muscle (Oz, Erbas et al. 1999, Wettstein and Haussinger 2000, Michalk, Hoffmann et al. 2003, Wang, Li et al. 2005). Wang et al. (2005) found that the administration of taurine reduced anterior compartment pressure, muscle edema, lactate dehydrogenase and lipid peroxidation products in a rabbit model of CS (Wang, Li et al. 2005).

Vitamin C (ascorbate) is an antioxidant that has been shown to decrease or prevent reperfusion injury in the lung and skeletal muscle, as well as to reduce oxidant production in neutrophils (Herbaczynska-Cedro, Wartanowicz et al. 1994, Lehr, Frei et al. 1995, Kearns, Kelly et al. 1999, Armour, Tyml et al. 2001, Kearns, Moneley et al. 2001). It has scavenging effects on hydrogen peroxide, which is an important component for neutrophils recruitment and adhesion (Armour, Tyml et al. 2001). Vitamin C also targets circulating neutrophils and lymphocytes (Levine, Daruwala et al. 1998). It is believed to exert its beneficial effects by reducing neutrophil recruitment and activation, as well as their extravasation into the tissues by altering the expression of adhesion molecules (e.g. ICAM-1). Vitamin C has also shown promising results in prophylactically treating complex regional pain syndrome (CRPS) or reflex sympathetic dystrophy (Zollinger, Tuinebreijer et al. 1999). While CRPS and CS are distinct pathological entities, they do share certain underlying physiological processes such as an exaggerated inflammatory response, peripheral nervous system dysfunction and an increase in circulating free radicals causing lipid membrane oxidation (Van der Laan 1997). A study by Kearns et al., 2004 using a rat cremasteric muscle model of CS, found

that pre-treatment with Vitamin C reduced intercellular adhesion molecule-1 (ICAM-1) expression and myeloperoxidase (MPO) activity as well as muscle swelling, while preserving muscle contractile function (Kearns, Daly et al. 2004). Although there is a concern that vitamin C may have pro-oxidant properties when administered at high doses for a prolonged period of time (Podmore, Griffiths et al. 1998), it has been shown to have a potent antioxidant effect without associated toxicity at doses less than 500mg per day (Bendich and Langseth 1995).

1. 3 PATHOPHYSIOLOGY OF CS

The pathophysiology underlying the onset, progression and muscle necrosis associated with CS is only partially understood. A bony or soft tissue insult, combined with an inherently rigid and unyielding fascia which prevents volume expansion leads to increased ICP, which, in turn, leads to microcirculatory dysfunction. This is followed by the activation of an inflammatory cascade and tissue edema, eventually leading to impaired gas exchange, restricted oxygen and nutrient delivery. The final common pathway is cellular anoxia, cell death and myonecrosis.

In 1881, Volkmann was the first to suggest that limb paralysis secondary to CS was due to the interruption in arterial blood supply, causing ischemia (von Volkmann 1881). He described the devastating hand deformity seen in the paediatric population following a supracondylar fracture, complicated by CS. The deformity still bears his name today. However, Volkmann was unable to describe the cause of the ischemia. Leser in 1884 expanded on this principle and confirmed, through well-designed animal

experiments, that muscle necrosis was a crucial part of the condition (Leser 1884). Hildebrand in 1906 demonstrated that nerve involvement also occurred, in addition to muscle necrosis (Hildebrand 1906). Over the last century, various theories have been offered and expanded upon to explain the pathophysiological basis of CS. These have included neurological injury (Thomas 1909), arterial injury and spasm (Griffiths 1940), venous obstruction (Murphy 1914, Brooks 1922), increased ICP and pressure-induced ischemia (Jepson 1926).

In 1940, while trying to expand on Volkmann's findings, Griffiths suggested that the paralysis and contractures seen were due to an underlying arterial injury with reflexive spasms (Griffiths 1940). The idea of arterial injury and spasm as a cause of ischemia and contractures was further supported by Watson-Jones in 1952 (Watson-Jones 1952). Foisie in 1942 believed that autonomic dysfunction mediated the arterial spasm. As a result, he suggested that autonomic sympathetic blockade could treat CS and prevent the complications (Foisie 1942). We now know that this was incorrect, on both a pathophysiological and clinical basis.

The connection between ICP and subsequent ischemia was first made by Hughes in 1948 (Hughes 1948) and in 1975, while considering the link between pressure, ischemia, muscle injury and the importance of compartmental decompression through fasciotomy, Matsen combined these relevant concepts into one unified theory of CS (Matsen 1975). Through his description, Matsen confirmed that CS could occur in any anatomical location, and was not a condition exclusive to the upper extremity. In addition, he suggested that the increase in tissue intracompartmental pressure was a critical underlying feature in the development of CS and furthermore, that surgical

fasciotomy was the only effective treatment (Matsen 1975). Whitesides (1975) then helped to define a methodology for directly measuring ICP (Whitesides, Haney et al. 1975). Initially, a threshold ICP was believed to exist above which irreversible changes and injury would occur (Heckman, Whitesides et al. 1993). Subsequently, rather than an absolute pressure threshold, others suggested that it is the difference between ICP and diastolic blood pressure that was relevant, and should be considered in the assessment of patients (Har-Shai, Silbermann et al. 1992, Heckman, Whitesides et al. 1994, Bernot, Gupta et al. 1996).

1.3.1 Ischemia

1.3.1.1 Microvascular Dysfunction

Three theories have attempted to describe the microcirculatory dysfunction and ischemia associated with increased tissue pressure seen in CS: microvascular occlusion theory, critical closing pressure theory and arterio-venous gradient theory.

The microvascular occlusion theory states that CS results from capillary occlusion caused by increased ICP. The theory postulates that increased ICP above capillary pressure leads to a reduction in the patency of capillaries and thus subsequent blood flow. This then creates an ischemic state, impairing gas exchange and nutrient delivery, leading to cellular anoxia and cell death. However, a study by Hartsock et al (1998) found that while compartment pressures could be experimentally raised well beyond the level to cause complete cessation of capillary blood flow, collapse of capillary vessels was not seen, essentially discrediting the microvascular occlusion theory (Hartsock, O'Farrell et al. 1998).

The critical closing pressure theory describes an absolute ICP above which arteriole closure occurs, caused by an elevated differential between tissue pressure and intravascular pressure (Burton and Yamada 1951). This would then lead to arteriolar collapse and tissue ischemia. The validity of this theory however was put into question by an experiment by Vollmar et al (1999) who assessed the response of arterioles, capillaries and venules to pressure elevation and found no signs of arteriolar spasm or collapse (Vollmar, Westermann et al. 1999).

Finally, the arterio-venous gradient theory states that CS is caused by increased tissue pressure, which reduces the pressure gradient from the high pressure seen in the arterial system to the low pressure on the venous side. As ICP rises, the gradient is reduced and blood flow decreases, causing cellular anoxia and tissue injury (Matsen, Winquist et al. 1980). This phenomenon also leads to pooling of venous blood, fluid extravasation, interstitial edema and swelling and causes a further rise in ICP (Matsen and Krugmire 1978). Although all three theories attempt to explain the link between raised ICP and microcirculatory dysfunction, the AV gradient theory provides the closest link, and is most easily reconciled with our current understanding of CS and microcirculatory dysfunction vis-à-vis pressure gradient changes.

1.3.1.2 Low Flow Ischemia

We now know that, rather than being due to a state of complete occlusive vascular ischemia and spasm, CS creates a microcirculatory "low flow" environment, occurring in the presence of patent arterial vessels. Under normal conditions, microvascular perfusion consists of predominantly continuously perfused capillaries (CPC). As the ICP rises,

there occurs a shift in perfusion toward intermittently perfused capillaries (IPC), and nonperfused capillaries (NPC) (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). Lawendy (2011) used intravital video microscopy (IVVM) to directly observe the microvascular perfusion changes seen in early CS. After artificially raising ICP in a rat model of CS, a decrease in the number of CPC (representing healthy perfusion) and an increase in intermittent and non-perfused capillaries was found. These changes in microvascular perfusion were accompanied by significant leukocyte activation, as well as parenchymal injury (Lawendy, Sanders et al. 2011).

Despite the presence of microvascular dysfunction, some degree of perfusion remains during CS, creating a "low flow" ischemic state, where CPCs are present in the same capillary bed as IPC and NPC. NPCs have no ability for nutrient or gas exchange, and represent a state of ischemia, unable to meet the metabolic demands of the tissue (Lawendy, Sanders et al. 2011). This creates a partial ischemic state, which triggers an early and significant inflammatory response (Gute, Ishida et al. 1998, Lum and Roebuck 2001, Schlag, Harris et al. 2001).

Heppenstall (1986) considered the ischemic process in CS in a canine model. They found that the low flow ischemic state (specifically associated with CS) caused tissue injury that was significantly greater than what was seen in a state of complete ischemia. This finding was believed to be due to the intense inflammatory reaction (Heppenstall, Scott et al. 1986). The association between partial ischemia and intense inflammatory response has been substantiated by Conrad (2005), who compared partial and complete ischemia in a murine model. Conrad's results indicated that a partial

ischemic state caused a significant early increase in pro-inflammatory mediators when compared to complete ischemia (Conrad, Stone et al. 2005).

There are important distinctions which must be drawn between CS and complete ischemia-reperfusion (I/R) injury: CS causes tissue injury and necrosis despite a patent macrocirculatory system in the face of a palpable distal pulse (Seddon 1966). In addition, the injury occurring as a result of CS is of greater magnitude compared to a complete ischemic insult of the same duration (Heppenstall, Scott et al. 1986). While our understanding of the pathophysiological basis underlying CS is not complete, microcirculatory dysfunction caused by an ongoing ischemia-reperfusion type injury, early leukocyte activation and a pro-inflammatory state appear to be the driving forces behind the generation of CS, and its potentially devastating sequelae.

1.3.2 Reperfusion and Inflammation

The greatest paradigm shift with respect to our understanding of the pathophysiology underlying CS came in 1941 during World War II. Researchers noted the systemic clinical collapse which occurred in otherwise stable patients following the revascularization of injured limbs. They noted a decrease in urine output, clinical deterioration and multi-organ failure, followed by death in certain cases. Interestingly, this occurred even when the injured limbs had been amputated (Bywaters and Beall 1941, Bywaters, Delory et al. 1941). This led to the concept of 'crush syndrome', defining the clinical entity associated with what we know today as 'reperfusion injury'.

Reperfusion injury occurs when tissues are perfused after a period of ischemia. This is accompanied by an intense inflammatory response, with both local and systemic

effects. The return of oxygen during reperfusion causes the formation of reactive oxygen species (ROS), which, along with activated neutrophils, cause the local and systemic injury seen following reperfusion.

It has been well documented, in complete ischemia-reperfusion (I/R), that increasing ischemia time leads to an increasing accumulation of activated leukocytes (particularly neutrophils) in the post-capillary venules. Neutrophils contain intracellular granules made up of various proteases and myeloperoxidase, which are very damaging to cellular and extracellular targets. Upon activation, these granules are released into the affected tissues. Thus, leukocyte activation leads to increased vascular permeability to plasma protein leakage, tissue edema, and increased interstitial pressure. An increase in interstitial pressure is believed to physically compress capillaries altering the arterial venous gradient, leading to further failure of capillaries to reperfuse upon restoration of blood flow. Correlation has been noted between the number of leukocytes in the capillaries of post-ischemic tissue and the percentage of capillaries exhibiting no-reflow (Engler, Dahlgren et al. 1986, Barroso-Aranda, Schmid-Schonbein et al. 1988, Gute, Ishida et al. 1998).

The I/R process also leads to the expression of cell surface ischemic antigens; this leads to complement activation cascade, which eventually results in the formation of the membrane attack complex (MAC). In addition, cytokines are also released, providing signals between the responding immunological cells, leading to adhesion, migration and extravasation (Ley 2008).

1.3.2.1 Reactive Oxygen Species (ROS)

ROS are small chemically reactive substances containing oxygen. These include peroxides, superoxide and hydroxyl radical. ROS are formed as a normal byproduct of the mitochondrial electron transport chain, peroxisomal fatty acid metabolism and oxygen metabolism, and play an important role in cellular signalling as well as maintenance of homeostasis (Toyokuni 1999).

During periods of ischemia, xanthine oxidase (XO) (an enzyme located in microvascular endothelial cells of skeletal muscle) is converted from its oxidized nicotinamide-adenone dinucleotide (NAD^+)-dependent dehydrogenase (XDH) state into XO (Korthuis, Granger et al. 1985, Korthuis, Grisham et al. 1988, Carden, Smith et al. 1990, Carden, Smith et al. 1991). Upon reperfusion (ie: the re-introduction of oxygen), molecular oxygen now acts as the substrate which XO converts to ROS, such as superoxide and hydroxyl radicals. The newly formed ROS will cause further tissue damage by attacking cell membrane lipids, proteins and glycosaminoglycans. Furthermore, the process will further stimulate the pro-inflammatory state, bringing leukocytes to the affected tissues.

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. During oxidative stress, when ROS overwhelm the anti-oxidant defense of the host, ROS will damage cellular membranes, and as a result, severe cellular/tissue damage ensues.

1.3.2.2 Endothelial Activation

Under normal circumstances, resting endothelial cells do not interact with leukocytes; on the contrary, they actually play a role in maintaining leukocyte quiescence (Ley, Laudanna et al. 2007). Leukocyte quiescence is due, in part, to adhesion molecules not being expressed (like E-selectin or VCAM-1), expressed at very low levels (like ICAM-1), or sequestered internally (like P-selectin). In response to reperfusion injuries, the activation of endothelial cells consists of three time-dependent stages: immediate (within minutes), acute (within hours) and chronic (within days) (Ley and Reutershan 2006). The immediate activation of endothelial cells is triggered by inflammatory chemokines, which leads to endothelial degranulation and contraction (Maier and Bulger 1996). P-selectin, which is normally stored within the cytoplasmic Weibel-Palade bodies, is delivered to the cell surface and functions to facilitate leukocyte recruitment (Weibel and Palade 1964), by interacting with the P-selectin glycoprotein ligand-1 (PSGL-1) found on leukocytes.

The acute endothelial activation is triggered by the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β); this leads to an upregulation of 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). The process appears to be reversible once the source of inflammation is resolved (Ley, Laudanna et al. 2007).

1.3.2.3 Cytokines/Chemokines

Cytokines and chemokines are a family of cell-derived secreted polypeptides that act as communication messengers between cells (Feghali and Wright 1997). They can communicate through autocrine, paracrine and/or endocrine mechanisms. Chemokines are a subset of cytokines possessing chemotactic properties. These messengers are responsible for cellular activation, communication, feedback loops and the initiation of the systemic response to inflammation. The majority of cytokines are multifunctional and, through their binding to cell surface receptors, can initiate a series of intracellular signal transduction pathways (Feghali and Wright 1997).

Cytokines can alter the expression of various transcription factors and, therefore, regulate gene transcription, further altering and modifying the production of cytokines and cell surface receptors. Their effects are varied, and include synergistic and antagonistic action, as well as exerting both negative and positive feedback regulatory loops. They provide signals between leukocytes and endothelial cells eventually leading to adhesion and transmigration of leukocytes (Gillani, Cao et al. 2012).

Acute inflammatory reactions, such as those seen in I/R injury and CS, are mediated by a number of pro-inflammatory cytokines, most notably IL-1β, TNF-α, IL-6, IL-8, thromboxane A2; these are produced in the acute phase of inflammatory response. Their upregulation stimulates downstream leukocyte activation and recruitment to the involved tissues. The end result is the effects on leukocyte activation, increased reactive oxygen species, the production and upregulation of adhesion molecules, phagocytosis and apoptosis.

