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Potential Use of Carbon Monoxide as a Non-Surgical Intervention in a Rat Model of Acute Compartment Syndrome

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ABSTRACT

Acute limb compartment syndrome (ACS), a potentially devastating complication of musculoskeletal trauma, results in muscle necrosis and cell death. Ischemia and inflammation both contribute to microvascular dysfunction and parenchymal injury. Currently, surgical fasciotomy remains the only first-line treatment.

Systemic application of carbon monoxide-releasing molecule-3 (CORM-3) in animal models of ACS has shown benefits when given in conjunction with fasciotomy; however, CORM-3 without fasciotomy has never been tested. The purpose of this thesis was to assess the effects of CORM-3 in ACS *without* surgical intervention.

Twenty-nine male adult Wistar rats were used to test the effects of CORM-3. Microvascular perfusion, tissue injury, and inflammatory response were measured at 24, 48, and 72 hours following intracompartmental pressure elevation and CORM-3 injection.

The results demonstrated partially restored microvascular perfusion, significantly reduced tissue injury, and significantly diminished leukocyte activation (inflammation), indicating the potential of CORM-3 as a pharmacological agent in the treatment for ACS.

Keywords: *acute compartment syndrome; elevated intracompartmental pressure; carbon monoxide; CORM-3; fasciotomy; microvascular perfusion, tissue injury; inflammation; non-operative management.*

SUMMARY FOR LAY AUDIENCE

Acute limb compartment syndrome (ACS) is a potentially devastating complication of limb trauma resulting in muscle and cell death. Lack of blood flow and white blood cell activation both contribute to vessel dysfunction and tissue injury. The treatment gold standard is an emergency surgery to relieve the tissue pressure.

Injection of carbon monoxide-releasing molecule-3 (CORM-3), a substance that gives off carbon monoxide, in animal models of ACS has shown benefits when given together with surgery; however, CORM-3 without surgery has never been tested. Therefore, the purpose of this thesis was to assess the effects of CORM-3 in ACS *without* surgical intervention.

Twenty-nine male adult Wistar rats were used to test CORM-3. Tissue perfusion, injury, and white blood cell response were measured at 24, 48, and 72 hours following tissue pressure elevation and CORM-3 injection.

Our results demonstrated that CORM-3 injection without surgery improved perfusion, reduced tissue injury, and diminished inflammation, indicating its potential in prolonging the surgical window and its use as a pharmacological treatment for ACS.

CO-AUTHORSHIP

Despite being the principal author of this thesis, where I performed the experiments, collected, analyzed, and interpreted the data, as well as wrote the dissertation, this project would not have been possible without the co-authors listed below.

Abdel-Rahman Lawendy, MD, PhD, FRCSC: It is through his previous research and publications that the conception of this project was even made possible. In his role as the principal investigator and supervisor of this thesis, his clinical expertise and intimate knowledge of compartment syndrome have provided crucial guidance, direction, and leadership over the course of my project; his insight into the pathophysiology of compartment syndrome and knowledge of reperfusion injury mechanisms helped to direct the project towards the end product. He also critically reviewed this work.

Aurelia Bihari, PhD: Dr. Bihari has been the driving force throughout this experiment. She has spent countless hours supervising and teaching me how to perform the experiments. She has played an instrumental role in setting up the lab, providing technical support, helping with data collection, data analysis, and interpretation, as well as critically reviewing the dissertation.

DEDICATION

To my parents, Shengrui and Zhanhong, who have made countless sacrifices throughout my childhood and young adulthood to help me reach my ambitions and pursue my dreams.

To my little brother, Samuel, who is also enrolled in a master's program at the time of writing this thesis. He has always had my back in every step of life. He has also tried to bully me at times to show who's boss, but that obviously never worked.

To my beautiful wife, Mylène, who has always supported me despite my busy schedules, and more importantly, whose unconditional love has brought me joy and happiness, even in the most difficult of times.

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Nothing would have been possible without the incredible efforts and extraordinary patience of Dr. Aurelia Bihari. Without her, this project does not come to fruition. She has been a workhorse of this experiment from its conception, laboratory set-up, data collection, and analysis, as well as critical dissertation review. How she remained calm and has never yelled at me throughout the year is, to me, a feat in itself.

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LIST OF ABBREVIATIONS

ACS, acute compartment syndrome

AIF, apoptosis-inducing factor

ANOVA, analysis of variance

APAF-1, apoptotic protease activating factor-1

ATP, adenosine triphosphate

AV, arteriovenous

BB, bisbenzimide

BR, bilirubin

BV, biliverdin

BVR, biliverdin reductase

CCAC, Canadian Council on Animal Care

cGMP, cyclic guanosine monophosphate

CM, centimetres

CO, carbon monoxide

COHb, carboxyhemoglobin

CO-RMs, carbon monoxide-releasing molecules

CORM-3, carbon monoxide-releasing molecule-3

COX, cyclooxygenase

CPC, continuously-perfused capillaries

DISC, death-inducing signalling complex

EB, ethidium bromide

EDL, extensor digitorum longus

EIU, endotoxin-induced uveitis

ERK, extracellular signal-related kinases

GRO, growth-regulated oncogene

HO, heme oxygenase

HSP32, heat shock protein 32

HUVEC, human vascular endothelial cells

ICP, intracompartmental pressure

ICAM-1, intracellular adhesion molecule-1

ICAM-2, intracellular adhesion molecule-2

Ig, immunoglobulin

IL-1β, interleukin-1 beta

IL-6, interleukin-6

IL-8, interleukin-8

IM, intra-muscular

IPC, intermittently-perfused capillaries

I-R, ischemia-reperfusion

IVVM, intravital video microscopy

JAM, junctional adhesion molecule

JNK, *c*-jun N-terminal kinases

LFA-1, lymphocyte function-associated antigen-1

LG, lateral gastrocnemius

LT, leukotriene

MAC-1, macrophage-associated protein-1

MADCAM-1, mucosal vascular addressin cell adhesion molecule type 1

MAPK, mitogen-activated protein kinases

MCP-1, monocyte chemoattractant protein 1

MIN, minute

MIP-1, macrophage inflammatory protein-1

ML, milliliters

MMP, matrix metalloproteases

MPO, myeloperoxidase

N.S., not significant

NA, neutralizing antibodies

NAC, N-acetyl cysteine

NAD^+, nicotinamide adenine dinucleotide

NADPH, nicotinamide adenine dinucleotide phosphate

NIRS, near infra-red spectroscopy

NF-κB, nuclear factor kappa B

NM, nanometers

NO, nitric oxide

NOS, nitric oxide synthase

- cNOS, constitutive NOS; eNOS, endothelial NOS; iNOS, inducible NOS

NPC, non-perfused capillaries

NSAIDs, non-steroidal anti-inflammatory drugs

PAF, platelet-activating factor

PCA, patient-controlled analgesia

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

PI3K, phosphatidylinositol 3-kinase

PL, peroneus longus

PMN, polymorphonuclear leukocytes

PSGL-1, P-selectin glycoprotein ligand-1

PVD, peripheral vascular disease

RIP, receptor-interacting protein

ROS, reactive oxygen species

sGC, soluble guanylate cyclase

SEC, seconds

SEM, standard error of the mean

SOD, superoxide dismutase

TA, tibialis anterior

TNF-α, tumor necrosis factor-alpha

TUF, tissue ultrafiltration

VCAM-1, vascular cell adhesion molecule-1

VLA-4, very late antigen-4

XD, xanthine dehydrogenase

XO, xanthine oxidase

Δp, pressure differential

μg, microgram

μm, micrometer

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 ACUTE COMPARTMENT SYNDROME

Acute compartment syndrome (ACS) in musculoskeletal trauma is a limb-threatening and, potentially, life-threatening injury. Without prompt surgical intervention, the elevation of pressure within a closed osseofascial compartment leads to tissue ischemia, myonecrosis, and nerve injury (McMillan et al. 2019; Mortensen et al. 2020; Schmidt 2017; von Keudell et al. 2015). The microvascular compromise that results in a lack of oxygen and nutrient delivery to the tissues then leads to cellular anoxia and cell death. Ultimately, this can cause severe functional impairment in extremity function, potential loss of limb, and even patient death. Although multiple therapeutic agents have been proposed, the treatment gold standard remains fasciotomy. However, the surgical window for the procedure is quite narrow before permanent damage develops (Guo et al. 2019; Mubarak & Hargens 1983). Risk factors for delayed diagnosis of ACS reported in the literature include poor communication between physicians, failure of early recognition of signs and symptoms, neurological injuries, altered levels of consciousness, children, and patient-controlled or regional anaesthetics (Bhattacharyya & Vrahas 2004; Garner et al. 2014; Mortensen et al. 2020; von Keudell et al. 2015).

1.2 ACS: A DIAGNOSTIC CHALLENGE

1.2.1 Aetiologies

There are numerous causes of increased compartmental pressures in the limbs. They can be divided into traumatic injuries, such as fractures, soft-tissue trauma without fracture, and penetrating trauma; vascular injuries, such as arterial/venous injuries, intracompartmental bleeding, reperfusion injuries following ischemia; soft tissue injuries, such as burns, crush injuries, hematomas, animal bites; iatrogenic causes including, but not limited to, tight casts, constrictive dressings, prolonged lithotomy position (flexion, elevation, and abduction of the leg) during orthopaedic surgery, intravenous drug use, tourniquet application, and anticoagulation (Gourgiotis et al. 2007; Olson & Glasgow 2005). Reported rare causes of ACS include hypothyroidism, diabetes, vascular abnormalities, nephrotic syndrome, statins, and ganglion cysts of the proximal tibiofibular joint (Chautems et al. 1997; Flamini et al. 2008). Although ACS can occur in the arm, hand, thorax, abdomen, buttock, thigh, foot, paraspinal muscles, and even in the ocular orbit, it is most commonly seen following injuries to the lower leg and forearm (Elliott & Johnstone 2003; Garner et al. 2014; McCallum et al. 2019; Rupprecht et al. 2019).

Bone fractures are the most common cause of ACS when compared to non-fracture aetiologies, and most commonly occur following tibial shaft fractures (Elliott & Johnstone 2003; Garner et al. 2014; McQueen et al. 2000; von Keudell et al. 2015). In a retrospective study, McQueen et al. (2000) found that tibial diaphyseal fractures were the most common fracture cause of ACS, at 36%,

followed by distal radius fractures with nearly 10% of cases. Nonetheless, 23.2% of ACS patients were a result of soft-tissue trauma without fracture. Risk stratification demonstrated that men under the age of 35 are at greatest risk of developing ACS following injury (McQueen et al. 2000).

1.2.2 Clinical Findings

Making the diagnosis of ACS is a subject of much debate. It relies on clinical acumen and monitoring. Intracompartmental pressure (ICP) monitoring is often used when the diagnosis is equivocal; however, there are no standardized guidelines for its use (Garner et al. 2014; Mabvuure et al. 2012). Furthermore, the quality and timing of symptoms can vary between injuries and patients. Studies have even suggested that muscle damage can occur before the diagnosis of ACS becomes evident (Frink et al. 2010; Nudel et al. 2016; Vaillancourt et al. 2004).

Classically, the five Ps (pain, pallor, pulselessness, paralysis, and paraesthesia) mnemonic has been used as a diagnostic tool for ACS (Donaldson et al. 2014; Gourgiotis et al. 2007). However, some authors have contended that they are unreliable. Von Keudell et al. (2015) argue that these represent arterial ischemia rather than increased compartment pressures. Furthermore, given that ACS is a dynamic process, the progression of symptoms can be variable (von Keudell et al. 2015).

Mubarak and colleagues (1978) found that swelling and palpable tenseness were the first signs of ACS as a result of ICP elevation. Alone,

however, they are not diagnostic of ACS. They also found that pain with passive stretch was subjective and unreliable, given that it could be a result of the traumatic injury rather than ischemia (Mubarak et al. 1978). Furthermore, Rorabeck and Macnab (1976) found that hypoesthesia and anaesthesia were the last clinical findings to develop (Rorabeck & Macnab 1976). Meanwhile, Ulmer (2002) showed that pain, pain with passive stretch, paraesthesia, and paresis are more useful in ruling out the diagnosis of ACS in the absence of these symptoms rather than making the diagnosis when they are present. He found that the individual sensitivities of each symptom ranged from 13% to 19%, whereas their positive predictive value ranged from 11% to 15%. Meanwhile, both their specificities and negative predictive values ranged between 97% and 98%. Nonetheless, in patients at risk of ACS, he demonstrated that the odds of diagnosing ACS increase with an increasing number of symptoms present; 19% to 26% with one clinical finding, 68%, 93%, and 98% with two, three, and four clinical findings respectively (Ulmer 2002).