1.3.2.3.1 TNF-α

TNA-α is a pro-inflammatory cytokine produced by macrophages following an appropriate stimulus such as trauma, inflammation or infection (Stein and Gordon 1991). It is one of the first cytokines released as the inflammatory process is initiated. It has multiple functions, including being a chemoattractant for surrounding neutrophils, and is involved in the upregulation of downstream cytokine production (Ascer, Gennaro et al. 1992). It binds to and exerts its effects through two transmembrane receptors, TNFR1 and TNFR2 (Banner, D'Arcy et al. 1993). Binding and subsequent activation leads to altered gene transcription and protein coupling, which has been shown to initiate programmed cell death pathways (Wallach 1997, Jiang, Wang et al. 2009). In a study by Jiang et al. looking at hind limb ischemia in TNFR1 knockout mice, by blocking the TNF-α/TNFR1 pathway, it was found that they could prevent the downstream actions of TNF-α and decrease programmed cell death (Jiang, Wang et al. 2009). In a rat model of CS, systemic levels of TNF-α, along with other cytokines such as Il-1β, GRO/KC, MCP-1, MIP-1α and IL-10 were found to increase in response to elevation of ICP (Donohoe 2015); however, unlike in complete I/R (characterized by TNF-α spike), TNF-α levels continued to increase following fascial decompression (Lawendy, Bihari et al. 2014, Lawendy, Bihari et al. 2016). This second rise in TNF-α levels is described by the authors as a second hit (Lawendy, Bihari et al. 2016) and likely due to the liberation of cellular debris, pro-inflammatory mediators and cytokines into the systemic circulation (Lawendy, Bihari et al. 2016). The second hit and washout of debris is also significant in that it suggests that CS, rather than being purely an I/R phenomenon is more consistent with a pro-inflammatory state. Their research, along with previous work on the systemic

effects of I/R, has shown that the release of TNF-α and other inflammatory cytokines in response to a local ischemic event can cause significant systemic injury (Brock, Lawlor et al. 1999, Lawlor, Brock et al. 1999, Lawendy, Bihari et al. 2016).

1.3.2.3.2 IL-1 β

IL-1β, like TNF-α, is a pro-inflammatory cytokine produced by activated macrophages, and is involved in cell proliferation, differentiation and programmed cell death (Gao, Madi et al. 2014). IL-1β has been shown to play a significant role in the I/R literature and multiple studies have shown significant increases in IL-1β levels associated with I/R events (Rothwell, Allan et al. 1997, Touzani, Boutin et al. 1999, Pomerantz, Reznikov et al. 2001, Furuichi, Wada et al. 2006, Simi, Lerouet et al. 2007). Studies utilizing IL-1β knockout mice have shown significant reduction in ischemia-induced inflammatory responses (Furuichi, Wada et al. 2006). The same authors have further lent credence to the idea that IL-1β mediates the injury seen in I/R injury from improvements seen with IL-1-targeted therapy in these experimental models (Touzani, Boutin et al. 1999, Pomerantz, Reznikov et al. 2001, Furuichi, Wada et al. 2006). A study by Kalns et al., looking at extremity CS in a pig model, in order to assess the impact of normobaric vs. hypobaric situations in relation to CS, found that levels of IL-1β, along with TNF-α, IL-6, FGF, IGF-1, IGF-BP4/BP5 and others were elevated in experimentally-induced CS (Kalns, Cox et al. 2011, Kalns, Cox et al. 2011). A study by de Franciscis (2016) assessed biomarker changes in patients with arterial occlusion and CS. All ischemic patients experienced a significant rise in levels of IL-1, IL-6, IL-8 and TNF-α, and patients

requiring a fasciotomy had higher levels of these pro-inflammatory mediators compared to the patients that did not require a fasciotomy (de Franciscis, De Caridi et al. 2016).

1.3.2.4 Leukocyte Activation

The increased ICP leads to a low flow ischemic state, which is associated with significant leukocyte activation that contributes to microcirculatory dysfunction and leukocyte accumulation in post-capillary venules (Lawendy, Bihari et al. 2015). The complex and regulated leukocyte activation cascade was first described in 1839 by Rudolph Wagner (Wagner 1839). The cascade is initialized by the release of inflammatory cytokines and chemokines, which then trigger the upregulation and differential expression of various classes of adhesion molecules such as selectins, integrins and Ig superfamily, on both the endothelium and leukocytes (Ley 2008).

The activation cascade is comprised of several highly regulated stages (Figure 1.2). The first step is the capture of the leukocyte by the activated endothelium. This process is mediated by selectins, such as L-selectin on leukocytes and P-selectin and E-selectin expressed on endothelial cells. The next step is leukocyte "rolling", whereby the leukocyte attaches itself to the surface of the endothelium. The process then progresses to the slow rolling stage, mediated by integrins. This is then followed by the firm adhesion of leukocytes to the endothelium. This adhesion is further strengthened and then followed by the clustering of integrins, which, under the appropriate stimuli, leads to leukocyte transmigration and extravasation into the interstitium (Ley, Laudanna et al. 2007).

The process of adhesion and extravasation is mediated by members of the Ig superfamily such as ICAM-1 and VCAM-1 on the endothelial surface, and PECAM-1 on

neutrophils (Albelda, Muller et al. 1991, Barreiro, Yanez-Mo et al. 2002, Yang, Froio et al. 2005). Following leukocyte extravasation into the sub-endothelial matrix and migration towards the site of injury, a positive feedback loop is created, increasing the permeability of the endothelium, leading to further intravascular protein extravasation. This manifests clinically as significant interstitial edema and swelling, which in itself will amplify the increase in ICP (Gute, Ishida et al. 1998).

Several studies have examined CS using intravital video microscopy (IVVM), a modern technique which allows for the direct visualization of microvascular perfusion (Manjoo, Sanders et al. 2010, Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2014). They have demonstrated a significant increase in activated leukocytes in the post-capillary venules of skeletal muscle. Furthermore, activated leukocytes also appear to contribute to the parenchymal injury directly, as demonstrated in neutropenic rats (Lawendy, Bihari et al. 2015).

1.3.2.5 Complement

The complement system forms part of the innate immunity which acts as a mediator between the innate and acquired immunity response pathways. The function of the complement system is to clear pathogens from the host organism (Ricklin, Hajishengallis et al. 2010). The complement cascade is made up of a series of proteins on cell surfaces and in plasma, many of which exist as precursors, and are activated at the site of inflammation. The complement system mediates a sequence of events that begins with inflammatory activation and ends with pathogen opsonisation and lysis.
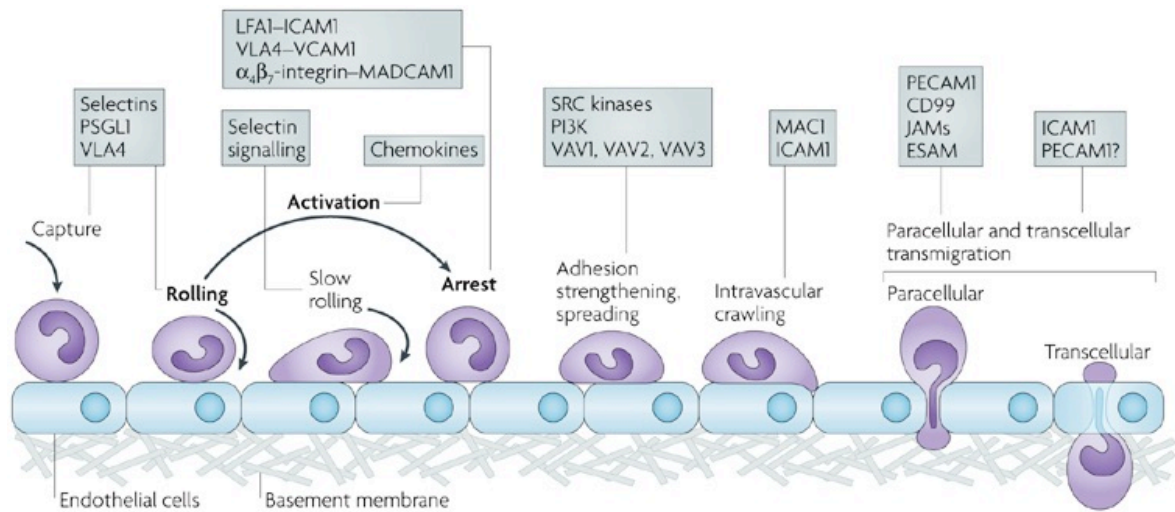
Figure 1.2. **Leukocyte activation cascade.** Leukocyte activation is an orderly process comprised of leukocyte capture, rolling, adhesion and extravasation. The individual steps are mediated by differential expression of various adhesion molecules on both the leukocyte and the endothelium.

Adapted from Ley, Laudanna et al. 2007.

The complement cascade is activated by IgG and IgM antibodies. There are three known biochemical pathways that activate the complement system: the classical pathway, the alternative pathway and the lectin pathway (Zipfel and Skerka 2009). During I/R, the presence of ischemia antigens on cell surfaces triggers binding of circulating IgM which subsequently leads to complement activation and formation of C3a/C3b. This is then followed by the formation of the membrane attack complex (MAC), mediating the local cellular injury response (Gillani, Cao et al. 2012). MAC is also involved in the inflammatory amplification through stimulation of arachidonic acid metabolism, release of prostaglandin E2, leukotriene B4, thromboxane B2, prostanoids, IL-1 and ROS (Hansch, Seitz et al. 1984, Hansch, Seitz et al. 1987, Gillani, Cao et al. 2012).

Inflammatory processes such as leukocyte activation, cytokine release and the complement cascade as well as ROS production serve a useful and protective function including cleanup of diseased/dead cells and initiate tissue repair mechanisms. Unfortunately, in *overwhelming* inflammation, as is seen in various states such as ischemia-reperfusion injury and CS, these pathways contribute to the extensive tissue and organ damage.

1.4 HEME METABOLISM AND OXIDATIVE STRESS

Oxygen is inherently toxic but living organisms have evolved the ability to utilize this gas, as well as coping mechanisms to deal with oxidative stress. Most notable of these strategies, is the presence of the heme oxygenase (HO) enzyme.

HO degrades heme, forming equimolar amounts of biliverdin, free iron and carbon monoxide (CO) (Ryter, Alam et al. 2006) (Figure 1.3). There are three separate HO isoforms: the constitutively expressed HO-2 and HO-3, and the inducible HO-1 (Maines, Trakshel et al. 1986, McCoubrey, Huang et al. 1997).

The importance of CO and the HO enzyme has been shown in case reports which detailed the death of individuals who lacked the enzyme (Yachie, Niida et al. 1999). Several studies have demonstrated that the inhibition of HO (by pharmacological or gene knockout means) was found to be a lethal mutation and detrimental to the host due to heightened sensitivity to cellular stress (Poss and Tonegawa 1997, Dungey, Badhwar et al. 2006). Conversely, the upregulation of HO (pharmacologically or through transfection with adenovirus containing HO gene construct) has been found to be protective against ischemia-reperfusion injury (Otterbein, Kolls et al. 1999, Otterbein, Lee et al. 1999, McCarter, Akyea et al. 2004, McCarter, Badhwar et al. 2004) Badhwar et al. 2004).

There are case reports which have suggested that individuals with higher expression of HO enzyme are less likely to develop diabetes, atherosclerosis, chronic obstructive pulmonary disease and arthritis (Yamada, Yamaya et al. 2000, Brydun, Watari et al. 2007, Wagener, Toonen et al. 2008, Song, Bergstrasser et al. 2009, Motterlini and Otterbein 2010). As upregulation of HO is not clinically feasible, further research has looked at the downstream byproducts of the heme degradation pathway, particularly CO, to examine its contribution to the observed protective effects.
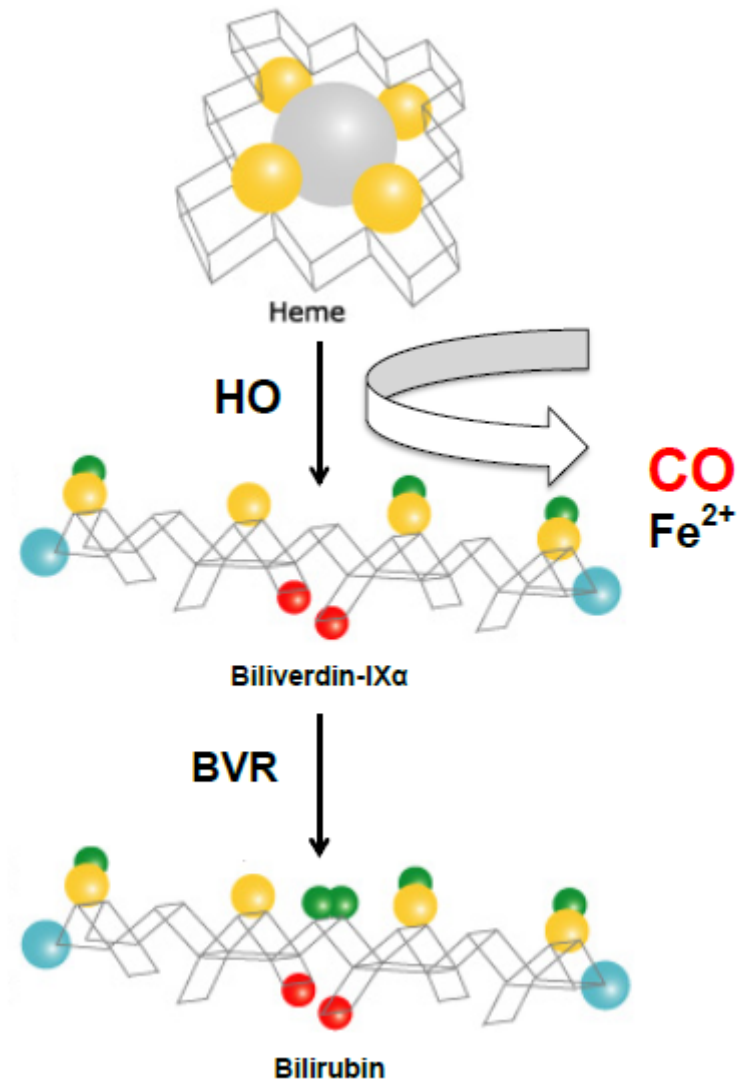
Figure 1.3. **Heme degradation pathway**. Heme, derived from hemoglobin, is broken down into biliverdin by heme oxygenase (HO). Biliverdin is then converted into bilirubin by biliverdin reductase (BVR). In this process, carbon monoxide (CO) and free iron (Fe^{2+}) are generated.

1.4.1 Carbon Monoxide

Carbon monoxide (CO) is a freely diffusible gas that traverses all cell membranes. Although considered a ubiquitous pollutant and a dangerous inhalation hazard, it is present in every mammalian cell (Coburn 1967). Small and regulated amounts of intracellular CO are continuously produced: the rate of endogenous CO production is approximately 0.42ml/hr (Coburn, Williams et al. 1967); approximately 86% of endogenous CO production comes from the metabolism of heme, while the remaining 14% is derived from lipid oxidation, xenobiotic metabolism and other metabolic processes (Vreman, Wong et al. 2000, Archakov, Karuzina et al. 2002). The endogenous production of CO has been found to increase under cellular stress arising from certain toxicological and pathological conditions (Zayasu, Sekizawa et al. 1997).