Heavy reliance on the five Ps can be misleading: pain out of proportion and pain with passive stretch are important early clinical signs and most reliable indicators in the diagnosis of ACS (Donaldson et al. 2014; Guo et al. 2019; von Keudell et al. 2015). However, they can be confounded by the nature of the injury(ies) suffered, analgesics, altered levels of consciousness, being uncooperative or unconscious, and pain tolerance (Garner et al. 2014; Mortensen et al. 2020). Serial and careful examinations thus become crucial. The other Ps (pallor, pulselessness, paralysis, and paraesthesia) are generally

considered late signs of persistent ischemia. Paraesthesia and muscle weakness can be a result of the injury itself rather than ACS. However, if true weakness is detected, then this could potentially indicate permanent muscle damage (Elliott & Johnstone 2003). Pulselessness is almost only noted in the very late stages of ACS. Tense compartments and increasing analgesic requirements are also important clinical signs that should increase the index of suspicion and must not be neglected (Garner et al. 2014; Matsen 1975; Mubarak et al. 1978).

1.2.3 Use of Compartment Pressure Monitoring

The concept of compartment pressure monitoring was first introduced by Whiteside and colleagues in 1975. Proper technique is critical: monitoring should be done within five centimetres from the zone of injury, and multiple pressure readings should be done rather than relying on one measurement (Heckman et al. 1994; Raza & Mahapatra 2015; Whitesides et al. 1975). Compartment pressure measurement should also be performed in all compartments of the extremity involved, to avoid missing ACS in adjacent compartments (Erdos et al. 2011; Nudel et al. 2016; Whitesides & Heckman 1996). Needle misplacement, faulty monitoring systems, and operator experience can all affect pressure reading (McLaughlin et al. 2014).

Commercial pressure monitoring systems, such as the Stryker Intra-Compartmental Pressure Monitor System (Stryker Instruments, Kalamazoo, MI) or the Synthes Electronic Pressure Transducer (Synthes USA, Paoli, PA) are readily available on the market. However, when resources are limited, ICP

monitoring is often performed using simple hospital equipment (Pechar & Lyons 2016; Raza & Mahapatra 2015). These include the simple needle manometry, wick catheter, slit catheter, central venous manometer, side-ported needle technique, fibreoptic transducer, and infusion technique (Gourgiotis et al. 2007; McLaughlin et al. 2014).

The routine use of ICP monitoring and the threshold for fasciotomy remain controversial. While previous authors have advocated for absolute pressures of greater than 30mmHg (Mubarak et al. 1978), the more recent literature favours the Δp value of less than 30mmHg (McQueen et al. 2000). The Δp is a measurement of the difference between the diastolic blood pressure and the intramuscular pressure. Thus, a value below 30mmHg suggests inadequate perfusion. A growing body of literature supports the role of blood pressure in the role of ICP monitoring, given that tissue perfusion and consequent viability are dependent on blood pressure (Gourgiotis et al. 2007; Whitesides et al. 1975). In patients with tibial fractures, McQueen and Court-Brown (1996) demonstrated that upwards of 43% of patients would have undergone unnecessary fasciotomies had they used ICP measurement alone (McQueen & Court-Brown 1996).

It has been demonstrated that routine compartment pressure monitoring can lead to unnecessary fasciotomies in the alert, oriented, and cognitively sound patient (Guo et al. 2019; Janzing & Broos 2001; Tiwari et al. 2002). In a prospective study of patients with tibial fractures, Janzing and Boos (2001) compared patients who had “true” ACS (i.e. those either having undergone

fasciotomy or having residual physical exam symptoms consistent with compartment syndrome 12 months after injury) with patients without ACS (i.e. those who did not undergo fasciotomy and having no residual symptoms 12 months after injury). The data revealed that unnecessary fasciotomies would have been performed in 6.2%, 3.1%, and 2.1% of patients based on symptoms alone, absolute ICP >30mmg, and Δp <30mmHg, respectively. Meanwhile, if decision to fasciotomy were based on ICP monitoring alone, missed ACS would have been found in 47.4% and 29.0% of patients based on absolute ICP >30mmg and Δp <30mmHg, respectively. Sensitivity and specificity for diagnosing ACS was 0.67 and 0.89, respectively, based on symptoms alone; 0.83 and 0.65, respectively, based on ICP >30mmHg; 0.89 and 0.65, respectively, based on Δp <30 mmHg (Janzing & Broos 2001). In another study of patients with lower extremity injuries but no fasciotomy, 18 out of 19 patients had at least one ICP reading >30mmHg, with 12 patients exceeding 45mmHg and 16 patients with at least one Δp measurement of <30mmHg, without any clinical evidence of ACS (Prayson et al. 2006). A mathematical and quantitative analysis of ACS was also performed, arguing that even though monitoring may be beneficial, it is not yet fully reliable, given the non-uniform pressure distribution in the compartments and that permanent damage to the soft tissues can occasionally occur even before the onset of ACS symptoms (Nudel et al. 2016). Therefore, ICP measurement is recommended as an adjunct, rather than a sole diagnostic tool (Garner et al. 2014).

Some authors have advocated for continuous ICP monitoring in patients at risk of ACS, arguing that it may detect it early, before the onset of clinical symptoms, hence reduce time to fasciotomy. This type of monitoring can be carried out by connecting the compartment catheter to an arterial line transducer. However, obtaining precise measurements can be technically challenging and has a learning curve associated with it (Matsen et al. 1976; McLaughlin et al. 2014; McQueen & Court-Brown 1996; von Keudell et al. 2015). In a review of patients with a tibial fracture in which their compartment pressures were continuously monitored coupled with careful clinical examination did not lead to an increase in the rate of unnecessary fasciotomies (Al-Dadah et al. 2008).

There is no question regarding the benefit of using ICP monitoring in patients with risk factors that could delay the diagnosis of ACS. These include young children, intoxicated patients, patients with equivocal symptoms or altered levels of consciousness, patients with nerve injuries, use of epidural anaesthesia or nerve block, polytrauma patients, and high-energy injuries (Janzing & Broos 2001; McQueen et al. 2000).

1.2.4 Alternative Diagnostic Tools

Other proposed diagnostic tools for ACS include intramuscular oxygen partial pressure monitoring (Doro et al. 2014; Weick et al. 2016), near-infrared spectroscopy (NIRS) (Mancini et al. 1994; Shuler et al. 2011), monitoring localized perfusion using ultrasound or laser Doppler (Abraham et al. 1998; Edwards et al. 1999), and intramuscular glucose and pH monitoring (Doro et al.

2014). In a canine model of ACS, oxygen partial pressure of <30mmHg was reported to have a 100% sensitivity and a 100% specificity as a diagnostic tool. In addition, intramuscular glucose concentrations of <97mg/dL had 100% sensitivity and 75% specificity (Doro et al. 2014). Although the data looks promising, these are still in the experimental phase in human adults.

NIRS has been proposed and has gained more traction in recent years. Its proponents explain that blood flow should increase at the site of injury. A decrease or absence of blood flow could be used as a diagnostic tool for ACS and could be used as a continuous monitoring device (McMillan et al. 2019; Shuler et al. 2011). However, a recent study revealed poor reliability in patients with lower leg injuries, as well as a high number of erroneous NIRS readings (Schmidt et al. 2018).

Magnetic resonance imaging has limited use in the early acute setting, as the swelling and edema are not differentiated between early signs of ACS or from the injury itself. It can, nonetheless, confirm ACS once the diagnosis is established. However, at that point, a significant delay in the treatment would occur (Mabvuure et al. 2012).

1.2.5 Missed or Delayed Diagnosis

Patients may also present with variable findings leading to missed or delayed diagnosis of ACS. Risk factors include patient-controlled or regional anaesthetics, poor communication between physicians, small children, failure of early recognition of signs and symptoms, neurological injuries, and altered levels

of consciousness (Erdos et al. 2011; Garner et al. 2014; Mortensen et al. 2020). The use of compartment pressure monitoring is highly recommended for patients with any risk factor for potential ACS; the lack of monitoring in high-risk patients is a common cause of missed ACS (Al-Dadah et al. 2008; Garner et al. 2014; McLaughlin et al. 2014).

A case report by Wright et al. (2011) described a 39-year-old male with a crush injury to the left lower leg, leading to a minimally displaced fracture of the fibular head. The patient was noted to have absent sensory function over the dorsal first web space (associated with a foot drop), an absent active extension of the great toe (extensor hallucis longus), as well as a firm anterior compartment to palpation. At the same time, very little pain and no significant pain to passive stretch were noted, with distal pulses palpable and normal. Following fasciotomy, the patient quickly started regaining his sensory and motor functions. In his case, the ACS presented in the form of isolated peroneal palsy and an anterior compartment under great pressure (Wright et al. 2011).

Additional case reports described patients who were given patient-controlled analgesia (PCA) following tibial intramedullary nail for tibial shaft fracture (Harrington et al. 2000; O'Sullivan et al. 2002). The PCA masked the pain, delaying the diagnosis of post-operative ACS. While one patient underwent fasciotomy of the lower leg with subsequent closure and no long-term sequelae, another patient ultimately required a below-knee amputation of the leg (Harrington et al. 2000; O'Sullivan et al. 2002).

A study conducted by Hope and McQueen (2004) that compared patients with ACS following fracture and those in the absence of fracture revealed a 13:1 male-to-female ratio in ACS following fracture compared to a 5:1 ratio in ACS without fracture. The patients without fracture were found to be significantly older and with more known medical comorbidities. Consequently, the time from admission to fasciotomy in the non-fracture related patients was delayed, on average, by more than 12 hours, leading to a significantly greater number of patients with muscle necrosis, compared to those in the fracture group (Hope & McQueen 2004).

The diagnosis of ACS in young children can also pose challenges due to communication difficulties. A case series of patients aged 2 to 18 years revealed a mean time from admission to fasciotomy greater than 27 hours (Erdos et al. 2011). Thus, careful serial examination and clinical vigilance to any potential signs of ACS are critical.

Missed or delayed diagnosis of ACS can have serious consequences. Care must be taken to monitor for signs of shock, renal failure, acidosis, hypotension, hyperkalaemia, and hypocalcaemia (Guo et al. 2019). Depending on the amount of muscle(s) involved, missed ACS can lead to ischemic contractures, secondary deformity, permanent neuromuscular deficits, sensory deficits, limb dysfunction, infections especially gram-negative sepsis, amputation, and even death (Donaldson et al. 2014; Elliott & Johnstone 2003; Whitesides & Heckman 1996).

Myonecrosis puts patients at high risk of myoglobinuria due to myoglobin released in the systemic circulation accumulating in the distal convoluted tubules in the kidneys, leading to acute renal failure requiring aggressive fluid hydration (Donaldson et al. 2014; Mabvuure et al. 2012; Whitesides & Heckman 1996). In severe cases of metabolic acidosis, hyperkalaemia, and renal failure, debridement of necrotic muscles is often indicated to prevent and minimize systemic failure as well as metabolic complications (von Keudell et al. 2015).

1.2.6 Time to Irreversible Damage

It is generally accepted that irreversible changes begin around 6 to 8 hours following the onset of ACS, although determining the time of onset of ACS symptoms in itself is often quite challenging (Elliott & Johnstone 2003; Gourgiotis et al. 2007; Guo et al. 2019; Mabvuure et al. 2012; von Keudell et al. 2015). However, reports indicate that irreversible damage can also be seen much earlier, e.g. within 3 hours of ACS onset (Vaillancourt et al. 2004). A study by Sheridan and Matsen (1976) found that early fasciotomies (within 12 hours of onset of ACS) resulted in 68% of extremities with normal function and 4.5% complication rate, while late fasciotomy (mean 37.1 hours from ACS onset) produced only 8 percent of extremities with normal function and a 54% complication rate (Sheridan & Matsen 1976). Another study reported on patients who were operated within three hours of injury already showing evidence of muscle necrosis and complete muscle necrosis in the affected compartment within four hours of injury (Vaillancourt et al. 2004). Moreover, in an animal model

of ACS, Labbe et al. (1987) demonstrated upwards of 90% leg muscle necrosis within 5 hours of ischemia (Labbe et al. 1987).

1.2.7 Surgeon Variability

It has been demonstrated that there is a degree of variability among surgeons in the diagnosis and treatment of ACS (O'Toole et al. 2009). A comparison of seven surgeons within the same institution found a wide variation of ACS diagnosis, ranging from 2% to 24% of the tibial fractures assessed. The use of compartment pressure monitors also greatly varied (1.7% to 28.6%), with similar variation to the percentage of ACS diagnosis (O'Toole et al. 2009).

1.2.8 Medical-Legal Implications

ACS is often subject to litigation. A review found that the factors leading to unsuccessful defences included poor documentation, poor physician communication, lack of action once neurovascular signs and symptoms were identified, increased number of concerning clinical findings, and increased time to surgery (Bhattacharyya & Vrahas 2004). Furthermore, given the magnitude and urgency in the treatment of ACS, information disclosed to obtain informed consent for surgery was often found to be incomplete. Risk of infection, risk of re-operation, the potential need for staged-operations, skin grafts, negative pressure vacuum dressing, renal failure, and long-term complications were often omitted when surgeons were rushed to obtain informed consent (Garner et al. 2014).

1.3 MANAGEMENT OF ACS

ACS constitutes a surgical emergency. When ACS is suspected, all external compressive forces, such as casts or occlusive dressings, should be removed immediately. The affected limb should be elevated to the level of the heart (but not higher) to maximize perfusion (Forsh & Wolinsky 2013; Matsen et al. 1980). If the signs and symptoms of ACS persist, surgical fasciotomy of all compartments remains the gold standard. Although numerous nonoperative treatments of ACS have been studied in various models (tissue ultrafiltration, hyperbaric oxygen therapy, non-steroidal anti-inflammatory drugs (NSAIDs), mannitol, antioxidants), there is no conclusive evidence to support their use as a first-line treatment (Donaldson et al. 2014; Harvey et al. 2012; McLaughlin et al. 2014; McMillan et al. 2019).

1.3.1 Limb Anatomy

1.3.1.1 Leg

The lower leg is the most commonly affected limb in ACS. It includes two bones (tibia and fibula) and is divided into four compartments: anterior, lateral, deep posterior, and superficial posterior (Thompson 2016) (Figure 1.1).

The anterior compartment includes the tibialis anterior, extensor hallucis longus, extensor digitorum longus, and peroneus tertius muscles. These muscles function primarily in foot and ankle dorsiflexion, are innervated by the deep peroneal nerve, and the blood supply mainly comes from the anterior tibial artery. The compartment is delineated by the tibia medially, the interosseous membrane

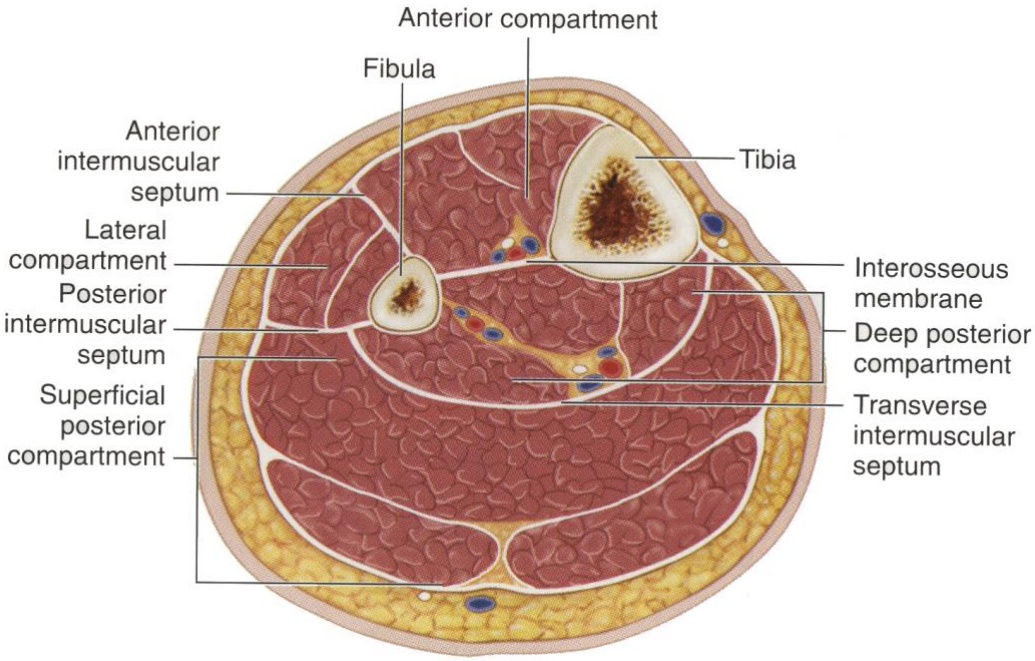


Figure 1.1 **Compartments of the leg**. Cross-sectional view of the leg including the tibia and fibula as well as muscles separated into four osseofascial compartments: anterior, lateral, deep posterior, and superficial posterior.

Reproduced with permission from Lawendy and Sanders (2010).

and fibula posteriorly, and the anterior intermuscular septum laterally (Thompson 2016).

The lateral compartment includes the peroneus long and brevis muscles. They are innervated by the superficial peroneal nerve and are supplied by the anterior tibial and fibular arteries. The muscles function as the primary foot evertor while having secondary functions in ankle plantarflexion. The fibula borders the compartment medially while the anterior and posterior intermuscular septum borders the front and back, respectively (Thompson 2016).

The muscles in the posterior compartments are mainly responsible for the plantarflexion of the foot and ankle. While the tibial and fibular arteries, as well as the tibial nerve, reside in the deep posterior compartment, they are responsible for vascularizing and innervating both posterior compartments. The deep posterior compartment includes the flexor hallucis longus, flexor digitorum longus, tibialis posterior, and popliteus muscles while the superficial posterior compartment is comprised of the gastrocnemius, soleus, and plantaris muscles. The compartments are separated by the transverse intermuscular septum (Thompson 2016).

1.3.1.2 Forearm

The forearm is the second most commonly affected limb in ACS. The forearm, along with the radius and ulna bones, contains three compartments: the anterior forearm (divided into superficial, intermediate, and deep layers), the

posterior forearm, and the mobile wad (brachioradialis, extensor carpi radialis longus and brevis) (Thompson 2016) (Figure 1.2).

The anterior forearm muscles are primarily responsible for hand and wrist flexion, as well as forearm pronation. These muscles are either innervated by the median nerve or by the anterior interosseous nerve, a branch of the median nerve. They are vascularized by both the ulna and radial arteries (Thompson 2016).

The posterior (or dorsal) forearm muscles are primarily responsible for hand and wrist extension as well as forearm supination. These muscles are innervated by the posterior interosseous nerve, a branch of the radial nerve. They are vascularized by branches of the radial and ulnar arteries (Thompson 2016).

The mobile wad muscles act as secondary elbow flexors proximally. While the brachioradialis also assists radioulnar joint supination and pronation during elbow flexion, the extensor carpi radialis longus and brevis allow wrist extension and hand abduction. The mobile wad is innervated by the radial nerve (brachioradialis and extensor carpi radialis longus) as well as the posterior interosseous nerve (extensor carpi radialis brevis). They are vascularized by the radial artery and brachii profunda (Thompson 2016).

1.3.2 Fasciotomy

The concept of fasciotomy surfaced early in the 1900s. As physicians attempted to provide treatment for Volkmann's ischemic contracture, multiple

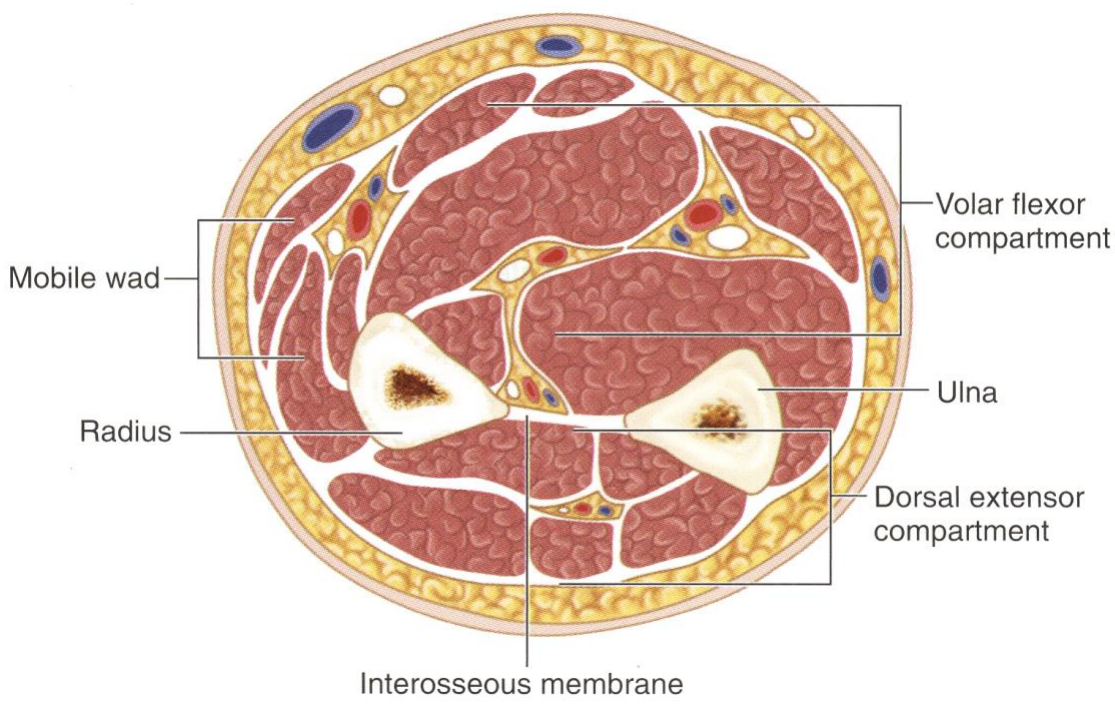


Figure 1.2 **Compartments of the forearm**. Cross-sectional view of the forearm including the radius and ulna as well as muscles separated into three different osseofascial compartments: dorsal, volar and mobile wad.

Reproduced with permission from Lawendy and Sanders (2010).

reports of myotomies and nerve release were published (Thomas 1909). In 1911, Bardenheuer described a fasciotomy-type technique, termed aponeurectomy, as an operative treatment for patients with impending Volkmann's contracture (Bardenheuer 1911). The procedure was then advocated by Murphy (1914), who argued that ischemic paralysis was caused by venous obstruction, hence splitting the deep fascia would relieve the pressure (Murphy 1914). Jepson (1926) also reported that decompression helped prevent ischemic contractures (Jepson 1926).

Fasciotomy has since become the forefront of treatment for ACS (Sheridan & Matsen 1976). By releasing the skin and fascia, the fasciotomy procedure decompresses the affected compartment(s), allowing the soft tissues to expand and increase in volume, immediately reducing the compartment pressures (Schmidt 2017).

1.3.2.1 Threshold for Fasciotomy

Controversy and variability exist when making decisions to intervene. While some authors advocate for continuous pressure monitoring or routine compartment pressure checks on all patients at risk, it is generally accepted that, in the absence of risk factors, the diagnosis of ACS and the decision to intervene surgically can be made based on clinical symptoms alone. However, if there are any equivocal symptoms or ambivalence, compartment pressure measurements should be added as a diagnostic tool (Garner et al. 2014; Mortensen et al. 2020).

The thresholds for compartment pressure measurements are also controversial. The evidence is shifting towards the use of Δp (McQueen & Court-Brown 1996); however, false positive rates may be more significant than previously believed. A study of patients with tibial shaft fractures but no clinical evidence of ACS and who did not undergo fasciotomy reported that if the threshold for surgery of Δp <30mmHg and <20mmHg had been applied, 35% and 24% of patients, respectively, would have met the criteria for fasciotomy based on compartment pressures alone (Whitney et al. 2014).

1.3.2.2 Techniques for Fasciotomy

The leg fasciotomy can be done either through a 1-incision technique laterally or two incisions medially and laterally (Forsh & Wolinsky 2013; von Keudell et al. 2015) (Appendix I). In the forearm fasciotomy, the volar incision is performed using either a radial or ulnar curvilinear incision. If needed, a dorsal incision extending from the lateral epicondyle to the centre of the wrist at the level of the radioulnar joint is made for the posterior forearm (Forsh & Wolinsky 2013; Gourgiotis et al. 2007) (Appendix I).

1.3.2.3 Delayed Fasciotomy

Some authors argue that fasciotomy should be considered even during late presentation due to the potential of salvaging any remaining muscle function (Olson & Glasgow 2005), as well as decreasing the risk of systemic complications such as rhabdomyolysis and renal failure (von Keudell et al. 2015).