Claude Bernard, in 1857, was the first to describe the binding of CO to heme within the hemoglobin molecule, leading to the formation of carboxyhemoglobin (COHb) (Bernard 1857). This was then followed in 1912 by Douglas, demonstrating that the binding of CO to heme was reversible (Douglas, Haldane et al. 1912). Importantly, the affinity of CO for heme is approximately 240 times that of oxygen (Weaver 1999). One molecule of hemoglobin has four oxygen binding sites. When two of the binding sites are occupied by CO molecules (i.e. half saturation), the release of oxygen from the remaining binding sites is inhibited, leading to a reduction in the oxygen-carrying capacity of hemoglobin and subsequent hypoxia (Weaver 1999). By exposing the body to supra-physiologic oxygen levels, such as is seen with hyperbaric oxygen therapy, oxygen can outcompete CO for the hemoglobin binding sites, and thus reverse the hypoxia seen with CO poisoning (Weaver 1999, Gorman, Drewry et al. 2003).

Under normal conditions, the majority of blood COHb comes from endogenous production, and corresponds to blood CO levels of 0.4-1% (Vreman, Wong et al. 2000). Symptoms of CO poisoning begin to manifest at around 20% COHb levels and include dizziness, drowsiness, headache, vomiting and loss of motor coordination. Prolonged exposure, and COHb levels of 50-80% will lead to respiratory difficulty, disorientation, chest pain, loss of consciousness, coma and even death (Weaver 1999).

1.4.2 Biological Effects of Carbon Monoxide

While history has focused mostly on the negative impact of CO, namely CO poisoning, CO plays an important and positive role in cellular communication. CO is an important mediator of cell signalling, and appears to possess anti-ischemic, anti-oxidant, anti-inflammatory, anti-apoptotic and vasodilatory properties (Kim, Ryter et al. 2006).

1.4.2.1 Cellular Signalling

There are currently a few known cellular mechanisms over which CO has particular influence. The modulation of soluble guanylate cyclase (sGC) by CO and the subsequent production of cGMP is currently the most commonly described mode of action (Ryter and Otterbein 2004). The binding of CO to the heme domain within sGC stimulates its activity and this leads to a significant increase in cGMP. Studies have shown that directly subjecting vascular smooth muscle cells to CO or hypoxia (via an increase in HO-1 and subsequently increased CO) leads to an increase in cGMP levels (Morita, Perrella et al. 1995).

In addition to the direct CO binding sGC signaling pathway, other indirect pathways have been identified such as modulation of mitogen-activated protein kinases (MAPK) and upregulation of calcium-dependent potassium channel activity (Ryter, Otterbein et al. 2002). We do know that there is a significant interaction and cross talk between CO and other endogenously produced gases (e.g. nitric oxide, hydrogen sulfide) in the biologic systems (Kajimura, Fukuda et al. 2010), although the intricacies of the underlying mechanism and signalling pathways have yet to be elucidated.

1.4.2.2 Vasodilation

Several cellular mechanisms are believed to be involved in the vasodilatory effect of CO. CO has both "direct" (i.e. endothelial independent) effects on vascular smooth muscles cells, as seen with the modulation of sGC and subsequent increase in cGMP, as well as endothelial-dependent changes in the expression of vasoconstrictor factors (Motterlini and Otterbein 2010). CO has been shown to target vascular smooth muscle cells and is, therefore, able to have a significant vasodilatory impact, which has been reported in the cardiac, renal, pulmonic and cerebral vasculature (Sylvester and McGowan 1978, McFaul and McGrath 1987, Abraham and Kappas 2008). In addition to the role played by sGC and the subsequent increase in cGMP in vasodilatory role of CO, CO has also been shown to directly activate calcium-dependent potassium channels in the peripheral vasculature, causing vasodilatation (Wang, Wang et al. 1997). Studies which attempted to block the pathway using ryanodine, a known calcium channel blocker, were able to inhibit CO-induced vasodilatation (Jaggar, Leffler et al. 2002). In addition to the direct peripheral role of CO, there is some evidence that neural CO plays an indirect role

in vasodilatation via signalling through the autonomic nervous system (Verma, Hirsch et al. 1993).

1.4.2.3 Anti-Inflammatory Effects

CO has been shown to be associated with significant anti-inflammatory effects which have been demonstrated in both *in vitro* and *in vivo* studies. For example, in an *in vitro* model of sepsis, Otterbein (2000) stimulated macrophages with LPS which induced an increase in TNF-α and other pro-inflammatory cytokines (Otterbein, Bach et al. 2000). The exogenous administration of low-dose CO inhibited this pro-inflammatory response. Furthermore, CO also inhibited the expression of other pro-inflammatory cytokines, such as IL-1β and MIP-1β, while increasing the expression of IL-10, an anti-inflammatory cytokine.

Beneficial effects of CO in relation to systemic inflammation have also been demonstrated in *in vivo* experiments. When examining the impact of I/R-induced systemic inflammatory response syndrome (SIRS), the exogenous administration of low-dose CO was able to prevent liver and small intestine microvascular dysfunction (Ott, Scott et al. 2005, Scott, Cukiernik et al. 2009). In another study, Song et al. (2003) performed orthotopic lung transplant in rats, which showed severe intra-alveolar hemorrhage, a significant increase in inflammatory cellular infiltration and intravascular coagulation. The response, however, was significantly attenuated with the exposure to 500ppm of CO; in these experiments, CO also downregulated pro-inflammatory genes such as MIP-1α and MIF (Song, Kubo et al. 2003).

1.4.2.4 Anti-Apoptotic Effects

To date, there have been multiple *in vivo* and *in vitro* studies demonstrating the anti-apoptotic effects of CO. In an *in vitro* mouse model, TNF-α-induced apoptosis of endothelial cells and fibroblasts was reduced following exposure to CO (Petrache, Otterbein et al. 2000). As HO-1 protects endothelial cells (EC) from apoptosis, Brouard (2000) were able to demonstrate that blocking the enzyme activity of HO-1 with tin protoporphyrin (SnPPIX) leads to EC apoptosis. By exposing EC to exogenous CO, they were able to once again prevent apoptosis in the face of HO-1 inhibition. The findings demonstrated that the anti-apoptotic effects of HO-1 were mediated by CO and more specifically, by the activation of the p38 MAPK pathway (Brouard, Otterbein et al. 2000).

In vivo research, examining tissue injury following I/R, found that pre-treatment with low-dose exogenously administered CO also had anti-apoptotic effects (Ryter, Alam et al. 2006). In a study by Abe et al. (2017), the exposure of rat kidney grafts to oxidative stress in the presence and absence of high pressure CO found that CO significantly improved graft function and inhibited tubular apoptosis (Abe, Yazawa et al. 2017).

1.4.3 Carbon Monoxide-Releasing Molecules (CO-RMs)

CO gas is known to be non-reactive, not expensive and easily produced. Due to these properties, it became desirable to develop a set of compounds that could safely carry and deliver CO to biological systems and tissues, bypassing inhalation-associated COHb formation. Considering the known beneficial effects of HO and exogenous CO administration, and knowing that CO binds strongly to transition metals in organic

solvents, Motterlini et al. (2002), 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) form carbonyl complexes, such as manganese decarbonyl and tricarbonyl-dichloro-ruthenium dimer, which can release CO upon activation. CO-RMs are, therefore, capable of delivering CO to the tissues in a controlled manner, without causing a dangerous increase in COHb formation (Motterlini, Clark et al. 2002). CO-RMs can thus be considered chemical delivery vehicles for CO.

The first CO-RM to be synthesised was CORM-1 (formula $[Mn_2(CO)_{10}]$). It contains manganese at its centre, and is a rapid CO releaser (Motterlini 2007), but requires photo-activation, thereby limiting its use to *in vitro* protocols. The second CO-RM synthesized was CORM-2 (formula $[Ru(CO_3Cl_2)]$), containing a ruthenium metal dimer at its core. CORM-2 requires organic solvent (e.g. DMSO) for CO release also limiting its clinical use (Motterlini 2007). CORM-3 (formula $[Ru(CO)_3Cl(glycinate)]$) was the first water-soluble ruthenium-based carbonyl that readily and rapidly releases CO under physiological conditions (Motterlini 2007), making it an attractive compound for clinical applications. The release of CO by CO-RMs has been validated spectroscopically, utilising the myoglobin-binding assay (conversion of myoglobin into carboxymyoglobin) and the biological and physiological effects of CO-RMs have been confirmed by numerous experiments (Motterlini 2007).

1.4.4 Carbon Monoxide-Releasing Molecule-3 (CORM-3)

CORM-3 is an equimolar CO releaser under physiological conditions. CORM-3 has been shown to have beneficial vasodilatory properties in aortas (Foresti 2004),

positive ionotropic and anti-ischemic effect in cardiac and renal tissues (Musameh 2006; Sandouka, 2006), and to cause mesenteric vasodilatation in cirrhotic rats (Bolognesi 2007). Furthermore, CORM-3 has been shown to have therapeutic potential in various inflammatory conditions, such as acute liver failure induced by lipopolysaccharide (LPS) (Yan, Yang et al. 2016), postmenopausal arthritis (Ibanez, Alcaraz et al. 2012) and also reduced tissue injury, inflammatory response and TNF-α levels in a rat model of hemorrhagic stroke (Yabluchanskiy, Sawle et al. 2012). The mechanism of action of CORM-3 is unclear, but it appears to regulate the production of TNF-α, fibrinogen/fibrin, cellular infiltration, ICAM-1 expression and the activation of transcription factors (NF-κB, MAPK) (Kramkowski, Leszczynska et al. 2012). CORM-3 has also been shown to have direct bactericidal properties against bacteria such as *Pseudomonas aeruginosa* (Desmard, Davidge et al. 2009).

CO-RMs and CORM-3 have been shown to have various positive therapeutic effects. In a rodent model of CS, the administration of CORM-3 at the time of fasciotomy was associated with a decrease in leukocyte activation, systemic TNF-α release, and diminished tissue injury, while improving microvascular perfusion (Lawendy et al, 2014). In a rat model of I/R injury following kidney transplantation, pre-treatment of donor grafts with CORM-3 was shown to improve recipient survival, graft survival and decrease serum creatinine levels compared to control (Caumartin, Stephen et al. 2011). A study by Bihari et al., (2017), looking at I/R injury in a hind limb rat model, found that treatment with CORM-3 improved tissue perfusion while decreasing tissue injury and inflammatory activation (Bihari, Cepinskas et al. 2017).

1.4.5 CORM-3 in Human CS

CORM-3 has never been tested in human patients. While the results of animal studies employing CORM-3 as a therapeutic/interventional agent in various inflammatory conditions look promising, CORM-3 would have to be thoroughly examined before applying it towards human clinical pathology. The first step would be testing CORM-3 in a relevant, reliable and reproducible *in vitro* model, such as that employing human vascular endothelial cells (HUVECs).

Jaffe et al. (1973) described the process of isolating HUVECs from umbilical cords by collagenase digestion, and used electron microscopy to demonstrate their monolayered growth (Jaffe, Nachman et al. 1973). Since then, HUVECs have been used extensively to study various pathological developments, such as the inflammatory processes underlying diabetes and atherosclerosis (Onat, Brillon et al. 2011). In a study by Caumartin et al. (2011), investigators pre-treated cultured human umbilical vein endothelial cells with CORM-2 and found altered inflammatory state, coupled with reduced levels of cytokines, ROS, and pro-inflammatory transcription factors (Caumartin, Stephen et al. 2011). In addition, in an *in vitro* model of CS, incubating HUVECs with serum isolated from CS patients, Bihari et al (2017) found that application of CORM-3 significantly diminished the CS-induced HUVECs monolayer breakdown, ROS production, apoptosis, leukocyte adhesion and transmigration (Bihari, Cepinskas et al. 2017).

1.5 AIM OF THE THESIS

Significant gains have been made towards understanding the pathophysiology of CS, yet much remains to be determined. Currently, surgical fascial decompression is the only definitive treatment for CS, with current pharmacological therapies limited as adjuncts to surgery. In order to deliver effective surgical and medical therapies, the pathophysiological basis for the underlying condition and treatments needs to be better understood.

The purpose of this thesis is to further expand upon the human *in vitro* model of CS, developed by Bihari et al (2017), using elevation of hydrostatic pressure (EHP). Previous experiments have demonstrated that a significant level of microvascular injury and dysfunction develops in response to CS, as demonstrated by the loss of continuously perfused capillaries, elevated tissue injury and a significant degree of tissue inflammation. Unlike complete ischemia, however, CS appears to cause myonecrosis in the face of patent vessels, implicating leukocytes as playing a primary role in both microvascular and parenchymal injury during CS. Activated leukocytes are a major source of inflammatory cytokines; as such, systemic neutralization of these was able to diminish the severity of observed tissue injury, however it was unable to restore the proper microvascular perfusion (Donohoe 2015). This would suggest that the elevated tissue pressure itself contributes a significant amount of damage to the muscle microcirculation (i.e. inflammation is not the only driving force behind the CS pathophysiology), creating physical changes within the endothelial monolayer of muscle blood vessels that lead to microvascular compromise.

In vitro modelling of CS allows us not only to explore the underlying pathophysiology in a mechanistic manner, but also permits interventions that are currently not possible (or unethical) in humans. Thus, the ultimate goal of this thesis is to further our understanding of CS pathophysiology, and to develop a safe medical adjunct (or standalone therapy) for patients presenting with CS, avoiding (or at the least, minimizing) the potential devastating complications of this complex condition.

This thesis is organized into four chapters. The general introduction and historical review of CS, highlighting the advances in our understanding of diagnosis (both clinical and through objective monitoring), therapeutic approaches and pathophysiology (describing the microvascular dysfunction, inflammation, as well as important differences between CS and ischemia-reperfusion injury), which have been made over the past 140 years, are summarized in Chapter 1. We introduce CO and its potential beneficial role by underlining its biological effects, and the recent development of carbon monoxide-releasing molecules.

The response of human vascular endothelial cells (HUVECs) to elevated hydrostatic pressure, as an *in vitro* model of CS, is described in Chapter 2. Outcome measures included changes to monolayer structure, endothelial activation (measured by assessing leukocyte adhesion to the endothelium), production of ROS and apoptosis.

The effects of CORM-3 on the magnitude of response of HUVECs to EHP are described in Chapter 3. Relative contribution of EHP versus inflammatory mediators (cytokines) was also assessed.

Finally, conclusions, study limitations and future directions are addressed in Chapter 4 (General Discussion).

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CHAPTER 2. ELEVATED HYDROSTATIC PRESSURE ALTERS ENDOTHELIAL CELLS IN AN *IN VITRO* MODEL OF COMPARTMENT SYNDROME

2.1 INTRODUCTION

Compartment syndrome (CS) is a true medical and surgical emergency, with potential devastating consequences, caused by an elevation in pressure within a closed osseofascial compartment (Matsen, Winquist et al. 1980). The intercompartmental fascia is unyielding (Gratz 1931) and as such, individual compartments have limited ability to expand; this makes them vulnerable to small increases in intracompartmental pressure (ICP) or decreases in volume. The increase in pressure within the closed compartment leads to microvascular dysfunction and compromise, thereby creating an ischemic environment within the compartment. This limits oxygen and nutrient delivery, as well as gas exchange, resulting in cellular anoxia, muscle necrosis and eventual cell death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen 1980, Hartsock, O'Farrell et al. 1998).

The importance of various inflammatory processes is increasingly being recognized as contributing to the pathophysiology of CS (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). The role of activated leukocytes and the direct negative impact they may have on capillary perfusion, including intravascular protein leakage, leading to tissue edema and parenchymal injury is progressively being documented in the pathophysiology of CS (Kurose, Anderson et al. 1994, Forbes, Carson et al. 1995, Forbes, Harris et al. 1996, Harris and Skalak 1996).