No guidelines exist, particularly for patients with clearly missed ACS, no viable muscle function following the onset of symptoms for more than 24 to 48 hours, and no nerve injury or nerve block to alter the examination. In this scenario, supportive care has been the main indication, including adequate fluid resuscitation, analgesics, and splinting, unless there is evolving limb necrosis or systemic involvement requiring source control (Mabvuure et al. 2012; Schmidt 2017; von Keudell et al. 2015).

Finkelstein et al. (1996) reported that delayed fasciotomy (patients underwent fasciotomies at an average 56 hours after lower limb ACS was established) can result in death due to multi-organ failure or amputation due to infection and sepsis (Finkelstein et al. 1996). Another study reported an infection rate of 28% in patients undergoing late fasciotomy (>12 hours) (Williams et al. 1997). Complication rates of 4.5% were reported for an early fasciotomy (less than 12 hours after onset of ACS, with a mean 5.3 hours) and 54% for late fasciotomy (more than 12 hours after onset of ACS, with a mean 37.1 hours). Additionally, only 8% of patients in the late group regained normal function (Sheridan & Matsen 1976).

1.3.2.4 Complications of Fasciotomy

Complication rates of fasciotomy are significant. In a study by Fitzgerald et al. (2000), long-term complications of fasciotomy included altered sensation around the wound margins (77%), persistent wound pain (10%), dry scaly skin (40%), pruritus (33%), discoloured wound (30%), swollen extremity (25%),

tethered scar (26%), recurrent ulceration (13%), muscle herniation (13%), and tethered tendon (7%). Furthermore, 12% and 28% of patients stated that they changed occupation and hobbies, respectively, following fasciotomy (Fitzgerald et al. 2000). Other complications of fasciotomy include delayed wound closure, need for surgical reconstruction with grafts or flaps, chronic pain, nerve injury, infection, permanent muscle weakness, chronic venous insufficiency, renal failure, and death (Schmidt 2017).

1.3.3 Alternative Treatments

To-date, open fasciotomy remains the gold standard in the treatment of ACS. Given the morbidity and potential complications, non-surgical management is rather used as an adjunct to fasciotomies or when fasciotomies are absolutely contraindicated (Donaldson et al. 2014; McMillan et al. 2019). Several different techniques have been tested in various models of ACS: tissue ultrafiltration, hyperbaric oxygen, mannitol, anti-inflammatory medications, and antioxidants.

1.3.3.1 Tissue Ultrafiltration

Odland and colleagues explored the use of tissue ultrafiltration (TUF) as a potential therapy for ACS. The technique consists of an insertion of small hollow fibers via catheters into muscle compartments to remove interstitial fluid and thus decrease compartmental pressures (Harvey et al. 2012). The technique was first tested in a porcine model of hindlimb ACS by Odland et al. (2005), demonstrating decreased intramuscular pressure and reduced muscle necrosis in the treated

limb (Odland et al. 2005). A small, randomized pilot study was then undertaken in 10 patients with tibial fracture. Although no patients suffered from ACS during the study period, one patient developed ACS in the lateral compartment of the lower leg and underwent a 4-compartment fasciotomy 22 hours after the TUF catheter was removed and 46 hours after fracture fixation (Odland & Schmidt 2011).

1.3.3.2 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy allows the delivery of 100% oxygen to the patient (normal atmospheric air contains 21% oxygen). In a case report of a 43-year-old male bitten by a Western diamondback rattlesnake who developed ACS and was recommended fasciotomy by the consulting orthopaedic surgeon, due to the patient's refusal of fasciotomy, the therapy consisted of antivenom, mannitol, and hyperbaric oxygen; the patient fully recovered from the envenomation without any long-term complications (Gold et al. 2003).

Given the high cost of equipment and paucity of evidence, hyperbaric oxygen therapy has been largely abandoned as a treatment of ACS (Greensmith 2004), although limited evidence shows that it does offer some value as an adjunct to standard ACS therapy.

1.3.3.3 Mannitol

Mannitol, a type of sugar alcohol, has previously been suggested to offer protective effects as an osmotic agent, thereby decreasing muscle edema (Daniels et al. 1998; Shah et al. 1996). Successful treatment of ACS using

mannitol was reported in some case reports: a patient presenting with a heat stroke who later developed ACS of the anterior compartment of the right lower leg was treated with mannitol alone and developed only mild residual weakness in the right lower extremity (Daniels et al. 1998); a snakebite that progressed into ACS was treated with mannitol as an adjunct (along with hyperbaric oxygen and antivenom) resulting in full recovery and no long-term complications (Gold et al. 2003).

1.3.3.4 Anti-Inflammatories

Significant leukocyte activation (i.e. inflammation) is one of the driving forces of ACS pathophysiology (Lawendy et al. 2015). As such, the potential of non-steroidal anti-inflammatory medications as a therapy for ACS has also been explored in animal models. Cyclooxygenase (COX) enzymes play a key role in prostaglandin formation, mediating inflammatory processes. While COX-1 is expressed constitutively, COX-2 enzymes are upregulated during pro-inflammatory state (Tsatsanis et al. 2006). In a rat model of ACS, Manjoo et al. (2010) demonstrated improved muscle perfusion and decreased tissue damage in animals treated with indomethacin, a COX-2 inhibitor. The observed effects were a result of indomethacin's inhibitory effects on activated neutrophils and its effects as a free radical scavenger. However, it did not affect the apoptotic activity seen during ICP elevation (Manjoo et al. 2010).

1.3.3.5 Antioxidants

Antioxidants have potential benefits in situations of oxidative stress-induced microvascular injuries such as trauma, ischemia-reperfusion, sepsis, and organ failure (Oudemans-van Straaten et al. 2014; Spoelstra-de Man et al. 2018). A previous study of limb ACS demonstrated that exogenous administration of superoxide dismutase (SOD) antioxidant (an intrinsic enzyme) and hydroxyl radical inhibitors decreased compartment pressure rise and improved muscle perfusion (Perler et al. 1990). Meanwhile, the administration of SOD and mannitol, a hydroxyl radical scavenger, in a canine model of ACS significantly reduced muscle necrosis compared to control groups but did not prevent compartment pressure rise (Ricci et al. 1990).

Vitamin C (ascorbic acid) has many antioxidant and immune-supporting effects such as helping the mediation of direct free radical scavengers, reducing reactive oxygen species (ROS) production, and increasing the immune response (Spoelstra-de Man et al. 2018). It has been shown that in a rat model of ACS within the cremaster muscle, vitamin C pre-treatment in conjunction with decompression (fasciotomy equivalent) helped maintain muscle function and decreased extracellular neutrophil infiltration and soft tissue edema (Kearns et al. 2004).

N-acetyl cysteine (NAC), a precursor of glutathione, is a potent antioxidant by acting as a free radical scavenger (Lejay et al. 2018). Kearns et al. (2010) demonstrated preserved muscle contractility, decreased oxidant injury, and

improved muscle viability following decompression and NAC treatment in a rodent cremasteric striated muscle model of ACS (Kearns et al. 2010).

1.4 PATHOPHYSIOLOGY OF ACS

1.4.1 Historical Perspective

The first description of ACS originates in 1881 by Volkmann. Following his observation on outcomes of bandages being applied too tightly, he suggested that ischemic contractures and limb paralysis secondary to ACS following traumatic injuries were a result of arterial obstruction rather than neurologic injuries (Volkmann 2007; von Volkmann 1881). Greater attention was brought in 1884 following Leser's report of seven upper extremity cases treated with plaster cast and consequent contractures (Leser 1884; Thomas 1909). However, much debate remained regarding the pathophysiology of ACS. Over the next century, multiple theories have been postulated, including arterial injuries and arterial spasms (Griffiths 1940; Plewes 1940), venous obstruction (Bardenheuer 1906; Brooks 1922; Jepson 1926; Murphy 1914; Plewes 1940), nerve injuries (Hildebrandt 1906; Thomas 1909), and thromboembolism (Lander 1895; Thomas 1909).

In the 1930s and 1940s, the correlation between increased tissue pressures and ischemia started surfacing (Hughes 1948; Lewis 1936). Although fasciotomy or fasciotomy-type procedures had been previously described, the concept of fascial splitting or decompression became more widely accepted and emphasized in the treatment of ACS in the 1950s (Benjamin 1957; Mavor 1956;

Reneman 1975). In 1975, Matsen unified the concepts from the scientific evidence gathered over the years. He emphasized that the underlying pathophysiology of ACS was the same, regardless of aetiology, and could occur within any closed space, regardless of location. Moreover, immediate surgical decompression of the affected compartments is crucial while suggesting that the pressure required to cause irreversible damage was not an absolute value but rather dependent on local factors such as time under elevated ICP, vascular tone, local blood perfusion, and metabolic rates (Matsen 1975).

While many theories have been postulated, the underlying mechanism leading to tissue ischemia, myonecrosis, and loss of limb function during sustained ICP elevation is not yet fully understood. However, it is widely accepted that increased compartment pressures due to inelastic fascia following a bony or soft tissue insult leads to progressive microvascular dysfunction and tissue hypoperfusion. This leads to impaired gas exchange and inability to meet tissue metabolic demands (Schmidt 2017; von Keudell et al. 2015). Furthermore, elevated ICP-generated ischemia is associated with leukocyte recruitment, as well as local and systemic pro-inflammatory cascades, ultimately leading to cellular anoxia and death, tissue necrosis, and potential loss of limb function (Donohoe et al. 2018; Lawendy et al. 2015). It is also important to note that unlike complete ischemia, the deleterious effects of ACS occur despite a patent macrocirculation where distal pulses are usually palpable unless there is presence of associated arterial injury. Furthermore, pulselessness is an end-

result of ACS rather than the cause of tissue necrosis (Gourgiotis et al. 2007; Hoekman et al. 2005; Leach et al. 1967; Seddon 1966; Whitesides et al. 1975).

1.4.2 Microcirculation in ACS

1.4.2.1 Normal Microcirculation

In normal skeletal muscle, arterioles, capillaries, and venules form the microcirculatory system (Figure 1.3). Gas exchange and nutrient delivery occur in the capillaries of terminal vascular beds. In the terminal vascular beds, arterioles divide into multiple closely spaced capillaries situated along parenchymal cells to allow oxygen and nutrient exchange. Following material exchange, the blood flows through the venules and into the systemic venous circulation. In the microcirculatory system, all vessels are comprised of a single layer of vascular endothelial cell lining sitting on a layer of extracellular matrix (basement membrane). The arterioles are surrounded by smooth muscle mesenchymal cells to allow contractility, while capillaries and post-capillary venules are surrounded by pericytes rather than smooth muscle cells. The post-capillary venules also have a rather larger cross-sectional area compared to the corresponding arterioles resulting in decreased luminal pressures. This allows perfusion through an arteriovenous pressure gradient (Brown et al. 2006).

1.4.2.2 Microcirculatory Dysfunction

The exact mechanism by which microcirculatory dysfunction and tissue ischemia occur remains poorly understood. Multiple theories have been

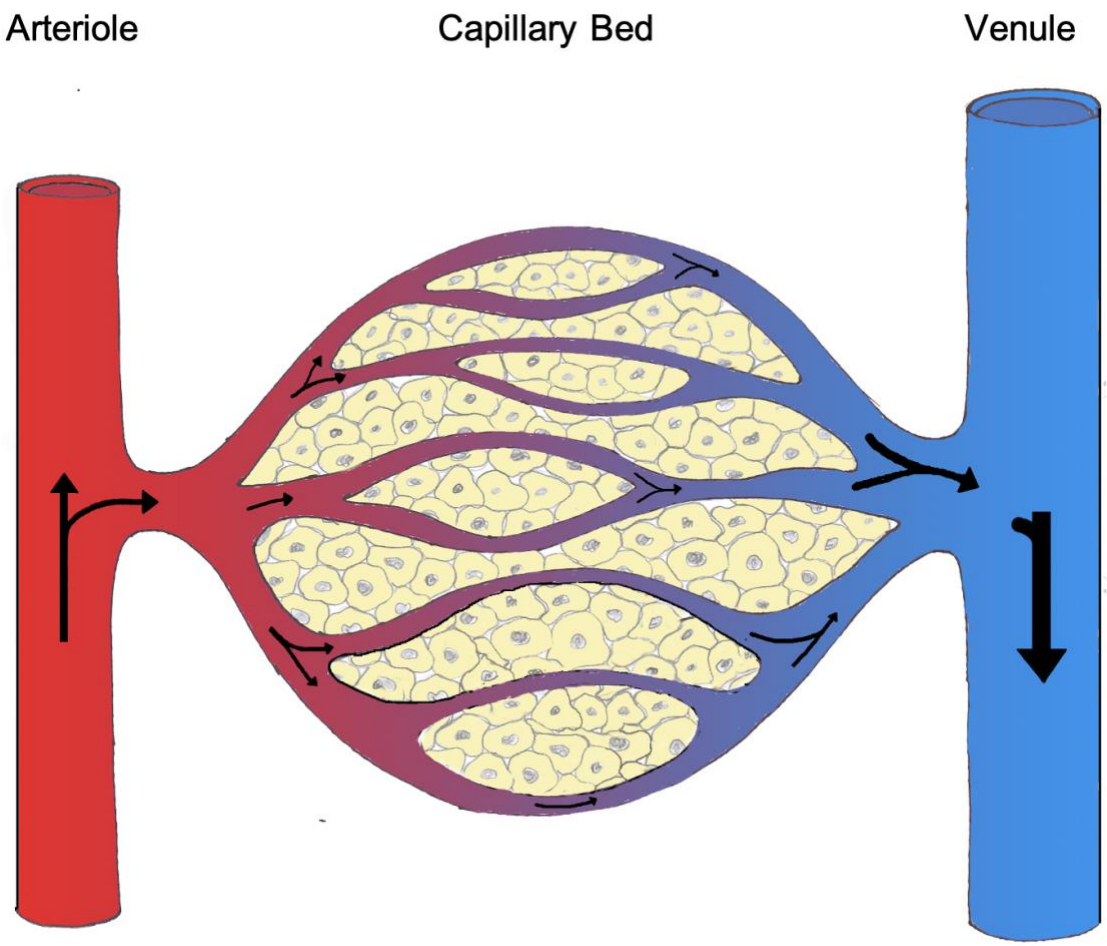


Figure 1.3 **Microcirculation of the skeletal muscle**. Normal microcirculation where an arteriole branches into capillaries. Oxygenated blood flows from the arterioles (red) into the capillary beds and deoxygenated blood flows from the capillary beds to collect into the post-capillary venules (blue).

Reproduced with permission from Bihari (2017).

postulated in an attempt to explain the phenomenon in relation to increased compartment pressures, including the critical closing pressure theory, microvascular occlusion theory, and arteriovenous (AV) gradient theory. The critical closing pressure theory suggests that the intravascular arteriolar mural tension must be sufficiently elevated to overcome extravascular tissue pressure. Disruption of this equilibrium and an extravascular ICP above the arteriolar mural pressure in compartment syndrome leads to abrupt arteriole occlusion resulting in ischemia (Burton 1951; Singh et al. 2004). Additionally, Ashton (1975) also postulated other mechanisms, including active arteriolar closure mediated by vasomotor tone due to a decrease in intravascular pressure or extravascular pressure increase (Ashton 1975). However, the theory has been questioned when a rabbit model of ACS revealed increased venous pressures and total intramuscular pressures, while arteriolar dilatation occurred (Reneman et al. 1980). Additionally, in a rodent model, Vollmar et al. (1999) demonstrated no signs of arteriolar collapse or spasm with elevated intracompartmental pressures despite blood flow cessation and decreased venular diameter (Vollmar et al. 1999).

In the microvascular occlusion theory, it is assumed that increased ICP leads to capillary collapse as tissue pressures overcome intracapillary pressures. Given the low baseline capillary pressures, small extravascular pressure increments can lead to a reduction in blood flow and subsequent ischemia (Ashton 1975; Hargens et al. 1978). However, Hartsock et al. (1998)

demonstrated, in a rat model, that capillaries did not collapse despite an increase in ICP and even complete cessation of capillary perfusion (Hartsock et al. 1998).

The assumption behind the AV gradient theory is that with rising intracompartmental pressures, the intraluminal venous pressures also increase, causing a decrease in the AV gradient and subsequent decrease in perfusion. This leads to a rise in interstitial pressure due to the extrusion of fluids into the interstitial space. While the continuous arterial perfusion contributes to increasing edema and swelling, the increasing extravascular pressures eventually decrease arterial perfusion, and the cellular metabolic demands are no longer met (Elliott & Johnstone 2003; Mabvuure et al. 2012; McLaughlin et al. 2014; McMillan et al. 2019). In animal models of ACS, it has been shown that increasing ICP and extravascular pressure resulted in the reduction of venule diameter, thus decreasing local perfusion and effectively decreasing the AV gradient, without complete closure of vessels (Hartsock et al. 1998; Reneman et al. 1980; Slaaf et al. 1987; Vollmar et al. 1999). The AV gradient theory is the most widely accepted theory explaining the relationship between the microcirculation and increased ICP. However, it only accounts for passive microvasculature changes in response to pressure changes and does not consider local adaptive responses, endothelial structural changes, and the inflammatory responses associated with ACS.

1.4.3 Ischemia and Reperfusion

The exact underlying pathophysiology of ACS still needs to be fully elucidated. However, the consequences of untreated ACS appear to share characteristics with ischemia-reperfusion (I-R) injuries. In general, morbidity and cellular death from ischemic events alone are either a result of prolonged ischemia (vasculopathy) or lack of oxygen supply due to interrupted blood flow for periods beyond the organs' hypoxic threshold (Grace 1994). In ischemic events, depletion of intracellular adenosine triphosphate (ATP) leads to cellular membrane damage and loss of transmembrane electrical potential. This leads to acidosis, osmotic shock, and intracellular calcium overload, with eventual irreversible mitochondrial damage, protein and enzymatic leakage, and cellular apoptosis (Grace 1994; Hamburg & Creager 2017; Sharfuddin & Molitoris 2011). However, histologic findings are often 'normal' with preserved tissue architecture and acellularity, while I-R injuries are characterized by tissue necrosis and neutrophil infiltration, also observed in ACS (Epstein & McCord 1985; Francis & Baynosa 2017; Lawendy et al. 2011, 2014). Therefore, understanding the inflammatory process of I-R injury may help elucidate the mechanisms that lead to myonecrosis and systemic complications in ACS.

1.4.3.1 Complete Versus Partial Ischemia

ACS is believed to be a type of I-R injury (Francis & Baynosa 2017; Gourgiotis et al. 2007; Lawendy et al. 2015). However, the degree of ischemia in ACS remains questioned. Perry and his colleagues have previously compared

the effects of 3 hours of partial ischemia and complete ischemia, followed by reperfusion in canine animal models. Although both the complete and partial ischemia induced cell membrane dysfunction, partial ischemia led to greater damage than complete ischemia (Perry 1988; Perry et al. 1984; Roberts et al. 1985). Heppenstall and colleagues (1986) demonstrated that increased ICP resulted in decreased levels of ATP, more severe muscle acidosis and cellular degeneration compared to tourniquet-induced I-R (Heppenstall et al. 1986). Conversely, Conrad et al. (2005) found that partial ischemia resulted in significantly greater early inflammatory cytokine production than incomplete ischemia. However, after 24 hours of reperfusion, less tissue viability was found following complete ischemia (Conrad et al. 2005).

1.4.3.2 Low-Flow Ischemia

Recent animal model studies have demonstrated ACS as a “low-flow” ischemic state (Lawendy et al. 2011, 2015) (Figure 1.4). Normal microvascular perfusion is predominantly of continuous type (Lawendy et al. 2011; Sadasivan et al. 1997). However, as the ICP increases, capillaries progress to intermittency and non-perfusion, although some capillaries remain continuously perfused, thus decreasing gas exchange and nutrient delivery overall. This partial ischemic state results in a significant degree of leukocyte activation, cytokine production, and tissue necrosis (Donohoe et al. 2018; Lawendy et al. 2011, 2015). Therefore, while the underlying pathophysiology of ACS may not be due to pure I-R mechanisms, it is thought to be a result of perpetual I-R injuries leading to severe

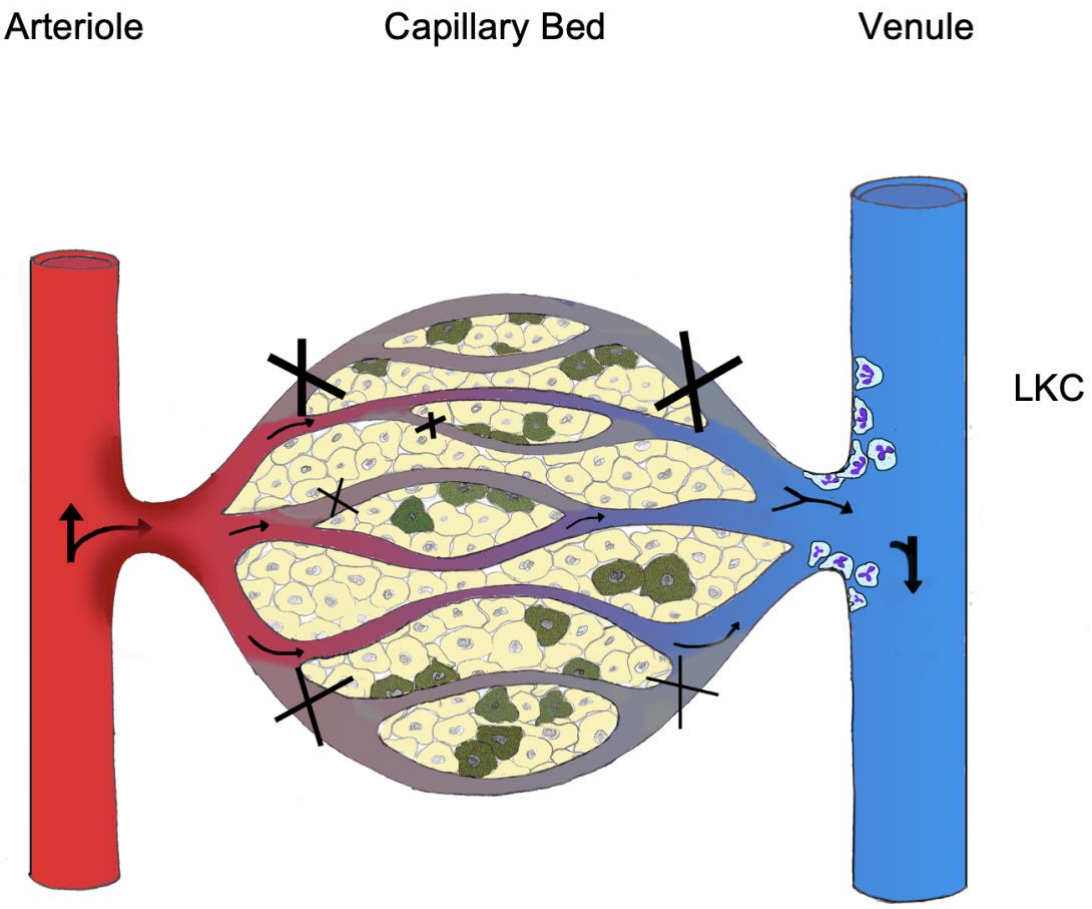


Figure 1.4 **Microcirculatory dysfunction in compartment syndrome**. Hypo-perfusion of the capillary beds (low-flow ischemia) causes tissue injury (brown cells). Subsequently, leukocyte activation occurs in post-capillary venules and results in inflammation. *LKC*: leukocytes. *Reproduced with permission from Bihari (2017).*

microvascular dysfunction, tissue ischemia, and eventual macrocirculation deficits.

1.4.4 Reperfusion Injury

Logically, reperfusion of ischemic tissues serves to restore blood flow, tissue oxygenation, nutrient delivery, and removal of toxic metabolites. Paradoxically, it further exacerbates tissue injury and cell death.

The concept of reperfusion injury surfaced in the early 1940s following air raids during World War II. Patients with crush injuries had localized symptoms but were systemically well. However, upon restoration of blood flow, they were found in shock within hours and many passed away within one week (Bywaters & Beall 1941; Bywaters et al. 1941). Reperfusion following ischemia results in multiple systemic disturbances, including metabolic acidosis, hyperkalaemia, myoglobinemia, myoglobinuria, and ultimately renal failure (Erdos et al. 2011). The systemic return of acidotic blood with venous blood pH usually below 7.2 causes metabolic acidosis, while intracellular potassium (cation) leakage leads to hyperkalaemia and could be severe enough to cause death. Additionally, intracellular enzymes, such as creatinine phosphokinase, lactic acid dehydrogenase, and glutamic-oxaloacetic transaminase, can lead to rhabdomyolysis (Grace 1994). Furthermore, ischemic muscles release myoglobin that can lead to acute renal failure (Matsen 1975; von Keudell et al. 2015).

Re-introduction of oxygen molecules leads to the formation of ROS, metabolites, endothelial alterations, increased leukocyte activation, neutrophil

infiltration, inflammatory cascades, and changes in microvascular permeability (Francis & Baynosa 2017). Several studies have shown that reperfusion of ischemic tissues with anoxic (without oxygen) blood or limited oxygenated blood produced considerably less tissue injury (Gute et al. 1998). Consequently, ischemic tissue injuries appear to be exacerbated by the formation of ROS (Grace 1994; Gute et al. 1998; Wright et al. 1988).