Currently, the only effective treatment for CS is surgical fasciotomy (Olson and Glasgow 2005), with few other treatment modalities or adjuncts. Although fasciotomy is effective, it is associated with significant (and not infrequent) complications. Some adjunctive therapies have shown limited but promising potential in animal models (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2014), but their use is hindered by both the lack of a thorough understanding of the pathophysiological basis of CS injury, and the lack of human models on which to develop research protocols and treatments. The purpose of this study was to attempt to translate the recent progress which has been made in animal studies to human subjects. To accomplish this, we developed an *in vitro* model of CS using human vascular endothelial cells and elevated hydrostatic pressure.

We hypothesised that elevation of hydrostatic pressure to 30mmHg would have a detrimental effect on the HUVECs, producing significant changes in the endothelial monolayer, thus contributing to the breakdown of endothelial barrier. The ultimate and hopefully eventual goal of the study is the development of a safe pharmacologic adjunctive treatment for CS, which would reduce the morbidity and disability in patients.

2.2 MATERIALS AND METHODS

2.2.1 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).

2.2.2 Cells

2.2.2.1 HUVECs

Human vascular endothelial cells (HUVECs) were isolated from human umbilical veins by collagenase treatment, as previously described (Yoshida, Granger et al. 1992, Cepinskas, Sandig et al. 1999, Cepinskas, Savickiene et al. 2003). Briefly, under sterile conditions, an umbilical cord was washed with PBS; a three-way stopcock was inserted into the umbilical vein and secured into position. Culture medium was then flushed through the cord to remove soft tissue and blood clots, followed by an injection of collagenase and incubation at room temperature for 30 minutes. The collected fluid and medium was centrifuged, discarding the supernatant while resuspending the pellet in the fresh medium.

The HUVECs were plated on parallel-flow multichannel slides (ibidi μ-Slides VI 0.4) in M199 supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (100i.u./ml penicillin, 100μg/ml streptomycin and 0.125μg/ml amphotericin B), and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cell were grown to confluence. HUVECs at passages 1-3 were used for all of the experiments.

2.2.2.2 Neutrophils

Human neutrophils, i.e. polymorphonuclear cells (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), followed by the lysing of red blood cells with ammonium chloride solution, as previously described

(Yoshida, Granger et al. 1992, Kuhns, Long Priel et al. 2015). Isolated leukocytes were reconstituted in 0.1M phosphate buffered saline (PBS), pH 7.4, adjusting the buffer volume to achieve the concentration of 1×10^9 leukocytes/ml. PMN viability was confirmed by Trypan blue dye exclusion test. This procedure yielded a PMN population that was 95–98% viable.

2.2.3 *In vitro* Pressure Model of CS

HUVECs were subjected to varied hydrostatic pressures (0mmHg and 30mmHg) for 4 hours via a gravity-fed system, closed off with 3-way stopcocks. The system was connected to a compartment pressure monitor (Synthes, Westchester, PA) to ensure constant and uniform pressures (Figure 2.1). Both pressure groups were compared to a control which was exposed to normal room pressure. The 0mmHg (i.e. sham group) was included, to control for the experimental pressure set-up. All experimentation was performed in an incubator, at 37°C. All experiments were repeated five times, on different days, to account for any variability in ambient conditions.

2.2.4 Cellular Morphology

Morphological changes in the cell shape and integrity, as well as the integrity of intercellular junctions were qualitatively assessed through F-actin and VE-Cadherin immunostaining, respectively. Confluent cells were exposed to elevated hydrostatic pressure (EHP) for 4 hours, followed by fixation in 3.7% formalin, washing three times in 0.1M PBS, pH7.4, and solubilisation with 0.1% Triton X-100 in PBS for 15min. Cells were then subjected to a standard immunostaining protocol for either F-actin (phalloidin

staining) or VE-cadherin. Both Alexa Fluor 594 and Texas Red fluorochromes were visualized with Zeiss Axiophot epifluorescence microscope using excitation/emission wavelengths of 590/617nm, while bisbenzimide was visualized with wavelengths of 343/383nm.

2.2.4.1 F-actin Staining

Slides were incubated with 1% bovine serum albumin for 30min, followed by 6.6μM Alexa Fluor 598-labelled falloidin (F-actin stain) (Thermo Fisher Scientific, Mississauga, ON) for 1 hour. After washing with PBS three times, slides were coverslipped with fluorescence mounting medium (Vectashield, Vector Labs, Mississauga, ON).

2.2.4.2 VE-cadherin Staining

Slides were incubated in 10% normal goat serum for 1 hour. After washing with 0.1M PBS, they were incubated with a polyclonal VE-cadherin antibody (Santa Cruz Biotechnology Inc., Mississauga, ON) at 1:100 dilution for 2 hours. Slides were then washed three times in PBS and incubated with goat-anti-mouse IgG, conjugated with Texas Red, at 1:1000 dilution for 1 hour. Slides were then washed with PBS three times. To visualize cell nuclei, slides were counterstained with Hoechst 33342 dye (bisbenzimide) for 5 minutes. Following three PBS washes, cells were then coverslipped with Vectashield and visualized by fluorescence illumination.
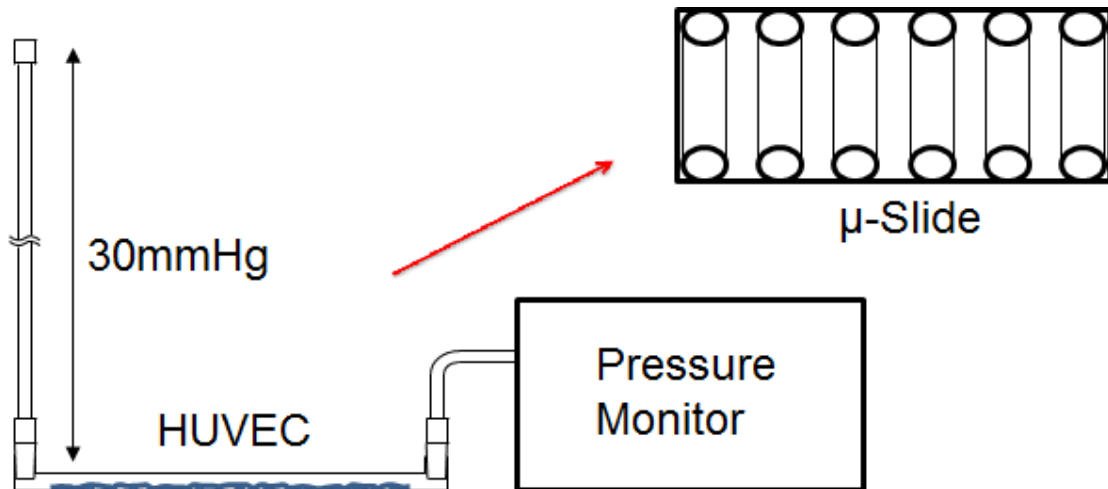
Figure 2.1 **The experimental setup for the elevation of hydrostatic pressure (EHP) in the endothelial cells as an *in vitro* model of CS.** HUVECs were plated on multichannel slides and exposed to culture medium of varied hydrostatic pressures via a gravity-fed system, with the system connected to a pressure monitor.

2.2.5 Reactive Oxygen Species (ROS) Production

The production of ROS in HUVECs was measured by intracellular oxidation of dihydrorhodamine-123 (DHR-123), a pan-oxidant-sensitive fluorochrome, as previously described (Mizuguchi, Stephen et al. 2009, Bihari, Cepinskas et al. 2017). DHR-123 is an uncharged and nonfluorescent reactive oxygen species (ROS) indicator that can passively diffuse across membranes, where it is oxidized to cationic rhodamine-123 localizing in the mitochondria and exhibiting green fluorescence. Briefly, HUVECs ($2.5x10^5$ cells), grown to confluence, were loaded with DHR-123 (10μM) for 45min. Following a wash with normal M199, the cells were then subjected to elevation of hydrostatic pressure (EHP) for 4 hours, with either 0 mmHg or 30 mmHg. Following this, the 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.

2.2.6 Quantification of Apoptosis

HUVECs were subjected to EHP of 30mmHg or 0mmHg for 4 hours, and the level of the activation of active caspases was 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, detached from the slides (0.025% Trypsin in M199), plated on Lumitrac 96-well black plates Greiner Bio-One) 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).

2.2.7 Leukocyte (PMN) Activation – Adhesion

HUVECs grown on the parallel-flow perfusion microslides (μ-slide VI 0.4; ibidi, Madison, WI) were subjected to EHP of 30mmHg or 0mmHg for 4 hours. Microslides with HUVECs were then placed into an air-heated chamber (37°C) attached to an inverted phase-contrast microscope (Diaphot 300, Nikon). Following the 10min wash with M199 at a shear stress of 1dyn/cm^2 using syringe pump (Harvard Apparatus, St. Laurent, QC), PMNs (1×10^6/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 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. Adhesion was defined as PMNs that remained stationary for at least 10s. PMN adhesion was expressed as a number of PMN/mm^2.

2.2.8 Statistical Analysis

All parameters were expressed as means ± standard error of the mean (SEM), and analyzed using one-way analysis of variance (ANOVA) (GraphPad Prism, v. 7.0, San Diego, CA), with Bonferroni post-hoc test as needed. $p < 0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Structural Changes to Endothelium Due to EHP

Exposure of HUVECs to EHP of 30mmHg produced marked changes in F-actin and VE-cadherin staining (Figure 2.2). Sustained EHP of 30mmHg led to prominent stress fiber formation, as indicated by the significant increase in phalloidin labelling (a specific marker of F-actin). Junctional VE-cadherin staining pattern was also significantly altered, indicating gap formation within the endothelial monolayer. EHP maintained at 0mmHg, however, did not result in any changes to F-actin or VE-cadherin staining, both of which remained virtually identical to those seen in control cells.

2.3.2 ROS Production

Exposure of HUVECs to EHP of 30mmHg for 4 hours induced a significant increase in the production of ROS within the endothelial cells, as shown in Figure 2.3. DHR-123 fluorescence intensity increased from 325.0±20.1 FI/mg protein in control and 340.1±38.0 FI/mg protein in 0mmHg to 556.9±71.4 FI/mg protein in 30mmHg group ($p<0.05$).

2.3.3 Apoptosis

Elevation of hydrostatic pressure to 30mmHg led to a significant increase in the activation of caspases, as shown in Figure 2.4. The caspases activation increased from 1.7±0.5 RFI in control and 1.6±0.4 RFI in the 0mmHg groups to 4.0±0.7 RFI in 30mmHg group ($p<0.05$).