1.4.4.1 Reactive Oxygen Species and Free Radicals

ROS are oxygen-containing reactive molecules that display much greater reactivity with other molecules than molecular oxygen (O_2) does. Free radicals are a type of ROS as they contain one or multiple unpaired electrons (Toyokuni 1999). ROS normally play an important role in regulating cell signalling, growth, and apoptosis. They are also vital in regulating immune, thyroid, and cognitive functions (Brieger et al. 2012). However, when ROS levels are supraphysiologic and overwhelm the antioxidant system, they become detrimental, leading to I-R injuries and various diseases, such as oncologic, cardiovascular, neurologic, and psychiatric disorders (Brieger et al. 2012; Francis & Baynosa 2017; Lushchak 2015; Toyokuni 1999).

Multiple sources of ROS have been described in biological systems, such as xanthine oxidase (XO) metabolism, by-products of leukocyte activation, mitochondrial electron transport chain, arachidonic acid metabolism, catecholamine autoxidation, endothelial cells, and prostaglandins (Cowled & Fitridge 2011; Gute et al. 1998). The evidence suggests that XO metabolism and

leukocyte activation are the main drivers of ROS production in I-R. Superoxide anion and hydrogen peroxide appear to be the most commonly produced ROS, while others include, but are not limited to, hydroxyl radical, nitric oxide, and peroxyl radical (Brieger et al. 2012; Cowled & Fitridge 2011; Grace 1994; Toyokuni 1999).

XO is a primary producer of ROS in I-R injuries: during the ischemic phase, glycogen breakdown in the mitochondria leads to increased lactic acid, resulting in a tissue pH drop, subsequently inhibiting ATP production and decreasing the transmembrane gradient. ATP is subsequently broken down into adenosis, inosine, hypoxanthine, and xanthine. Concomitantly, as the transmembrane gradient diminishes, intracellular sodium rises while potassium escapes into the extracellular space, and calcium is released from the mitochondria into the cytoplasm (Cowled & Fitridge 2011). Calpain, a calcium-dependent cytosolic protease, is then activated, converting xanthine dehydrogenase (XD) to XO. During reperfusion, molecular oxygen reacts with XO, catalysing the formation of uric acid and superoxide anion. Superoxide anion can further be converted to hydrogen peroxide and hydroxyl radicals (Cowled & Fitridge 2011; Epstein & McCord 1985; Perry & Fantini 1987) (Figure 1.5). In contrast, in its physiologic role, XD reacts with nicotinamide adenine dinucleotide (NAD^+), converting hypoxanthine to xanthine and NAD^+ to nicotinamide adenine dinucleotide phosphate (NADPH) rather than reacting with molecular oxygen, and does not produce ROS (Gute et al. 1998).

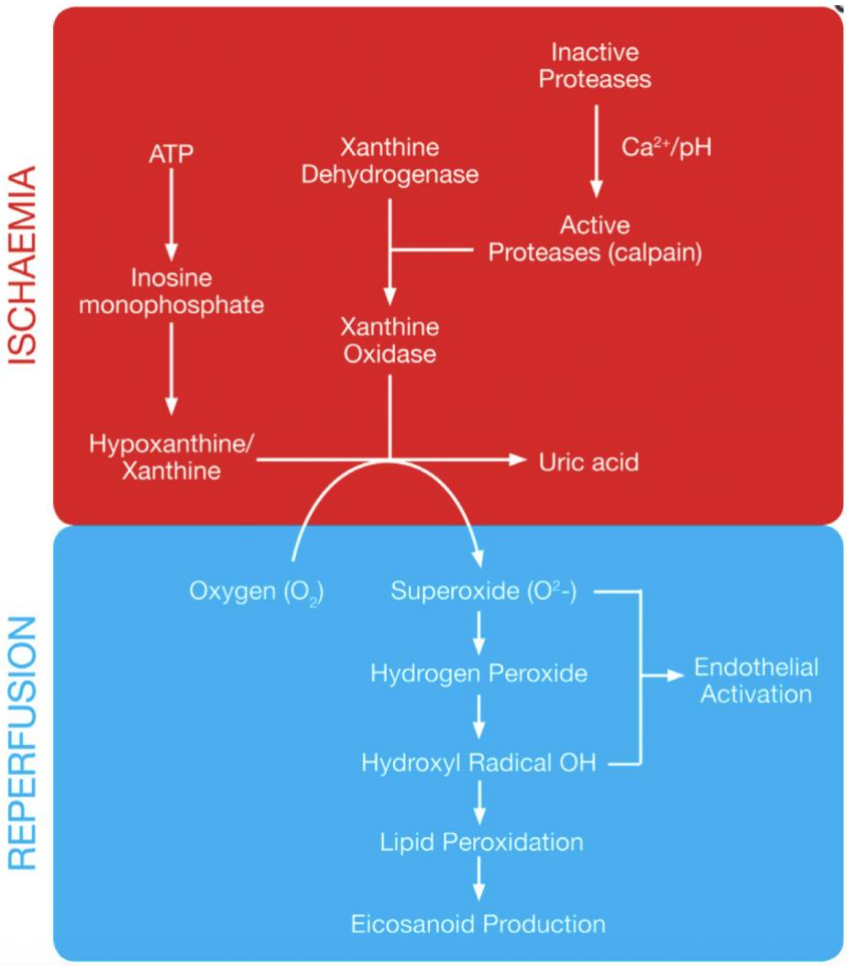


Figure 1.5 Generation of reactive oxygen species in ischemia-reperfusion. During ischemia, ATP is degraded and XD converted to XO. Upon reperfusion, XO catalyses the conversion of hypoxathine to superoxide anions; these then initiate the production of hydrogen peroxide and superoxide radical, leading to lipid peroxidation and tissue damage.

Reproduced with permission from Cowled and Fitridge (2011).

The evidence that free radicals play a major role in I-R has been shown in multiple experiments. Superoxide dismutase (SOD), a free radical scavenger, has been shown to decrease free radical levels and tissue damage (Gute et al. 1998; Perler et al. 1990; Perry & Fantini 1987; Salvemini & Cuzzocrea 2003).

Other endogenous antioxidants, such as catalase, glutathione peroxidase, histidine, and vitamin E play a significant role in maintaining equilibrium and physiologic levels of ROS; however, during I-R, the antioxidant system becomes overwhelmed and is unable to maintain its scavenging function (Francis & Baynosa 2017).

1.4.4.2 Lipid Peroxidation

A major consequence of ROS production is structural and functional damage to the phospholipid-derived cell membrane (Gaschler & Stockwell 2017; Grace 1994). Following reperfusion, superoxide contributes to free ferrous ion release, catalysing the formation of hydroxyl radicals and peroxyl radicals from hydrogen peroxide. These radicals then remove a hydrogen atom in the methylene group (-CH_2-) between two unsaturated bonds (double covalent bonds) in the lipid molecule, which creates a new carbon-centred radical. When this new carbon-centred radical reacts with molecular oxygen, a lipid-peroxyl radical (ROO^-) is formed; it then abstracts another hydrogen atom from another methylene group in the cell membrane, forming lipid peroxide (ROOH). Molecular oxygen then reacts with the newly created carbon-centred radical, creating a chain reaction, ultimately leading to cellular permeability and cell death (Cowled & Fitridge 2011; Gaschler & Stockwell 2017; Grace 1994).

1.4.4.3 Thromboxane

Plasma thromboxane A_2 is derived from arachidonic acid that is released from lipid peroxidation. Released within minutes of I-R injuries, it promotes vasoconstriction and platelet aggregation (Mazolewski et al. 1999). A rat model of I-R with administration of thromboxane A_2 receptor antagonist demonstrated a significant decrease in vasoconstriction (Mazolewski et al. 1999).

1.4.4.4 Leukocyte Activation

The production of ROS has a downstream effect on leukocyte activation and endothelial injury. Superoxide promotes the production of inflammatory mediators such as platelet-activating factor and leukotrienes. It also activates leukocyte chemoattraction and endothelial adhesion proteins, leading to neutrophil infiltration (Kubes et al. 1990a; Lehr et al. 1991; Lewis et al. 1988; Rubanyi & Vanhoutte 1986). Leukocyte chemoattraction creates an additive effect of ROS production through NADPH oxidase (Gute et al. 1998).

Leukocytes contain NADPH oxidase and can oxidize O_2 into superoxide; superoxide is further converted to hydrogen peroxide (Cowled & Fitridge 2011; Gute et al. 1998). Activated neutrophils undergo degranulation and release myeloperoxidase (MPO), which then catalyses the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions. This ROS activates the secretion of matrix metalloproteinases (MMPs). MMP secretion is tightly controlled and plays an essential role in angiogenesis, arteriogenesis, and extracellular matrix remodelling (Dejonckheere et al. 2011; Hobeika et al. 2008).

However, during I-R, MMP upregulation leads to further basement membrane and tissue destruction (Cowled & Fitridge 2011; Dejonckheere et al. 2011; Gute et al. 1998; Hobeika et al. 2008).

Leukocyte proteinases, such as neutrophil elastase and MMPs, further amplify leukocyte adherence and endothelial transmigration (Owen 2008; Stowe et al. 2009). Previous mice studies with either neutrophil elastase or MMP-9 deficiency demonstrated diminished leukocyte adherence, decreased brain infarct size and blood brain barrier deficiency, and reduced extracellular matrix destruction in cerebral I-R (Gidday et al. 2005; Stowe et al. 2009). Other animal studies showed significant decrease in liver injury (Okajima et al. 2004; Soejima et al. 1999), renal injury (Matsuyama et al. 2008), and pulmonary injury (Ishikawa et al. 2003) following neutrophil elastase inhibition in the context I-R. MMP inhibition also showed similar effects in animal studies of cardiac (Cheung et al. 2000; Wang et al. 2002), cerebral (Chen et al. 2018), and renal as well as other tissue models (Kunugi et al. 2011) of I-R. Neutrophil elastases have been shown to independently promote neutrophilic and endothelial receptors that allow leukocyte adherence and transmigration (Woodman et al. 1993; Yamaguchi et al. 1998) while MMPs have degrading effects on the basement membrane, extracellular matrix, and interstitial tissue (Cheung et al. 2000; Wang et al. 2002, Kunugi et al. 2011).

Platelet-activating factors also play detrimental roles in causing interstitial edema and leukocyte adhesion to the endothelium. Free radicals promote the activation of phospholipase A_2, which then leads to the formation of platelet-

activating factors, a leukocyte chemoattractant. Lewis (1988) demonstrated that hydrogen peroxide catalysed the synthesis of platelet-activating factors via the endothelium, inducing leukocyte-endothelial adhesion (Lewis et al. 1988). Platelet-activating factor antagonists have also been found to play a role in attenuating leukocyte adhesion and cellular extravasation (Kubes et al. 1990a,b).

Lipid peroxidation of the cell membrane leads to the release of arachidonic acid. Arachidonic acid then leads to the production of leukotrienes (LTs) (Gaschler & Stockwell 2017). LTs affect the endothelial cytoskeleton by increasing membrane permeability, smooth muscle contraction, and vasoconstriction (Cowled & Fitridge 2011), as well as activation of endothelial adhesion molecules, such as CD18 (Grace 1994). Furthermore, leukotriene B_4 also mediates neutrophil-generated hydrogen peroxide and elastase (proteolytic) production (Welbourn et al. 1991).

1.4.4.5 Interaction Between the Endothelium and Leukocytes

During reperfusion, neutrophils are recruited to the ischemic areas, adhere to the endothelium, and transmigrate into the extravascular space (Ley et al. 2007). This is a multi-step process, initially described by Du Trochet in 1824 (Du Trochet 1824), known as the leukocyte adhesion cascade (Figure 1.6). While knowledge surrounding the leukocyte adhesion cascade is still evolving, the current model of the cascade features nine distinct steps: capture, rolling, slow-rolling, arrest, adhesion, intravascular crawling, paracellular transmigration, and transcellular transmigration (Eltzschig & Collard 2004; Ley et al. 2007).