0mmHg   30mmHg
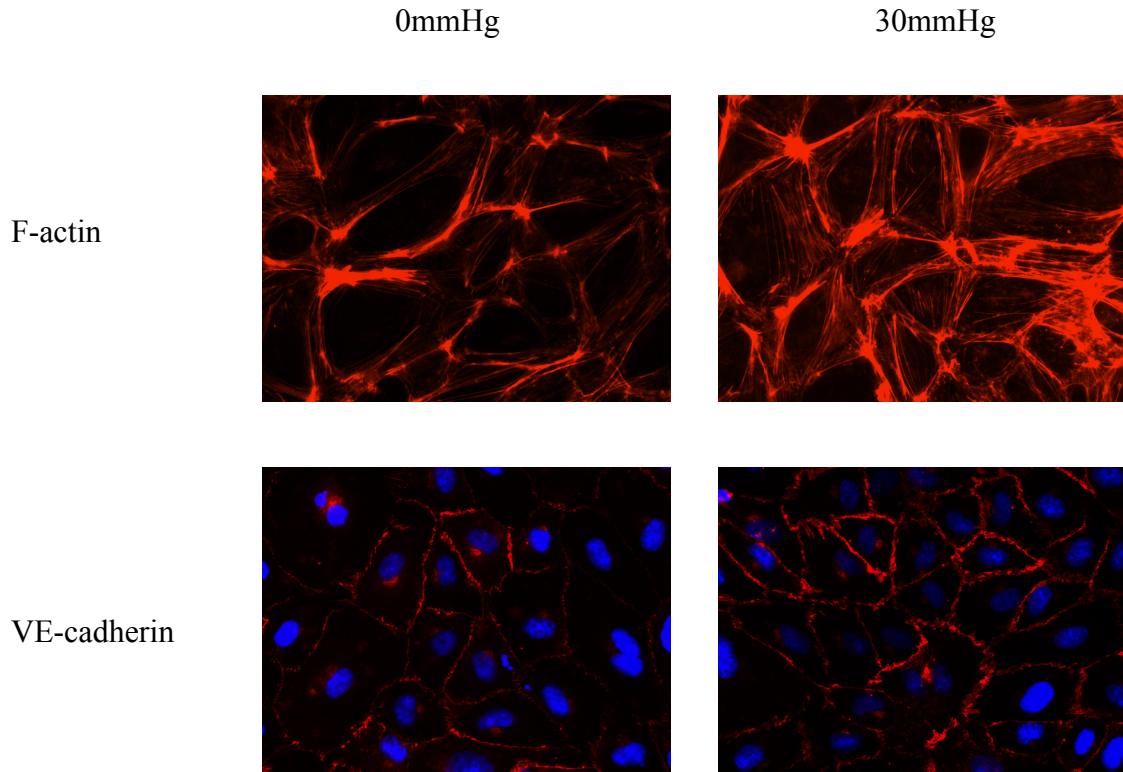
F-actin

VE-cadherin

Figure 2.2 **The effect of EHP on the expression of F-actin and VE-cadherin in an *in vitro* model of CS.** CS stimulus, in the form of a sustained EHP at 30mmHg, led to an increase in stress fiber formation and changes to junctional staining pattern of HUVECs.
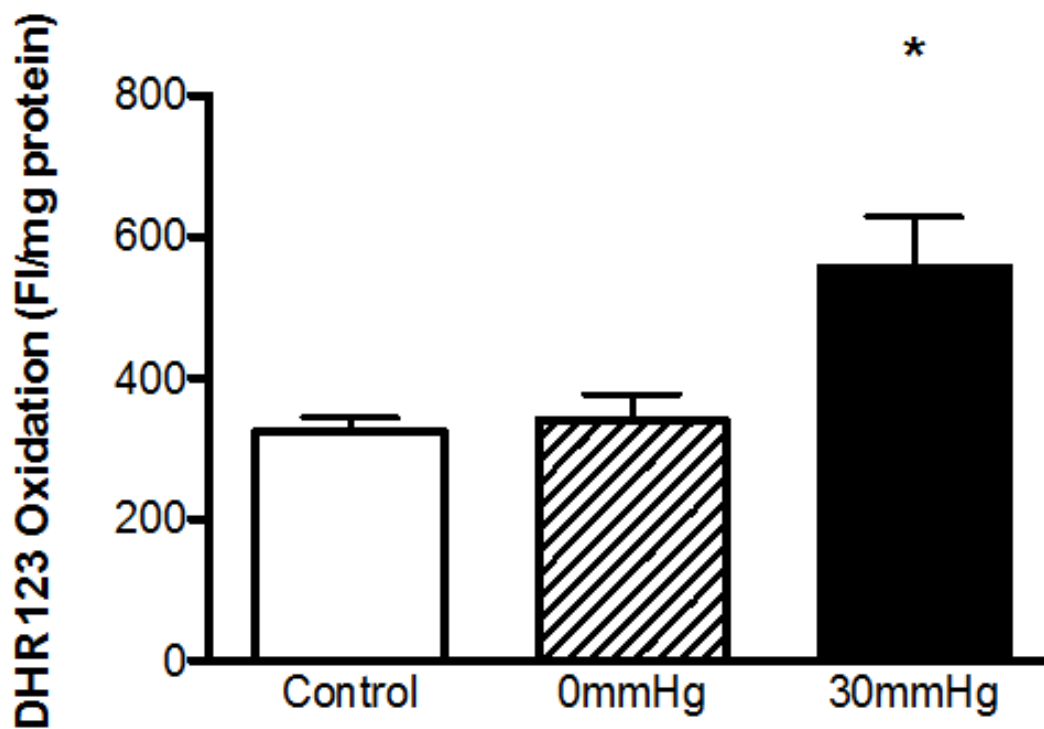
Figure 2.3 **The effect of EHP on the level of oxidative stress within the endothelial cells in an *in vitro* model of CS.** CS stimulus, in the form of sustained EHP at 30mmHg for 4 hours, led to a significant increase in reactive oxygen species formation within HUVECs. (One-way ANOVA, *$p<0.05$; N=5 per group).
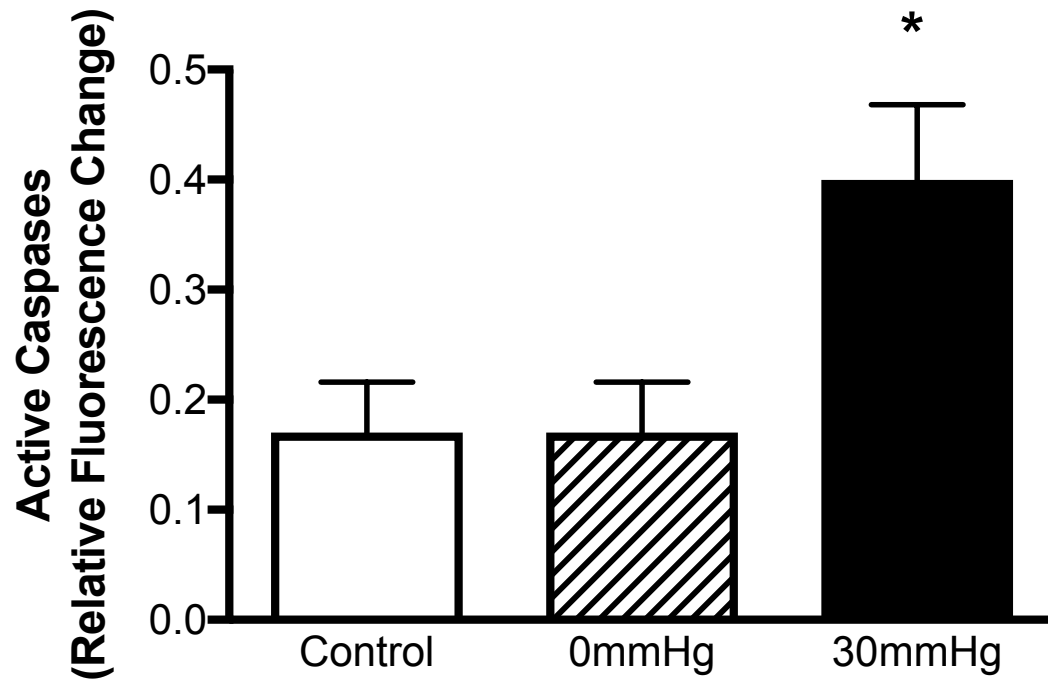
Figure 2.4   **The effect of EHP on level of apoptosis within the endothelial cells in an *in vitro* model of CS.** CS stimulus, in the form of sustained EHP at 30mmHg for 4 hours, led to a significant increase in the activity of caspases within HUVECs. (One-way ANOVA, *p<0.05; N=5 per group).

2.3.4 PMN Adhesion

Elevation of hydrostatic pressure to 30mmHg resulted in a marked increase in PMN activation, as demonstrated by an increase in PMN adhesion to HUVECs monolayer under the conditions of flow. Adhesion increased from 4.0 ± 1.5 PMNs/$0.1mm^2$ in the control and 3.8 ± 1.2 PMNs/$0.1mm^2$ in the 0mmHg groups to 12.2 ± 1.7 PMNs//$0.1mm^2$ in the EHP of 30mmHg group ($p<0.05$) (Figure 2.5).

2.4 DISCUSSION

CS is associated with a complex and often times confusing diagnostic picture, limited therapeutic options, significant complications and, unfortunately, significant patient disability (Giannoudis, Nicolopoulos et al. 2002). While certain non-operative adjuncts such as NSAIDs, tissue ultrafiltration and hyperbaric oxygen therapy have shown therapeutic potential in certain animal models and small human case series (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2014), their clinical use has been limited by a lack of understanding of the pathophysiology underlying both CS and the individual therapies as well as the lack of an effective human model on which to test therapeutic treatments.

In the present study, we attempted to mimic the CS conditions *in vitro* by subjecting *human* vascular endothelial cells to elevated hydrostatic pressure (the CS-relevant stimulus), directly assessing cellular response, while attempting to clarify the role in which EHP contributes to the endothelial activation and pro-inflammatory potential of CS. To our knowledge, this is the first study to model CS in cell culture using
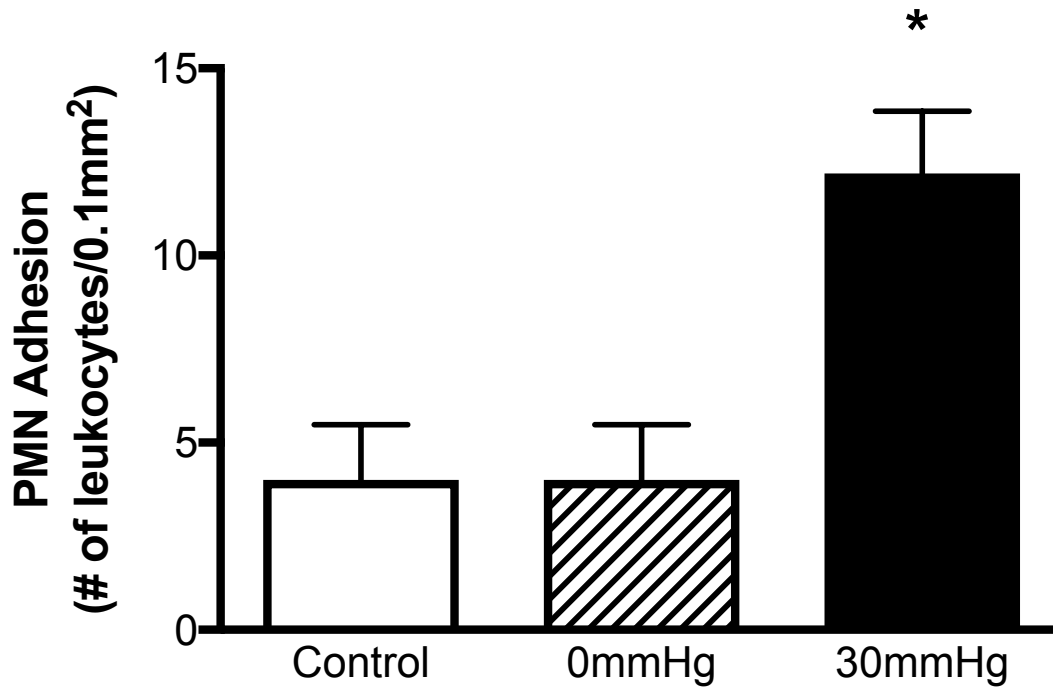
Figure 2.5. **The effect of EHP on the endothelial cell activation in an *in vitro* model of CS.** CS stimulus, in the form of sustained EHP at 30mmHg for 4 hours, led to a significant endothelial activation, as measured by an increase in PMN leukocyte adhesion to HUVECs. (One-way ANOVA, *p<0.05; N=5 per group).

EHP as a CS-relevant stimulus. The primary goal of these studies is to further our understanding of the pathophysiology leading to CS, as well as to allow for the development and testing of potential interventions currently not possible (or even unethical) in human subjects.

CS is comprised of a complex cascade of intracellular and extracellular events, eventually creating a pro-inflammatory milieu, with the subsequent activation of vascular endothelial cells and leukocytes. This leads to impaired cell-to-cell integrity, increased vascular permeability, leukocyte extravasation and the formation of interstitial edema (Sabido, Milazzo et al. 1994). The cellular and molecular basis underlying the parenchymal injury/organ dysfunction associated with CS are also poorly understood. In addition, there are no studies using human material to assess the health of vascular endothelial cells in response to stimulation with a CS-like stimulus.

In the current study, exposure of HUVECs to EHP led to a rearrangement of stress fibers and a breakdown of endothelial barrier, as evidenced by changes in F-actin and VE-cadherin expression (Figure 2.2). This is in keeping with previous research which found that EHP led to changes in endothelial cell morphology (Acevedo, Bowser et al. 1993). Furthermore, alterations in cellular structure in response to EHP are not seen to occur only in endothelial cells. In chondrocytes, EHP leads to F-actin thinning and disorganization, as well as a decrease in cell stiffness (Cao, Xia et al. 2015). Furthermore, Tokuda (2009) were able to show that the exposure of epithelial cells to EHP induced dynamic changes in cell height, actin structure and intercellular junctions (Tokuda, Miyazaki et al. 2009). Finally, Ohashi et al. (2007) found lower levels of VE-cadherin

and changes to cell structure, as well as loss of contact inhibition in bovine aortic endothelial cells exposed to EHP (Ohashi, Sugaya et al. 2007).

Under normal circumstances, the intact endothelium provides a semi-selective barrier between the vessel lumen and the interstitial tissue, controlling the passage of fluid, proteins, leukocytes and other material between the blood and the interstitium. When the endothelium is subjected to cellular stress and inflammation, it experiences an increase in permeability, eventually leading to tissue edema. This also creates non-perfused segments within the capillary system, further contributing to ischemia and microvascular dysfunction (Sabido, Milazzo et al. 1994). A study by Kataoka et al. (1998) found that subconfluent bovine aortic endothelial cells responded differently to shear stress than confluent cells, suggesting that cell shape change is dependent on cell to cell contact with neighbouring cells (Kataoka, Ujita et al. 1998). It has been postulated that changes in hydrostatic pressure may enhance the synthesis of VEGF by the endothelial cells, leading to dephosphorylation of catenins and a decrease in the formation of adherens junctions dependent on VE-cadherin (Ohashi, Sugaya et al. 2007). A study by Martin (2005) in renal cell lines subjected to pathological hydrostatic pressures found that EHP leads to shortening of actin fiber length (Martin, Brown et al. 2005). Previously, Ingber proposed the "tensegrity" model, which stipulates that different mechanical forces applied to a cell will be transmitted by actin filaments to proteins, like integrins, which anchor the cell to the extracellular matrix; these forces can cause reorganization of integrins into a different pattern, in order to resist those forces (Ingber 1993). In the context of CS, it is plausible to assume that the elevation of intracompartmental pressure results in EHP, directly producing changes in the 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), thus further contributing to the breakdown of endothelial barrier.

When endothelial cells were subjected to sustained EHP, a significant increase in intracellular production of ROS was observed (Figure 2.3). Increased ROS production is most likely due, at least in part, to changes in the mitochondrial respiratory chain. This is in agreement with previous research in deep dive physiology, suggesting that elevated hydrostatic pressure has direct impact on the mitochondrial respiratory chain or the inner mitochondrial membrane (Wang, Guerrero et al. 2015). Furthermore, it can also be surmised that an increase in ROS, as a result of EHP, will trigger downstream signalling, stimulating inflammation (Toyokuni 1999). Acute inflammatory reactions, such as those seen in ischemia-reperfusion and CS, are mediated by a number of pro-inflammatory cytokines (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). Interestingly, previous research has shown that EHP leads to an upregulation of cytokine levels in various cell types, such as periodontal ligament cells (Yamamoto, Kita et al. 2006), bladder smooth muscle cells (Liang, Xin et al. 2016), mesenchymal stem cells (Becquart, Cruel et al. 2016), human tumour cells (Fucikova, Moserova et al. 2014) and human chondrocytes (Fioravanti, Moretti et al. 2007). It has been demonstrated, using vascular endothelial cells, that an increase in intraluminal pressure leads to an induction of nuclear factor κB (NF- κB) (Lemarie, Esposito et al. 2003), a transcription factor controlling the expression of various cytokines, chemokines and adhesion molecules (De Martin, Hoeth et al. 2000). In

addition, application of cyclic hydrostatic pressure to HUVECs found an increase in CD95, a member of the TNF receptor family that is known to induce apoptosis, as well as elicit cytokine release (Hasel, Durr et al. 2005).

In addition to activation of the pro-inflammatory pathways, ROS also play a critical role in activation of various caspases (e.g. caspase-3), cytosolic enzymes responsible for the induction of cell apoptosis (Elmore 2007). In the current study, EHP resulted in an increase in endothelial apoptosis (Figure 2.4). This is consistent with the observations of Ju et al. (2009), who found that an exposure of cultured retinal ganglion cells to EHP of 30mmHg led to mitochondrial fissuring and increased release of cytochrome c into the cytoplasm, thus activating caspase-3 (Ju, Kim et al. 2009). Additionally, Lee et al. (2010) also found an increase in intracellular calcium elevation followed by elevated caspase-3/7 activation, suggesting that calcium, through its impact on ion channel activity and water efflux, is responsible for signalling the onset of the apoptotic cell changes (Lee, Lu et al. 2010). 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, in response to EHP, both intrinsic (mitochondria/cytochrome c-mediated) and extrinsic (TNF-α receptor-mediated) apoptotic pathways would be activated (Elmore 2007).

One of the essential features of CS appears to be the production of pro-inflammatory mediators, and subsequent infiltration of activated leukocytes, particularly, neutrophils, into the affected tissue (Sadasivan, Carden et al. 1997, Lawendy, Sanders et al. 2011). Leukocyte activation, recruitment and accumulation at the site of injury are

normal host responses intended to assist with pathogen and dead cell removal. However, in pathological situations, response to cellular stress, the accumulation of PMNs and subsequent production of cytotoxic ROS, coupled with a release of proteolytic enzymes can overwhelm the system, thus contribute to the parenchymal damage. Activated leukocytes, and the pro-inflammatory state they create will impair the viability of surrounding cells (Toyokuni 1999), further contributing to tissue injury and organ dysfunction.

Recent research indicates that the pathophysiology underlying CS is, at least in part, driven by ischemia-reperfusion injury. The cellular and clinical effects of complete ischemia on the skeletal muscle are well documented (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 creates a shift in cellular metabolism towards a mainly oxidative mode, while restoration of previously ischemic tissue will produce a pro-inflammatory state (Gute, Ishida et al. 1998, Gillani, Cao et al. 2012), characterized by an influx of activated leukocytes from the circulation into the surrounding tissues (Hernandez, Grisham et al. 1987, Kubes, Suzuki et al. 1990, Schlag, Harris et al. 2001). PMN recruitment involves a series of complex and well-coordinated cell-to-cell interactions by differential expression of various adhesion molecules on both the endothelium and the leukocyte; these include selectin-mediated PMN rolling, integrin-mediated firm adhesion, followed by PECAM-1-, CD99- and JAM-mediated transmigration across the endothelial barrier into the interstitial space (Ley, Laudanna et al. 2007). Furthermore, activated leukocytes themselves are also a significant source of ROS and proteolytic enzymes, contributing to interstitial 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, the fluid accumulation leads to a significant increase in the interstitial pressure, further compressing the neighbouring capillaries and exacerbating the perfusion deficit (Lawendy, Sanders et al. 2011).

In the current study, EHP applied to endothelial cells led to a significant increase in leukocyte adhesion (Figure 2.5), a key feature of leukocyte and/or vascular endothelial cell inflammatory activation (Butcher 1991, Ley, Laudanna et al. 2007). These results suggest that EHP not only induces ROS production, apoptosis and cytokine release, but it also has the potential to induce endothelial cell activation directly. It is plausible to assume that changes in hydrostatic pressure will cause changes in the expression of adhesion molecules on the vascular endothelium (e.g. P-selectin, E-selectin, ICAM-1, VCAM-1), allowing them to interact with their ligands on the PMNs (e.g. L-selectin, sialyl-LewisX, β2 integrins).