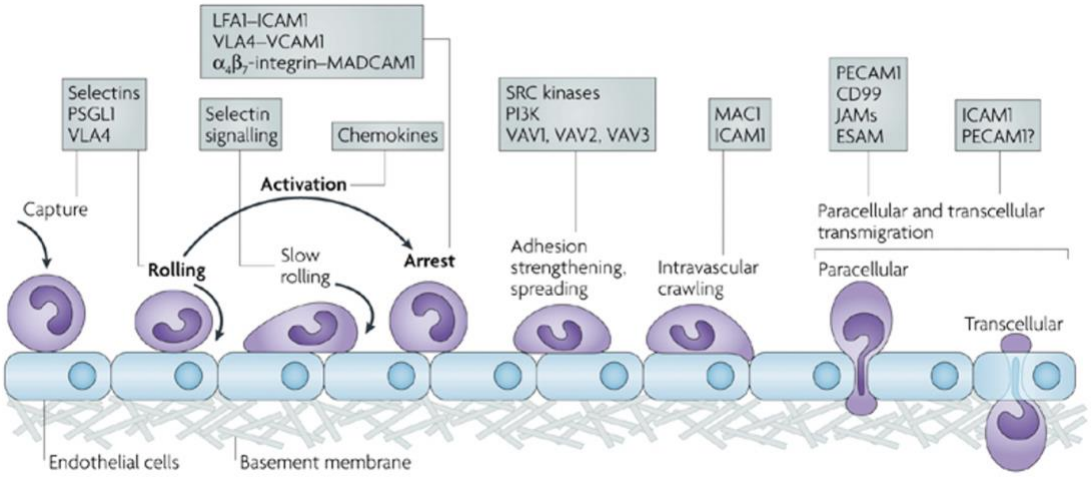


Figure 1.6 **Leukocyte activation sequence in inflammation**. Activated leukocyte sequence includes leukocyte capture/tethering, rolling, firm adhesion, arrest and extravasation. It is mediated by various adhesion molecules such as selectins, integrins, and Ig superfamily.

Adapted with permission of Springer Nature from Ley, Laudanna et al. (2007).

The process of neutrophil capturing (known as endothelial tethering, where the neutrophil makes first endothelial contact) and rolling (slow movement along the endothelial lining) are mediated by selectins and integrins. Initially, L-selectin is expressed on leukocytes; P-selectin and E-selectin are expressed on the endothelium (Ley et al. 2007). During reperfusion, P-selectin (normally stored in α-granules of platelets and Wiebel-Palade bodies of the endothelium) is quickly expressed on to the endothelial cell surface (Cowled & Fitridge 2011; Lum & Roebuck 2001). Inflammatory mediators promote de novo transcription and production of E-selectin, taking several hours until full expression (Ley et al. 2007).

Leukocyte rolling is then mediated by integrins, leading to eventual firm adhesion and arrest. Leukocyte cell surface receptors $\alpha_4\beta_7$ integrin and very late antigen-4 (VLA-4) contribute to leukocyte rolling along the endothelium, interacting with mucosal vascular addressin cell-adhesion molecule-1 (MADCAM-1) and vascular cell-adhesion molecule-1 (VCAM-1), respectively. (Cowled & Fitridge 2011; Ley et al. 2007).

Leukocyte adhesion is mediated by integrins and immunoglobulin superfamily (Ig). The CD11/CD18 αβ-integrin complexes mediate adhesion: CD11a/CD18, also known as lymphocyte function-associated antigen-1 (LFA-1), is expressed on all leukocytes, while CD11b/CD18, also known as macrophage-associated receptor-1 (MAC-1), is expressed on certain monocytes, macrophages, lymphocytes, natural killer cells, neutrophils, and granulocytes (Ley et al. 2007; Lum & Roebuck 2001). CD11a and CD11b are constitutively

expressed. Both neutrophilic-integrin complexes interact with immunoglobulin superfamily, including intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2) on the endothelium, whereas VLA-4 binds with VCAM-1 during the rolling phase to initiate arrest. ROS, along with pro-inflammatory cytokines, further promote CD11/CD18 complex and ICAM-1 interaction (Cowled & Fitridge 2011; Eltzschig & Collard 2004; Ley et al. 2007; Lum & Roebuck 2001).

Neutrophil transmigration is triggered by a chemoattractant transendothelial gradient. Emigrating leukocytes must pass through three distinct barriers: endothelial cell (rapid penetration, <5 minutes), endothelial cell basement membrane (penetration time around 5 to 15 minutes), and pericytes. During I-R, β-integrins promote greater leukocyte-endothelial interactions, leading to the transmigration of neutrophils. Platelet-associated cell adhesion molecule-1 (PECAM-1), also a member of the immunoglobulin superfamily, is constitutively expressed on endothelial cells, leukocytes, and platelets. It is a major protein facilitating transmigration of leukocytes through both the paracellular route (at the junctions between endothelial cells) and the transcellular route (within the body of the endothelium) (Albelda et al. 1991; Ley et al. 2007; Woodfin et al. 2007). The leukocyte-bound PECAM-1 interacts with endothelial PECAM-1 promoting transendothelial leukocyte movement, upstream integrin activation (including MAC-1), and upregulates neutrophil integrin $\alpha_6\beta_1$, which enables PECAM-1-mediated migration through the perivascular basement membrane. (Berman & Muller 1995; Privratsky et al. 2010). Studies of pro-inflammatory conditions have previously revealed diminished severity of disease,

reduced leukocyte endothelial transmigration, and reduced migration into the interstitium when PECAM-1 was inhibited (Privratsky et al. 2010; Ley et al. 2007; Woodfin et al. 2007). Other endothelial mediators of transmigration are junctional adhesion molecules (JAMs), including JAM-A, JAM-B, JAM-C, as well as ICAM-1, ICAM-2, CD99 antigen, and endothelial cell-selective adhesion molecules (Ley et al. 2007; Lum & Roebuck 2001). Junctional adhesion molecules interact with leukocyte ligands LFA-1, VL-4, and MAC-1. (Lum & Roebuck 2001; Tuma et al. 2008; Woodfin et al. 2007).

1.4.4.6 Cytokines/Chemokines

Cytokines and chemokines play a significant role in the pro-inflammatory response of I-R injury. During I-R, calcium phosphate complexes and uric acid levels increase and bind onto inflammasomes, assisting with the upregulation/production of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by tissue macrophages and neutrophils (Kalogeris et al. 2016; Martinon et al. 2002). These cytokines enable toll-like receptor stimulation through intracellular pathways, leading to an upregulation of nuclear factor kappa light-chain of activated B cells (NF-κB) transcription factor activity. As a result, platelet-activating factors, monocyte chemoattractant protein-1 (MCP-1), chemokine ligand 5 (also known as RANTES), and other interleukins (IL-) including IL-6 and IL-8 are produced and released. Cytokines and chemokines work synergistically with leukocyte-endothelial adhesion proteins, causing further

neutrophil chemoattraction, cellular infiltration, and edema (Kalogeris et al. 2016; Lum & Roebuck 2001; Lutz et al. 2010; Martinon et al. 2002).

Functions of TNF-α include chemoattraction of neutrophils, upregulating ICAM-1 expression on the vascular endothelium, stimulating production of downstream cytokines, and apoptosis. It has a high affinity for TNF-α receptor-1 and TNF-α receptor-2 (Cowled & Fitridge 2011). Studies have demonstrated that inhibition of TNF-α receptor-1 can prevent downstream effects and programmed cell-death (Jiang et al. 2009). Unlike complete ischemia followed by reperfusion (Bihari et al. 2017), ICP elevation in a rat model of ACS led to a sustained increase in serum TNF-α levels, with a second peak (biphasic TNF-α elevation) occurring after fasciotomy (Lawendy et al. 2016). Meanwhile, IL-1β is released by mast cells and macrophages to stimulate the production of downstream cytokines. IL-1β inhibition studies have shown to lessen the pro-inflammatory response, chemokine expression, and apoptotic events in I-R injuries (Furuichi et al. 2006; Lutz et al. 2010).

In a rat model of ACS, neutralizing TNF-α alone or both TNF-α and IL-1β together significantly reduced tissue injury but not with IL-1β neutralization alone. TNF-α with or without IL-1β neutralization significantly decreased leukocyte activation and inflammation, while IL-1β neutralization alone had partial suppressive effects (Donohoe et al. 2018).

1.4.4.7 Nitric Oxide

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) enzyme existing in three forms: constitutive (cNOS), inducible (iNOS), and endothelial (eNOS). During the ischemic phase, there is a surge of eNOS within minutes. However, during reperfusion, eNOS functions decline and NO levels, important in maintaining vascular tone, decrease. During later stages of reperfusion, cytokine-mediated upregulation of iNOS from mast cells, neutrophils, and macrophages leads to an increase in NO production. However, NO function is bimodal. While its production from eNOS during ischemia appears to be cytoprotective and acts as an antioxidant, the late surge from iNOS expression results in further oxidative stress and interacts with superoxide to form peroxynitrite free radical (Cowled & Fitridge 2011; Khanna et al. 2005).

1.4.4.8 Complement

I-R has also been shown to lead to an activation of the complement cascade. Active peptides, such as C3a and C5a, increase microvascular permeability and exert chemotactic effects on neutrophils. They also increase CD18 expression and increase neutrophil adhesion to the endothelium (Grace 1994). Heideman et al. (1988) demonstrated increased serum C3a and C5a as well as elevated serum terminal complement complexes in patients with acute limb ischemia. Following amputation, these peptide levels returned to normal range. Multiple complement inhibitors, at various points of the complement pathway, have been found to decrease tissue injury and organ damage in I-R

(Heideman et al. 1988). However, none were able to completely prevent tissue injury, suggesting that complement activation plays only a partial role in the underlying pathophysiology of I-R (Arumugam et al. 2006).

Despite their important physiologic functions in maintaining homeostasis, inflammation and ROS production in the context of ACS and I-R injuries contribute to extensive tissue damage, organ dysfunction, and systemic complications.

1.5 HEME METABOLISM AND OXIDATIVE STRESS

Despite the dependence of living organisms on oxygen to survive, the oxygen required to maintain life is intrinsically toxic due to its capabilities of inducing oxidative stress. Therefore, compensatory mechanisms have evolved to allow for survival in such toxic environment. One of the most ubiquitous mechanisms is heme oxygenase (HO) metabolism (Morse & Choi 2005).

The importance of HO was highlighted by Yachie et al. (1999), who reported on the first case of HO deficiency: a 2-year-old child presented with growth retardation, developmental delay, hepatomegaly, asplenia, and persistent hemolytic anemia requiring regular erythrocyte transfusions. The mother had two previous intrauterine death that could have been related to HO deficiency; the child did not survive beyond the age of 6 (Kawashima et al. 2002; Yachie et al. 1999).

1.5.1 Heme Oxygenase

HO is an enzyme that catalyses heme degradation (Figure 1.7). It was first described in 1968 by Tenhunen and colleagues (Tenhunen et al. 1968). Subsequently, three isoforms of HO were isolated: the inducible form, HO-1, and constitutively expressed HO-2 and HO-3 (Hayashi et al. 2004; Maines et al. 1986; Mccoubrey et al. 1997).

HO breaks down the heme porphyrin ring and cleaves the α-methene bridge, releasing carbon monoxide, biliverdin, and iron. Oxidation reaction between HO, NADPH-Cytochrome P450 reductase complex, and oxygen further converts biliverdin to bilirubin (catalysed by biliverdin reductase) and iron to ferritin (Ryter & Choi 2016; Wu & Wang 2005).

Bilirubin is increasingly recognized as an antioxidant. For example, it has been shown that small amounts of bilirubin can protect neural cells from hydrogen peroxide oxidant *in vitro* (Baranano et al. 2002). There appears to be a correlation between low bilirubin levels and increased risk of coronary artery disease and myocardial infarction (Djoussé et al. 2003; Hopkins et al. 1996; Schwertner et al. 1994).

The regulatory effects of HO-1 on iron levels have also been shown to prevent cell death by controlling intracellular iron levels (Ferris et al. 1999; Li & Stocker 2009). An *in vitro* study also demonstrated that ferritin itself might have endothelial antioxidant properties (Balla et al. 1992).