In summary, this is the first study demonstrating the impact of increased hydrostatic pressure on the human vascular endothelial cells in an *in vitro* model of CS. It represents the first step in an attempt to further elaborate to mechanisms driving the pathophysiology of CS.

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CHAPTER 3.   CARBON MONOXIDE-RELEASING MOLECULE-3 (CORM-3) IMPROVES ENDOTHELIAL CELL DYSFUNCTION IN AN *IN VITRO* MODEL OF COMPARTMENT SYNDROME

3.1 INTRODUCTION

Compartment syndrome (CS) is a devastating complication of musculoskeletal trauma. CS occurs following a rise in intra-compartmental pressure within a closed osseofascial compartment. If unrecognized, and/or untreated, the condition will lead to a muscle-threatening and limb-threatening ischemia (Whitesides, Haney et al. 1975, Matsen, Winquist et al. 1980, Mubarak and Hargens 1983). Currently, the only definitive treatment is surgical fasciotomy, with the goal of fully decompressing all of the affected compartments (Eaton and Green 1972, Rorabeck 1984). Fasciotomy must be carried out within 6-8 hours of CS onset, or the resulting tissue damage, neurovascular injury and limb ischemia become permanent.

The increase in compartmental pressure seen in CS leads to microcirculatory compromise restricting oxygen and nutrient delivery to the tissues, thus leading to cellular anoxia, tissue necrosis and limb dysfunction (Whitesides, Haney et al. 1975, Hargens, Schmidt et al. 1981). However, unlike situations of complete ischemia, CS causes tissue necrosis in the presence of patent vessels. Although the full pathophysiology of CS has not been fully elucidated, the contribution of inflammation is increasingly being recognized as one of the important driving forces (Lawendy, Sanders et al. 2011, Bihari, Cepinskas et al. 2015). Lawendy et al (2011) and others (Sadasivan,

Carden et al. 1997, Kalns, Cox et al. 2011) have shown that leukocytes appear to play an important role in producing both the microvascular and the parenchymal injury seen in CS.

Unfortunately, there are currently a limited number of effective therapeutic options when it comes to treating CS. Recently, carbon monoxide (CO), a byproduct of heme oxygenase (HO) activity, has been shown to improve microvascular perfusion, and to convey anti-inflammatory benefits during systemic inflammation. The exogenous administration of CO via inhalation leads to increased carboxyhemoglobin (COHb) levels, toxic to the host in the form of CO poisoning. However, transitional metal carbonyls, such as CO-releasing molecules (CO-RMs), have been synthesized and can be used experimentally, to deliver CO to the tissues in a controlled manner without significantly raising COHb levels (Motterlini, Clark et al. 2002, Clark, Naughton et al. 2003). While most CO-RMs are soluble in organic solvents, carbon monoxide-releasing molecule-3 (CORM-3) is water soluble (Motterlini and Otterbein 2010), making it an ideal choice for clinical applications. In a rodent and porcine models of CS, the application of CORM-3 at the time of surgical fasciotomy was shown to diminish tissue injury, leukocyte activation and block the systemic release of TNF-α, a very potent pro-inflammatory cytokine (Lawendy, Bihari et al. 2014, Bihari, Cepinskas et al. 2015). While these results are promising, more experimental testing is required before CORM-3 could be used as a therapeutic agent in human patients. Furthermore, the underlying mechanism and the actual beneficial effects of CORM-3 protection, as well as its potential side effects still remain to be elucidated.

The primary purpose of this study was to provide a transition between the previous animal studies and future testing of CO on human subjects. We attempted to evaluate the relative contribution of both elevated tissue pressure and inflammation to the pathophysiology of CS, as well as to examine the beneficial effect of CORM-3-derived CO. To this end, we used two clinically relevant *in vitro* models of CS: elevation of hydrostatic pressure (EHP) and the exposure of human vascular endothelial cells (HUVECs) to a cytokine/chemokine cocktail (representing the serum levels of inflammatory mediators previously detected in our experimental animal models of CS) ("CS cocktail") (Donohoe 2015). We hypothesized that the exposure of endothelial monolayer to inflammatory cytokines/chemokines will result in similar response to that obtained by the application of EHP, characterized by the activation of the endothelium, endothelial barrier breakdown and dysfunction. In addition, given that CORM-3 appears to provide beneficial effects in various animal models (Bihari 2017), we surmised that we could replicate these results *in vitro*, and also explore some of the mechanisms through which CORM-3 protects the endothelium. The ultimate goal of this research is the development of a safe pharmacologic adjunct therapy aimed at human patients with CS, in order to reduce the morbidity and disability resulting from this devastating condition.

3.2 MATERIALS AND METHODS

3.2.1 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). Human recombinant TNF-α, IL-1β and GRO were purchased from Invitrogen Canada (Life Technologies Inc., Burlington, ON). A water-soluble CORM-3 (tricarbonylchloro-glycinate-ruthenium(II), $[Ru(CO)_3Cl$-glycinate]; molecular weight 295 $gmol^{-1}$) was synthesized by this laboratory (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).

3.2.2 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 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.

3.2.3 *In vitro* Models of CS

To assess the relative contribution of physical tissue pressure elevation versus pro-inflammatory conditions to the severity of tissue injury seen in CS, two different models were employed: elevation of hydrostatic pressure (EHP), and cytokine/chemokine cocktail stimulation.

3.2.3.1 Elevation of Hydrostatic Pressure

Confluent HUVECs were exposed to EHP of 30mmHg, obtained via a gravity-fed system, for 6 hours, as previously described (Chapter 2, Figure 2.1). The system was connected to a compartment pressure monitor (Synthes, Westchester, PA) to ensure that constant and uniform pressures were obtained for the duration of the experiment.

Cells exposed to the same environmental conditions (i.e. 37°C, 5%CO_2) but normal pressure (0mmHg), served as a control for all experimentation.

3.2.3.2 Cytokine Cocktail Stimulation

Confluent HUVECs were stimulated with a cytokine/chemokine cocktail representing the serum levels of inflammatory mediators previously detected in our experimental model of CS ("CS cocktail") (Donohoe 2015) (Table 3.1) for 6 hours. The CS cocktail contained human recombinant TNF-α (1ng/ml), IL-1β (100pg/ml) and GRO (1ng/ml).

3.2.4 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 (1×10^6 cells), grown to confluence in 12-well fibronectin-coated plates, were loaded with DHR-123 (10μM) for 45min, and then subjected to either EHP of 30mmHg or stimulation with CS cytokine cocktail, for 6 hours, 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.

3.2.5 Measurement of the Endothelial Monolayer Integrity

HUVECs were grown to confluence on fibronectin-coated transwell inserts (1μm diameter pores) (BD Falcon). Cells were subjected to 6 hours of either EHP of 30mmHg, or stimulation with CS cytokine cocktail, in the presence of CORM-3 (or iCORM-3). The integrity of the endothelial layer was assessed by measuring the transendothelial electrical resistance (TEER) using EndOhm chamber method (EndOhm-6, World Precision Instruments) following 1hr, 3hr and 6hr EHP or CS cytokine cocktail exposure, and expressed as Ωcm^2. Changes in TEER from the baseline were evaluated at each time point.

Table 3.1   **Serum levels of cytokines/chemokines detected in a rat model of CS.**

Twenty-four different cytokines and chemokines were tested; those with detectable values are shown here. Cytokines showing significant upregulation in response to CS were utilized to model CS *in vitro*. *p<0.05.

Cytokine/Chemokine	Baseline (pg/ml)	CS (pg/ml)	Fold change	p-value
Eotaxin	228.0±161.3	175.7±144.3	-0.2	0.4431
TNF-α	33.7±6.8	1223.1±483.0*	36	0.0002
IL-1α	473.9±291.7	434.7±179.3	-0.1	0.8887
IL-1β	47.9±35.7	141.3±18.6*	3	0.0481
MIP-1α	69.8 ±66.4	84.8±41.0	0.2	0.7012
MCP-1	313.4±35.1	399.6±42.4	0.3	0.8737
GRO/KC	990.2±213.3	2984±1310*	3	0.0194
IFN-ϒ	326.6±98.6	360.4±159.3	0.1	0.8463
Leptin	9596±1038	8606±1353	-0.1	0.5344
IL-6	2566±1758	2351±1494	-0.1	0.9084
IL-13	225.3±60.0	254.9±106.9	0.1	0.7161
IL-12p70	196.1±68.1	199.2±83.5	0	0.9718
IL-18	86.6±7.3	76.4±6.1	-0.1	0.1621
IP-10	145.4±108.6	155.7±108.9	0.1	0.9424
RANTES	1673±250	1680±193	0	0.3560

3.2.6 Quantification of Apoptosis

HUVECs grown on black fibronectin-coated 96-well plates with clear bottom (Greiner Bio-One) were subjected to either EHP of 30mmHg or stimulation with CS cytokine cocktail, 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).

3.2.7 PMN Rolling/Adhesion Assay

HUVECs grown on the parallel-flow perfusion microslides (μ-slide VI0.4; ibidi, Madison, WI) were subjected to either EHP of 30mmHg or stimulation of with CS cytokine cocktail, 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). After 10min wash with M199 in the presence of CORM-3 or iCORM-3 at a shear stress of 1dyn/cm^2 obtained by syringe pump (Harvard Apparatus, St. Laurent, QC), PMNs (1×10^6/ml) isolated from healthy adult volunteers 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 were expressed as a number of PMN/mm^2.

3.2.8 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. All experiments were repeated four times (on 4 different days), to account for variability in ambient environmental factors.

3.3 RESULTS

3.3.1 ROS Production

Exposure of HUVECs to EHP, or their incubation with CS cytokine cocktail induced a significant increase in the production of ROS, as shown in Figure 3.1. DHR-123 fluorescence intensity increased from 578.0±43.4.5 FI/mg protein in unstimulated control cells to 791.5±64.4 and 1169.1±155.8 Fl/mg protein in cells exposed to EHP or CS cytokine cocktail, respectively ($p < 0.05$). CORM-3 treatment significantly decreased ROS production in both the EHP and the CS cytokine cocktail groups, to 516.8±48.0 and 468.3±37.8 Fl/mg protein, respectively ($p < 0.05$), while it had no effect on the unstimulated control endothelial cells (Figure 3.1).
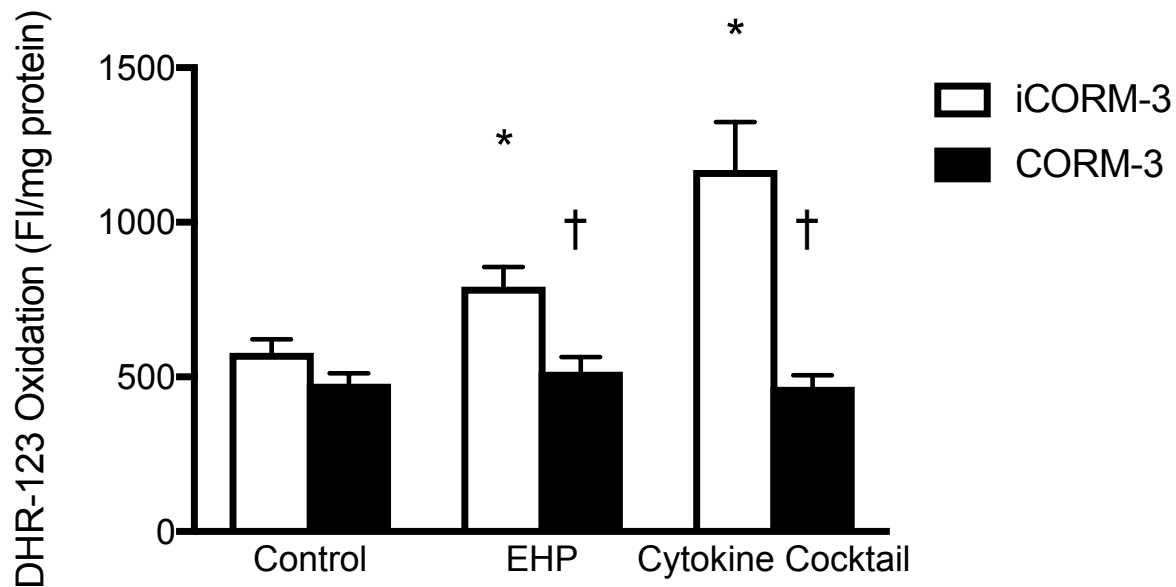
Figure 3.1   **The effect of CORM-3 on the oxidative stress response in human vascular endothelial cells in an *in vitro* model of CS.** HUVECs were subjected to 6 hours of sustained 30mmHg EHP or cytokine cocktail stimulation in the presence of CORM-3 (or iCORM-3), and the levels of ROS production were assessed by DHR-123 assay (two-way ANOVA, $p < 0.05$, *$p < 0.05$ from Control, †$p < 0.05$ from EHP+iCORM-3 and Cytokine Cocktail+iCORM-3, N=4 per group).

3.3.2 Transendothelial Electrical Resistance (TEER)

Exposure of HUVECs to EHP led to a gradual decrease in the integrity of the endothelial monolayer, as indicated by a decrease of TEER from the baseline of $63.4\pm3.3\Omega cm^2$ to $48.6.5\pm4.3\Omega cm^2$, $38.7\pm3.2\Omega cm^2$ and $35.3\pm3.5\Omega cm^2$ at 1hr, 3hr and 6hr EHP, respectively ($p<0.05$). Incubation of HUVECs with cytokine cocktail produced a gradual decrease in TEER to $24.5\pm7.0\Omega cm^2$, $16.1\pm7.1\Omega cm^2$ and $13.6\pm6.2\Omega cm^2$ at 1hr, 3hr and 6hr exposure, respectively ($p<0.05$ from control) (Figure 3.2).

CORM-3 treatment of cells exposed to EHP significantly diminished the magnitude of changes in TEER to $52.0\pm5.0\Omega cm^2$, $52.1\pm2.3\Omega cm^2$ and $47.0\pm3.2\Omega cm^2$ at 1hr, 3hr and 6hr, respectively ($p<0.05$ from EHP+iCORM-3). In parallel, CORM-3 treatment of cells stimulated with CS cytokine cocktail significantly diminished the magnitude of changes in TEER to $44.4\pm3.0\Omega cm^2$, $44.2\pm4.4\Omega cm^2$ and $39.5\pm8.7\Omega cm^2$ at 1hr, 3hr and 6hr, respectively ($p<0.05$ from CS cytokine cocktail+iCORM-3) (Figure 3.2).

3.3.3 Apoptosis

Exposure of HUVECs to EHP, or incubation with the CS cytokine cocktail led to a significant increase in the activation of caspases, as shown in Figure 3.3. Apoptosis increased from 2.4 ± 0.1 RFU in control to 8.8 ± 0.4 RFU in EHP and 10.3 ± 1.0 RFU in cytokine cocktail groups ($p<0.05$). CORM-3 treatment significantly decreased the activation of caspases to 4.0 ± 0.4 RFU in cytokine cocktail group ($p<0.05$), while having no effect on caspases activity in EHP group (8.6 ± 0.3 RFU, n.s.) (Figure 3.3). CORM-3 or iCORM-3 had no effect on the activity of caspases in control cells.
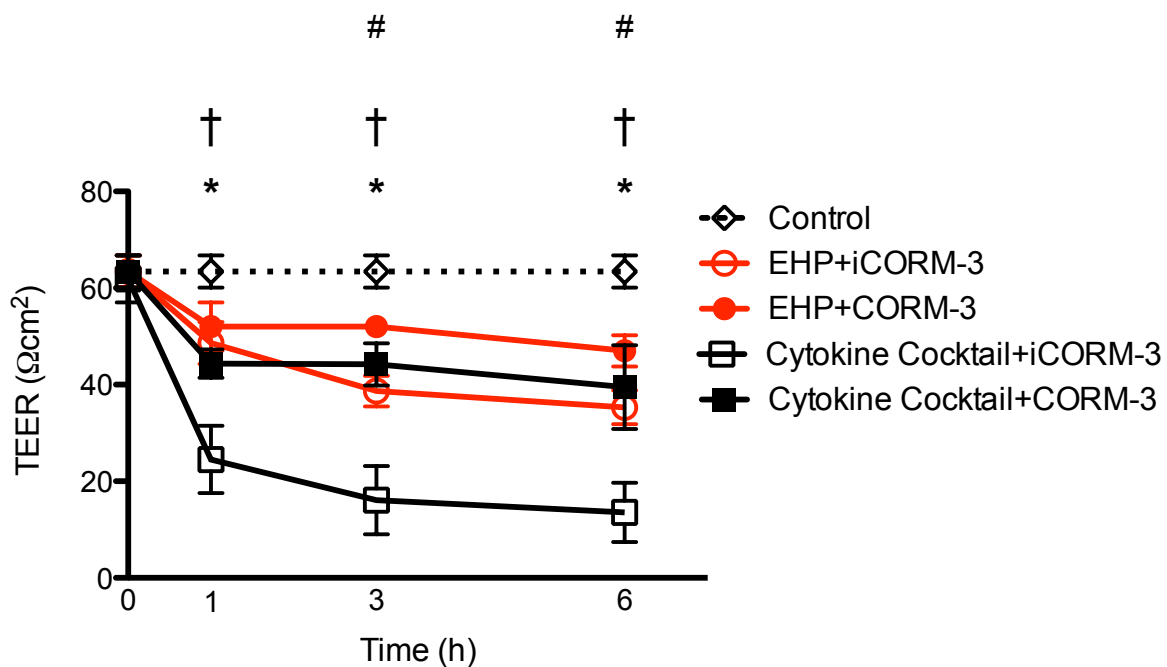
Figure 3.2 **The effect of CORM-3 on the integrity of human vascular endothelial cell monolayer in an *in vitro* model of CS.** HUVECs were subjected to 6 hours of sustained 30mmHg EHP, or cytokine cocktail stimulation, in the presence of CORM-3 (or iCORM-3), and the integrity of monolayer was assessed by measuring the transendothelial electrical resistance (TEER) (two-way repeated measures ANOVA, $p<0.05$, *$p<0.05$ from Control, †$p<0.05$ from Cytokine Cocktail+iCORM-3, #$p<0.05$ from EHP+iCORM-3, N=3 per group).

Figure 3.3. **The effect of CORM-3 on the level of apoptosis in human vascular endothelial cells in an *in vitro* model of CS.** HUVECs were subjected to 6 hours of sustained 30mmHg EHP or cytokine cocktail stimulation in the presence of CORM-3 (or iCORM-3), and the levels of active caspases were assessed by FAM-FLICA (two-way ANOVA, $p<0.05$, *$p<0.05$ from Control, †$p<0.05$ from Cytokine Cocktail+iCORM-3, N=4 per group).

3.3.4 Leukocyte Activation

Exposure of HUVECs to EHP resulted in a marked increase in PMN activation, from 0.4 ± 0.3 rolling PMNs/$0.1mm^2$ and 4.0 ± 1.5 adherent PMNs/$0.1mm^2$ in control to 2.2 ± 0.6 rolling PMNs/$0.1mm^2$ and 12.8 ± 1.8 adherent PMNs/$0.1mm^2$ in EHP group ($p<0.05$) (Figure 3.4). Incubation of HUVECs with the CS cytokine cocktail resulted in a significant increase in PMN activation to 2.1 ± 0.3 rolling PMNs/$0.1mm^2$ and 21.0 ± 4.5 adherent PMNs/$0.1mm^2$ ($p<0.05$) (Figure 3.4).

CORM-3 treatment of HUVECs exposed to EHP did not have significant effect . with 1.5 ± 0.5 rolling PMN/$0.1mm^2$ and 8.0 ± 1.5 adherent PMN/$0.1mm^2$. CORM-3 treatment of HUVECs exposed to CS cytokine cocktail resulted in a significant decrease in PMN activation to 0.5 ± 0.1 rolling PMN/$0.1mm^2$ and 4.4 ± 0.7 adherent PMN/$0.1mm^2$ ($p<0.05$) (Figure 4.4). There was no difference in PMN rolling or adhesion in unstimulated control HUVECs between the CORM-3 and iCORM-3 groups.

3.4 DISCUSSION

CS is associated with significant morbidity and patient disability (Giannoudis, Nicolopoulos et al. 2002) and often poses a significant clinical challenge for the physician and surgical team. Currently, the gold standard for definitive management of CS is surgical fasciotomy, with few other proven adjuncts (Olson and Glasgow 2005). While some supportive therapies have shown limited success in animal models (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2014), their use is hindered by the lack of understanding the full underlying pathophysiological basis of CS.
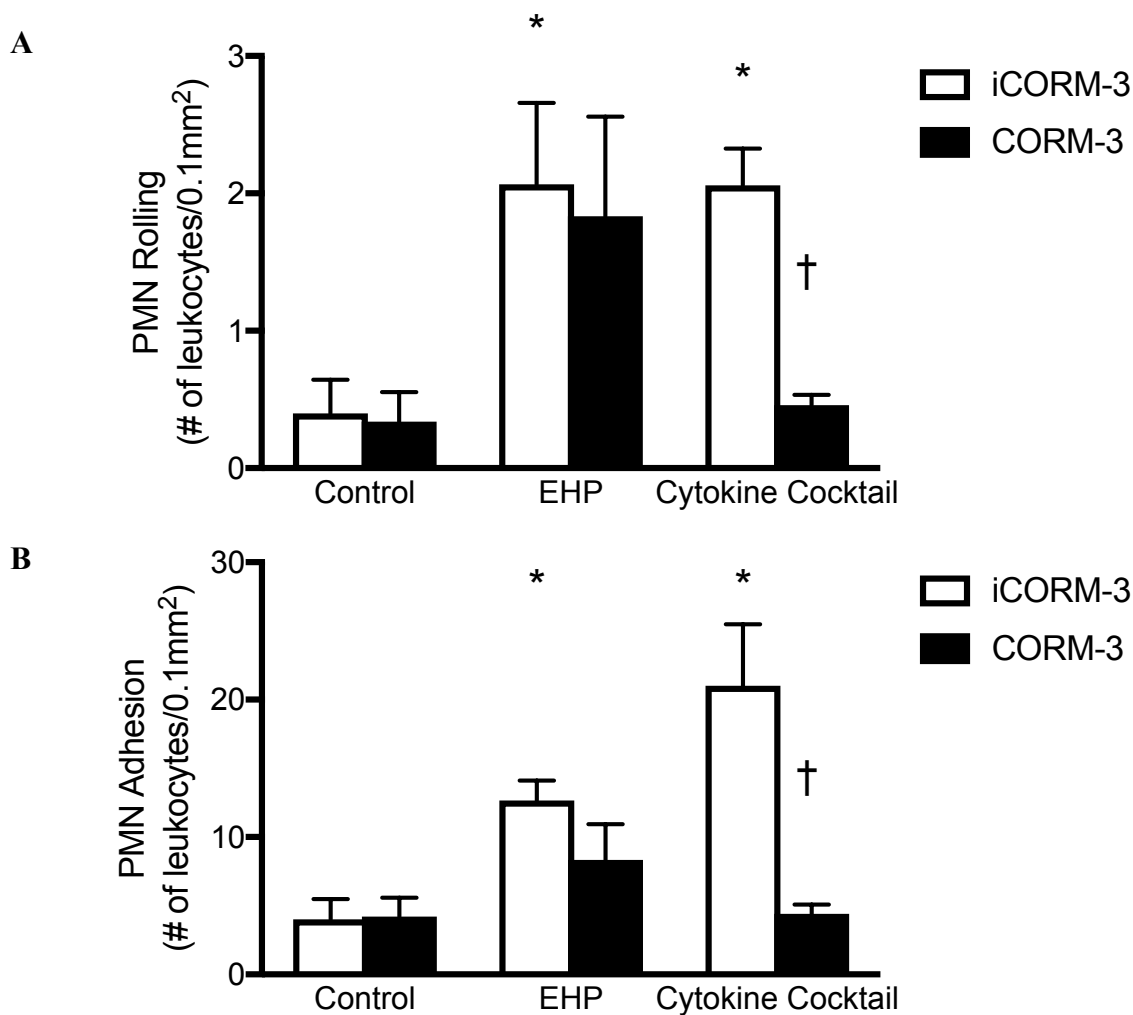
Figure 3.4 **The effect of CORM-3 on leukocyte (A) rolling and (B) adhesion in response to EHP, or cytokine cocktail stimulat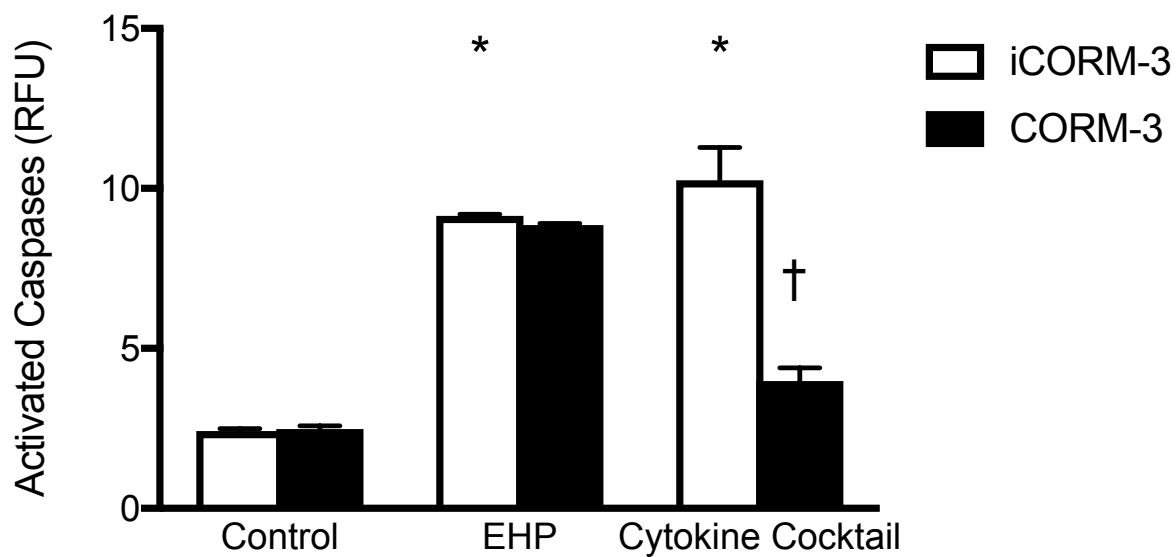ion of human vascular endothelial cells in an *in vitro* model of CS.** HUVECs were subjected to 6 hours of sustained 30mmHg EHP, or cytokine cocktail stimulation, in the presence of CORM-3 (or iCORM-3), followed by application of naïve PMNs, while being superfused at a constant rate of $1dyn/cm^2$ (two-way ANOVA, $p<0.05$, *$p<0.05$ from Control, †$p<0.05$ from Cytokine Cocktail+iCORM-3, N=4 per group).

CS is a complex pathophysiological process, leading to upregulation pro-inflammatory phenotype, followed by subsequent interactions between vascular endothelial cells and activated leukocytes. This leads to impaired vascular cell integrity, increased vascular permeability and leukocyte extravasation, followed by the clinical appearance of edema (Sabido, Milazzo et al. 1994). The production of pro-inflammatory mediators, coupled with leukocyte accumulation within the affected organ, is one of the key features of CS. While leukocyte recruitment at the site of injury is a normal physiological response, the overwhelming recruitment of PMNs, with the subsequent release of cytotoxic ROS and proteolytic enzymes, further exacerbates the parenchymal damage already seen in CS. Activated leukocytes are known to impair surrounding cell viability (Toyokuni 1999), further contributing to the already existing tissue injury and dysfunction.

Recent findings indicate that exogenous application of CO has potent anti-inflammatory effects (Motterlini and Otterbein 2010). However, inhalation of CO may lead to the formation of toxic levels of COHb (i.e. >10%), thus limiting its clinical use (Ryter, Alam et al. 2006, De Backer, Elinck et al. 2009). In order to address this, CO-releasing molecules (CO-RMs) - transitional metal carbonyls that can release CO on demand - have been synthesized. CO-RMs allow for CO delivery to the tissues without the associated rise in COHb and offer various routes of administration (IV, IP, SC or tissue superfusion) (Motterlini, Mann et al. 2005). Multiple studies have shown beneficial anti-inflammatory effects of CO-RMs: ischemia/reperfusion injury (Katada, Bihari et al. 2010), pulmonary hypertension (Zuckerbraun, Chin et al. 2006), organ transplant (Song, Kubo et al. 2003) and sepsis (Cepinskas, Katada et al. 2008).

Currently, there is a dearth of knowledge when it comes to understanding the role of CO-RMs on modulation of the response seen in CS, as well as the changes experienced at the cellular and molecular levels. Recently, we developed and tested a relevant experimental animal models of CS (Lawendy, Sanders et al. 2011, Bihari 2017). The studies found that CORM-3 had a significant therapeutic impact by reducing the inflammatory response and the resulting tissue injury, while preserving microvascular perfusion (Lawendy, Bihari et al. 2014, Bihari 2017). However, there are currently no human patient studies which have evaluated the role of elevated hydrostatic pressure, or the response to CORM-3 in CS.

In the present study, we attempted to mimic the CS conditions *in vitro*, by utilizing *human* vascular endothelial cells and two clinically-relevant CS stimuli, in order to examine the relative contribution of physical changes associated with EHP or cytokine-induced inflammation to the pathophysiology of CS, as well as to determine how CORM-3 offers protection upon exposure to these stimuli. To our knowledge, this is the first studies to model CS in such manner in humans.

Exposure of HUVECs to EHP resulted in a significant increase in intracellular production of ROS (Figure 3.1). This can most likely be linked to changes in the mitochondrial respiratory chain (Wang, Guerrero et al. 2015), and upregulation of downstream signalling molecules, which in turn, triggers inflammation (Toyokuni 1999). In addition, incubation of HUVECs with CS cytokine cocktail also resulted in a similar response; the data is consistent with prior research findings in CS and ischemia-reperfusion injury (Zhang, Hu et al. 2005, Cavaillon and Annane 2006, Lawendy, Bihari et al. 2014, Lawendy, Bihari et al. 2016). Cell stimulation by cytokines is known to

induce oxidative stress, leading to compromise of the cellular membrane, intercellular junctions and internal protein structures (Sprague and Khalil 2009). Our previous findings indicate that EHP also leads to significant stress fiber formation, changes in in F-actin and VE-cadherin expression, leading to a breakdown of endothelial barrier (Acevedo, Bowser et al. 1993, Tokuda, Miyazaki et al. 2009, Cao, Xia et al. 2015).

Increased oxidative stress, through ROS-based signalling, also leads to alterations in endothelial cell cytoskeleton and adherence junctions (Corada, Liao et al. 2001). In addition, previous reports found that EHP leads to an upregulation of cytokine levels in various cell types, such as periodontal ligament cells, bladder smooth muscles cells, mesenchymal stem cells, human tumour cells and human chondrocytes (Yamamoto, Kita et al. 2006, Fioravanti, Moretti et al. 2007, Fucikova, Moserova et al. 2014, Becquart, Cruel et al. 2016, Liang, Xin et al. 2016). The effect occurs through the upregulation of various downstream transcription factors, resulting in increased expression of various chemokines and cytokines (De Martin, Hoeth et al. 2000).

In our experiments, application of CORM-3 (but not iCORM-3) was able to diminish ROS release in both the EHP and cytokine cocktail stimulation groups. It has been demonstrated that there is a significant cross-talk between ROS and various enzymes, such as matrix metalloproteinases (MMP) and cell surface membrane proteins (various integrins) (Svineng, Ravuri et al. 2008). In addition, it has been shown that EHP is able to modulate various cellular pathways, including the induction of MMP (Inoue, Arai et al. 2015). Interestingly, previous studies found that exogenous application of CO, in the form of CORM-2, was able to diminish the induction of MMP expression and its downstream effects (Tsai, Lee et al. 2017). These reports further support the notion that

the protective effect of CORM-3 is likely mediated through its effects on downstream transcription factors, upregulated by both the direct effect of EHP and the presence of various inflammatory cytokines.

Exposure of human endothelial cells to EHP or CS cytokine cocktail led to a significant breakdown of the endothelial barrier, evidenced by a decrease in trans-endothelial electrical resistance (Figure 3.2). Previously, EHP has been found to lead to changes in endothelial cell morphology and diffusion barrier characteristics in multiple cell types (Acevedo, Bowser et al. 1993, Ohashi, Sugaya et al. 2007, Tokuda, Miyazaki et al. 2009, Cao, Xia et al. 2015). Cellular permeability and leukocyte extravasation are controlled by adhesive interactions between various endothelial cell surface proteins (Ley, Laudanna et al. 2007). EHP appears to not only have an effect on cell-cell junctions, but it also affects the upregulation of adhesion molecules that are involved in microvessel integrity, as demonstrated by a rapid decrease in TEER, correlated with a transient loss and/or redistribution of PECAM-1 (Sharma, Templin et al. 2013, Souvannakitti, Peerapen et al. 2016). CORM-3 treatment preserved endothelial monolayer integrity; a plausible explanation being that CORM-3 can modulate F-actin expression/distribution and various transcription pathways (particulary MAPK) (Inoue, Patterson et al. 2017). Another possibility is that CORM-3 may modulate mitochondria-based pathways. Previous reports have found EHP leads to mitochondrial fission and a reduction in cellular ATP (Ju, Liu et al. 2007); CORM-3 has been shown to uncouple mitochondrial respiration and activate phosphate carriers, enhancing mitochondrial phosphate uptake (Long, Salouage et al. 2014). Additionally, CORM-3 also appears to have a significant impact on membrane potential and permeability, through its effects on

the transmembrane movement of K^+ and Na^+, causing changes in cellular respiration and H^+ pumping (Wilson, Jesse et al. 2013).

Treatment of endothelial cells with EHP or CS cytokine cocktail resulted in a significant increase in caspase activation. Surprisingly, while CORM-3 treatment prevented apoptosis in the CS cytokine cocktail group, it had no effect on apoptosis levels in the cells exposed to EHP. Increased oxidative stress, through ROS-based signalling (Toyokuni 1999), plays an important role in caspase activation and eventual apoptosis (Elmore 2007). Multiple studies, both *in vivo* and *in vitro,* have demonstrated the anti-apoptotic effects of CO (Brouard, Otterbein et al. 2000, Petrache, Otterbein et al. 2000, Bihari, Cepinskas et al. 2014, Lawendy, Bihari et al. 2014). In the clinical and experimental scenario, elevated intracompartmental pressure leads to a significant rise in the inflammatory cytokine TNF- α (Lawendy, Bihari et al. 2015, Lawendy, Bihari et al. 2016, Bihari 2017) which is an important mediator of apoptosis via modulation of the expression of various proteolytic caspases (Seekamp, Warren et al. 1993, Roebuck, Carpenter et al. 1999, Ley 2008). Previously, treatment with CORM-3 has been shown to lead to a significant decrease in circulating TNF-α levels (Cepinskas, Katada et al. 2008, Lawendy, Bihari et al. 2014, Donohoe 2015, Bihari 2017). Heme oxygenase inhibitors have also been shown to lead to a significant decrease in TNF-α-induced apoptosis in an *in vitro* model of both mouse fibroblasts and endothelial cells (Petrache, Otterbein et al. 2000). In our study, the lack of CORM-3 effect upon exposure of HUVECs to EHP could be due to a relatively high dose of CORM-3 delivered to the HUVECs, changing its relative effect. In several experimental models of disease and/or tissue injury such as ischemia-reperfusion and lung transplantation, pre-treatment with low dose CO led to a

net anti-apoptotic effect (Ryter, Alam et al. 2006), although higher concentrations of CO produced pro-apoptotic effects (Piantadosi, Zhang et al. 1997). In addition, apoptotic effects of EHP have been shown to be time dependent (Hasel, Durr et al. 2005); it is plausible to assume that the lack of response to CORM-3 could have been due to time dependent changes in activated caspase levels.

The exposure of endothelial cells to EHP or CS cytokine cocktail led to a significant increase in leukocyte adhesive interactions (Figure 3.4), which remains a key feature of leukocyte and/or vascular endothelial cell inflammatory activation (Butcher 1991, Ley, Laudanna et al. 2007). The process is mediated by various cytokines, chemokines and cell surface proteins on the activated endothelial cells (Albelda, Muller et al. 1991). Changes in hydrostatic pressure are likely to directly cause alterations in the expression of adhesion molecules on the vascular endothelium (e.g. P-selectin, E-selectin, ICAM-1, VCAM-1), allowing them to interact with their ligands on the PMNs (e.g. L-selectin, sialyl-LewisX, β2 integrins).

CORM-3 treatment was able to inhibit both leukocyte adhesion and rolling (Figure 3.4) in the cytokine cocktail group, but the beneficial effects were not reproduced in the EHP group. Previously, the application of CORM-2 significantly reduced levels of vascular endothelial adhesion molecules such as E-selectin and ICAM-1 (Nizamutdinova, Kim et al. 2009, Katada, Bihari et al. 2010). A decrease in E-selectin and VCAM-1 expression has also been shown by pre-treatment with CORM-3 in HUVECs (Song, Bergstrasser et al. 2009). These results however, have not been universally found, as other studies have failed to show the ability of CORM-3 to suppress vascular endothelial cell adhesion protein expression (Urquhart, Rosignoli et al. 2007). Perhaps the lack of

statistically significant response in the EHP group following exposure to CORM-3 may be explained by its relative abilities to modulate adhesion on the endothelium when compared to the activated leukocytes. Although the effects of CORM-3 have been shown to modulate adhesive interactions on both activated leukocytes and the endothelium, its effect may be more significant on the former (Urquhart, Rosignoli et al. 2007).

To our knowledge, this is the first study demonstrating the beneficial effects of carbon monoxide, delivered in the form of CORM-3, in two clinically relevant human *in vitro* models of CS. While the exact mechanisms of CORM-3 protective action remain to be determined, the obtained data helps translate previous animal model research to human trials, and suggests a potential therapeutic application of CORM-3 in CS.

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CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 OVERVIEW OF RESULTS

CS is a true medical and surgical emergency associated with potentially devastating complications. Currently, the only proven treatment is surgical fasciotomy, to decompress all involved osseofascial compartments. The procedure, however, is associated with relatively frequent and significant complications leading to patient morbidity and dysfunction (Whitesides, Hirada et al. 1977). Certain adjunct and supportive therapies have shown limited therapeutic potential in various animal models (Manjoo, Sanders et al. 2010, Lawendy, Bihari et al. 2014, Bihari 2017), but their use in a clinical setting is hindered by our current lack of understanding of the underlying pathophysiology of CS, as well as their method of action. Recent reports utilizing animal models of CS have shown that unlike complete ischemia, CS creates a microcirculatory "low flow" environment, occurring in the presence of *patent* arterial vessels. This leads to early significant leukocyte activation, which in turn further contributes to parenchymal injury, cellular ischemia and limb dysfunction (Lawendy, Sanders et al. 2011).

CO has emerged as an important mediator of cell signalling while also possessing anti-ischemic, anti-oxidant, anti-inflammatory, anti-apoptotic and vasodilatory properties (Kim, Ryter et al. 2006). In order to clinically utilize the beneficial attributes of this gas, a novel class of transition metal carbonyls capable of releasing CO on demand, the carbon monoxide-releasing molecules (CO-RMs), was experimentally synthesized (Motterlini, Clark et al. 2002). CO-RMs are capable of delivering safe levels of CO to the tissues in a

controlled manner, without causing a corresponding dangerous rise in COHb (Motterlini, Clark et al. 2002).

Recent studies using animal models of CS (rat and pig) found that the administration of CORM-3 at the time of fasciotomy was associated with a decrease in leukocyte activation, systemic TNF-α release and diminished tissue injury, while improving microvascular perfusion (Lawendy, Bihari et al. 2014, Bihari 2017), but it has never been tested in human patients. *In vitro* modelling of CS allows to not only explore the underlying pathophysiology in a mechanistic manner, but also permits the development and testing of interventions (e.g. CO-RMs) that are currently not possible (or unethical) in humans. The goal of this thesis was to develop and test a relevant *in vitro* model of CS, using *human* cells, in order to expand our understanding of CS pathophysiology, as well as the development a safe medical adjunct (or standalone therapy) for patients presenting with CS.

4.1.1 EHP As an *In Vitro* Model Of CS

In Chapter 2, an attempt was made to mimic the CS conditions *in vitro* by subjecting *human* vascular endothelial cells (HUVECs) to the CS-relevant stimulus, in the form of elevated hydrostatic pressure (EHP), directly assessing cellular response, while attempting to clarify the role that EHP contributes to the endothelial activation and pro-inflammatory potential of CS. We found that exposure of HUVECs to EHP led to a rearrangement of endothelial monolayer, characterized by significant stress fiber formation, as evidenced by changes in F-actin and VE-cadherin expression. Cells exposed to EHP also experienced a significant increase in intracellular production of

ROS, most likely due, at least in part, to changes in the mitochondrial respiratory chain and the inner mitochondrial membrane. It can also be surmised that this increase in ROS would then go on to trigger downstream signalling molecules, further stimulating inflammation (Toyokuni 1999).

EHP applied to endothelial cells also led to a significant increase in leukocyte adhesion, a key feature of leukocyte and/or vascular endothelial cell inflammatory activation (Butcher 1991, Ley, Laudanna et al. 2007). These results suggest that EHP not only induces ROS production and apoptosis, but it also directly promotes endothelial cell activation. Changes in hydrostatic pressure most likely influence the transcription and expression of adhesion molecules on the vascular endothelium (e.g. P-selectin, E-selectin, ICAM-1, VCAM-1), which then allows the endothelium to interact with their ligands on the PMNs (e.g. L-selectin, sialyl-LewisX, β2 integrins), leading to increased endothelial permeability, extravasation, and *in vivo*, tissue edema and parenchymal injury. Thus, our study demonstrated that direct physical effects caused by elevated hydrostatic pressure within the injured limb also significantly contribute to the pathophysiology of CS.

4.1.2 Effect of CORM-3

In Chapter 3, we attempted to build on our results, by testing the effect of CORM-3 on HUVECs exposed to elevated hydrostatic pressure, and compare the findings to the results obtained by CS cytokine cocktail stimulation. We studied the effects of CORM-3 on ROS production, endothelial monolayer integrity, apoptosis and endothelial activation (leukocyte adhesion/rolling).

Application of CORM-3 was able to decrease intracellular ROS production in both the EHP and cytokine cocktail groups. In addition, CORM-3 also prevented the hydrostatic pressure-induced decrease in endothelial monolayer integrity, most likely by modifying EHP-induced cytoskeletal changes in F-actin or VE-cadherin. Surprisingly, while CORM-3 prevented apoptosis in the CS cytokine cocktail group, it had no effect on the level of apoptosis in the cells exposed to EHP, despite multiple studies previously demonstrating the anti-apoptotic effects of CO (Brouard, Otterbein et al. 2000, Petrache, Otterbein et al. 2000, Bihari, Cepinskas et al. 2014, Lawendy, Bihari et al. 2014).

Both EHP and CS cytokine cocktail stimulation led to a significant endothelial activation, as demonstrated by an increase in leukocyte rolling and adhesion to the endothelial monolayer. CORM-3 treatment was able to inhibit this response in the CS cytokine cocktail group but did not have a similar effect in the hydrostatic pressure group. The difference in magnitude could be explained by the ability of CORM-3 to modulate the expression of various adhesion molecules on both activated leukocytes and the endothelium, with the effect being more significant on the former (Urquhart, Rosignoli et al. 2007).

4.2 LIMITATIONS AND FUTURE DIRECTIONS

Although we were able to demonstrate detrimental consequences of elevated hydrostatic pressure on endothelial cells, and the beneficial effects of CORM-3 in this novel *in vitro* human CS model, there were several limitations to these studies. We arbitrarily chose to expose HUVECs to a hydrostatic pressure of 30mmHg, as this level is

clinically relevant and feasible in the laboratory setting using a gravity-fed system. However, the ideal pressure to which HUVECs should be exposed to in order to mimic CS most effectively, has yet to be discerned. In addition, we did not expose endothelial cells to different pressure changes, which (presumably) could have led to a graded cellular response. Furthermore, we chose 4 to 6 hours of EHP based on previous reports and clinical relevancy; in the future, comparing longer or shorter periods of exposure may reveal interesting results in terms of cellular response and the effects of CORM-3.

Another limitation is the fact that we used only one concentration of CORM-3, which we based on previous related reports. However, different concentrations may prove to be more effective, without compromising cell survival. Although the goal of this specific model was to test the effect of elevated hydrostatic pressure on HUVEC and the potentially protective effect of CORM-3, other compounds with anti-oxidant and anti-inflammatory properties, such as taurine, N-acetyl cysteine and vitamin C could potentially be utilized, individually or in combination.

CORM-3 is in the early experimental stage, yet its effect on CS in various animal models has been significant. Although the purpose of our experimental design was to examine the effect of CORM-3 on CS specifically, in the future, CORM-3 could be tested in other conditions, such as Charcot changes in the diabetic foot. Charcot changes and ulceration have been associated with ischemia/reperfusion, elevated cytokine levels, reactive oxygen species, apoptosis and significant inflammatory changes (Folestad, Alund et al. 2015, Petrova, Petrov et al. 2015); A condition which appears to share many similarities with ischemia reperfusion and CS models and which may also respond favourably to CORM-3 treatment.

4.3 CONCLUSIONS

The data in this thesis indicates that the effects of elevated hydrostatic pressure on endothelial cells is not inconsequential, and changes in hydrostatic pressure itself significantly contribute to the pathophysiology of CS. We demonstrated the beneficial effects of CORM-3 in an *in vitro* model of CS. Although certain outcomes were unexpected, such as the lack of the effect on caspase activation in response to CORM-3 in EHP, our results suggest CORM-3 may be of benefit to patients suffering with CS. While the exact mechanisms of CORM-3 action remain to be elucidated, CORM-3 may have a significant potential as a pharmacological treatment to supplement and/or replace fasciotomy, thus avoid the devastating complications associated with CS.

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APPENDICES

APPENDIX I. PERMISSION TO USE COPYRIGHTED MATERIALS

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Type of Publication: Book

Book Title:Operative Techniques: Orthopaedic Trauma Surgery
Book ISBN: 978-1-4160-4935-7
Book Author: Lawendy A, 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 II: <u>HUMAN RESEARCH ETHICS BOARD APPROVAL LETTER</u>

Use of Human Participants – Ethics Approval Notice

Research

Western

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.

███████████████████

Signature

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