HO-1, normally expressed at low levels, is upregulated in response to stressors, such as fever, heat, heavy metals, infection, lipopolysaccharides,

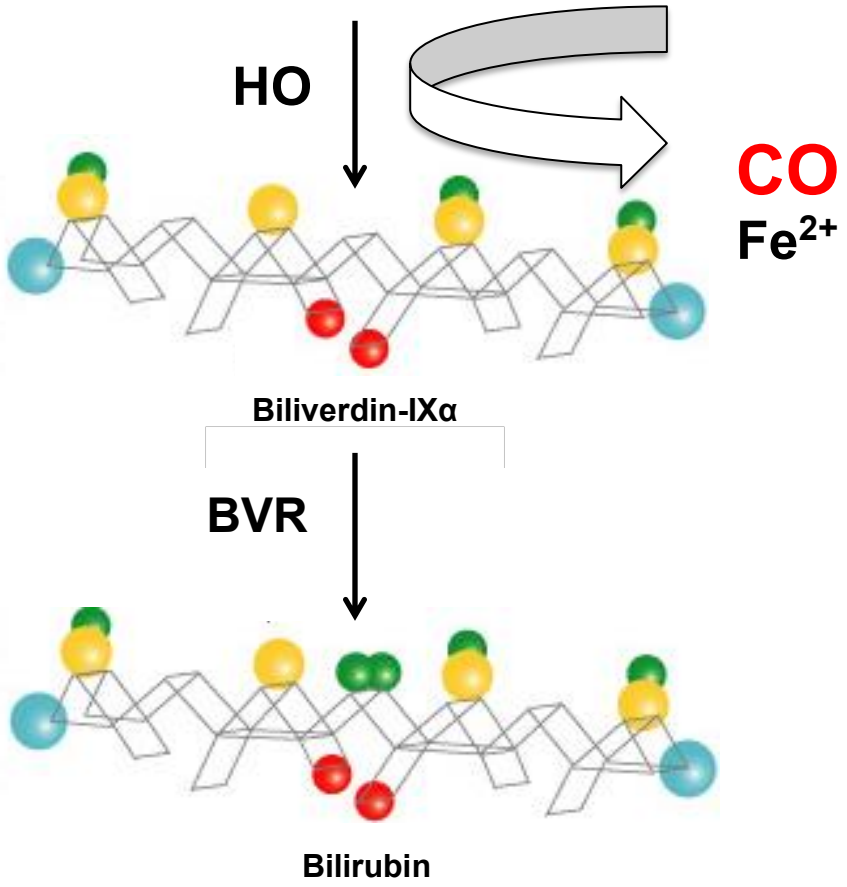


Figure 1.7 **Heme degradation pathway**. Heme derived from hemoglobin is broken down into biliverdin, carbon monoxide (CO), and free iron (Fe^{2+}) by heme oxygenase (HO). Biliverdin is then rapidly converted into bilirubin by biliverdin reductase (BVR).

Reproduced with permission from Bihari (2017).

oxidative agents, stress, radiation, toxins, and ultraviolet light (Choi & Otterbein 2002; Intagliata et al. 2019; Waza et al. 2018). Previously, stress-induced proteins have been shown to not only protect cells from immediate stress but also to fortify the organism to withstand future stresses originating from a different source. In the late 1980s, a 32kD mammalian stress protein, known as the heat shock protein-32 (HSP32), had been identified (Shibahara et al. 1987); it was later proven to be HO-1, the inducible form of HO (Keyse & Tyrrell 1989; Taketani et al. 1989). Benefits of HO-1 include anti-inflammatory properties, neuroprotection, and protective effects against cardiovascular, diabetic, hepatic, and renal diseases (Waza et al. 2018; Wu & Wang 2005).

Although HO-2 is constitutively expressed and its gene expression remains fairly consistent under stress conditions, it also plays important roles in neuroprotection of the brain and neurologic disease; protection against cardiovascular, pulmonary, and renal diseases as well as diabetes; protection against male reproduction disease and pregnancy-related complications; protection against oxidative stress and deleterious effects of inflammation (Intagliata et al. 2019; Wu & Wang 2005). Studies of HO-2 knockout mice have demonstrated elevated levels of inflammatory cytokines, increased oxidative stress, and decreased healing potential (Bellner et al. 2009; Chen et al. 2018).

The upregulation of HO has been extensively studied. While HO-1 is ubiquitously produced and found in virtually all tissues affected by stressors such as oxidative stress and inflammation, HO-2 is predominantly expressed in the endothelium, testes, and brain (Dunn et al. 2014; Motterlini & Foresti 2017;

Yachie et al. 1999). Current techniques involve the use of various protoporphyrin (Maines & Kappas 1977), adenoviral vector transfer of HO gene construct for short-term effects, and retroviral vectors for long-term upregulation (Abraham et al. 2007). Despite its potential, there are currently no clinical trials of HO upregulation gene therapy in humans and is currently not clinically feasible (Abraham et al. 2007; Kim et al. 2012; Luo et al. 2018; Motterlini & Foresti 2014). Therefore, the attention shifted towards the by-products of HO activity, notably carbon monoxide (CO).

1.5.2 Carbon Monoxide

CO is a diatomic molecule occurring naturally as a colourless and odourless gas (Ryter & Otterbein 2004). Although CO has a negative connotation due to CO poisoning, carbon monoxide plays a vital role in cell signalling, cellular communication, vasodilation, as well as peripheral and central nervous system neurotransmission (Kim et al. 2006). It also possesses anti-inflammatory, anti-proliferative, and anti-apoptotic properties (Kim et al. 2006; Ryter & Otterbein 2004; Weaver 2009).

CO binds to hemoglobin to form carboxyhemoglobin (COHb). CO affinity for hemoglobin is nearly 200 times greater than that of oxygen (Weaver 2009). Exogenous inhalation of CO not only increases levels of COHb but also shifts the oxygen dissociation curve to the left, thus decreasing the ability of the remaining oxyhemoglobin to release its oxygen into the tissues (Ryter & Otterbein 2004). Normal endogenous COHb levels from heme metabolism range between 1% and

3% in non-smokers and less than 10% in smokers (Weaver 2009). Symptoms of CO intoxication include headache, visual disturbances, dizziness, nausea, fatigue, decreased dexterity, vomiting, seizures, coma, and death if left untreated. Although the onset of symptoms starts around COHb levels of 15% and progresses as the CO levels increase, CO intoxication becomes lethal around COHb levels of 50-60% (Bleecker 2015). Since oxygen and CO are direct competitors for hemoglobin binding, the mainstay of CO poisoning treatment is oxygen therapy, which can include both normobaric and hyperbaric oxygenation (Kim et al. 2006; Ryter & Otterbein 2004; Weaver 2009).

The majority of endogenous CO is produced from heme metabolism. Daily production, in the absence of external stressors, reaches upwards of 10mL (Coburn et al. 1964). However, pathological conditions due to pro-inflammatory processes, external stressors, and environmental triggers that upregulate HO-1 expression lead to an increase in endogenous CO concentration. Multiple animal studies have confirmed that upregulating HO-1 expression increases endogenous CO production, while HO inhibition decreases CO synthesis (Intagliata et al. 2019; Wu & Wang 2005).

1.5.3 Effects of Carbon Monoxide

Multiple animal studies have demonstrated the benefits of exogenous CO application. CO therapy has shown inhibition of inflammatory mediators, decreased rates of apoptosis, decreased edema, and improved organ function in various animal models of I-R, including rodents (Neto et al. 2004), mice (Zhang

et al. 2003), and pigs (Lavitrano et al. 2004). Clinically, CO therapy has demonstrated improved graft healing, function, and survival following cardiac, intestinal, renal, and pulmonary transplantation (Akamatsu et al. 2004; Lavitrano et al. 2004; Nakao et al. 2003; Neto et al. 2004; Song et al. 2003; Zhang et al. 2003). Exogenous CO application also appears to decrease hyperoxic and ventilator-induced pulmonary injuries, increases survival, and inhibits inflammatory cytokines and leukocytes (Dolinay et al. 2004; Otterbein et al. 2003b). CO also decreased apoptosis and structural damage in mice with acute hepatic injury (Zuckerbraun et al. 2003).

1.5.3.1 Cellular Signalling

CO has a high binding affinity for heme proteins and transition metals, such as hemoglobin and myoglobin. Therefore, other potential hemoprotein targets include guanylate cyclase, cytochrome *c* oxidase, and cytochrome P450 (Ryter & Choi 2016; Ryter & Otterbein 2004).

CO appears to be a modulator of proteins with important implications on downstream smooth muscle relaxation, vasodilation, ion channel conductance, glycogenolysis, and cellular apoptosis (Morita et al. 1995; Motterlini & Otterbein 2010; Ryter & Otterbein 2004). CO has been shown to modulate soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP). These have downstream effects on vasodilation and anti-proliferation. However, it is a weaker agonist of sGC than NO (Ryter & Otterbein 2004). CO is also a

modulator of calcium- and voltage-gated potassium channels that are involved in vasoregulation (Peers 2011; Ryter & Choi 2016; Wang 1998).

CO modulates signalling that controls inflammatory and apoptotic processes. It is an agonist to mitogen-activated protein kinases (MAPK) such as p38, *c*-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK), leading to downstream inhibition of pro-apoptotic cytokines such as TNF-α, although the exact role of JNK and ERK kinases need further elucidation (Ryter & Choi 2016; Ryter & Otterbein 2004; Zuckerbraun et al. 2007). Animal and *in vitro* studies have shown CO as a potential inhibitor of NADPH oxidase- and mitochondria-dependent ROS, as well as a modulator of iNOS (Nakahira et al. 2006; Ryter et al. 2007; Sarady et al. 2004; Zuckerbraun et al. 2007).

1.5.3.2 Vasodilation

CO plays an important role in vasoregulation. Although NO is a much more potent sGC modulator (Ryter & Otterbein 2004), its effect on vascular smooth muscle cells appears to be independent of NO (Morita et al. 1995). Suematsu and colleagues (1994, 1995) demonstrated increased perfusion and vasorelaxation when either CO or cGMP was administered, whereas inhibition of cNOS and iNOS did not affect this response (Suematsu et al. 1994, 1995). CO may also have a role in promoting cGMP-independent vasodilation, controlling calcium-activated potassium ion channels, as illustrated by cerebral arteriolar dilation in response to CO (Jaggar et al. 2002). Wang et al. (1997) demonstrated

that vasodilation is induced by both cGMP-dependent and calcium-activated potassium ion channel-dependent effects simultaneously (Wang et al. 1997).

1.5.3.3 Anti-Apoptotic Effects

Apoptosis, also known as programmed cell death, plays a vital role in maintaining cellular and tissue homeostasis under normal physiological state and disease conditions. It is characterized by cell shrinkage, pyknosis (chromatin condensation), karyorrhexis (fragmentation of the nucleus), and plasma membrane blebbing, which eventually breaks off into apoptotic bodies and are engulfed by phagocytes (Linkermann et al. 2013; Van Cruchten & Van den Broeck 2002). Its mechanism can be separated into extrinsic and intrinsic pathways. Both pathways lead to downstream caspase -3 and -9 release as well as proteolytic enzymes that induce cell death. In the extrinsic pathway, TNF-α induces the pro-apoptotic pathway by initiating death-inducing signalling complex (DISC), including *Fas*, *Fas*-associated death domain, and caspase-8, which ultimately activates caspase-3 activity. The intrinsic pathway, usually upregulated in response to irreparable intracellular and DNA damage, is activated through mitochondrial translocation of *Bax* protein from the *Bcl-2* family, which then releases pro-apoptotic mediators such as cytochrome *c* and binds onto apoptotic protease activating factor-1 (APAF-1), thus triggering caspase activity (Bano & Prehn 2018; Green & Llambi 2015; Kluck et al. 1997; Soares et al. 2002; Wang et al. 2011; Zou et al. 1997). Various caspase-independent pathways lead to apoptosis. Increased intracellular calcium triggers calpain enzyme activation,

which leads to lysosomal-induced cell death. Calpain also functions as a cleavage system for apoptotic-inducing factors (AIF) causing DNA damage, while ROS stimulate AIF release through the activation of poly(ADP-ribose) polymerase-1 (Bano & Prehn 2018; Hongmei 2012; Kang et al. 2004; Kim et al. 2018).

Apoptosis is biologically distinct from cellular necrosis, also termed necroptosis. While TNF-α is a strong potentiator of both necroptosis and apoptosis, they have distinct pathways. In necroptosis, TNF-α mediates receptor-interacting protein (RIP) activation forming the RIP-1/RIP-3 complex. Cell death is then induced by mixed lineage kinase domain-like protein phosphorylation leading to cell membrane damage (Linkermann & Green 2014; Liu et al. 2019a; Vanlangenakker et al. 2011a,b).

Many studies have demonstrated that CO displays anti-apoptotic properties. Brouard et al. (2000) demonstrated the anti-apoptotic effects of CO, mediated through activation of p38 MAPK, in an *in vitro* murine model (Brouard et al. 2000). A murine model of pulmonary I-R injury also showed an inhibitory effect of CO on mitochondrial cytochrome *c* release (Zhang et al. 2003). Additional studies identified synergistic interaction between the HO-1/CO pathway, NF-κB-dependent anti-apoptotic genes (A1 and c-IAP2), and p38 MAPK activation in preventing TNF-α-mediated apoptosis (Brouard et al. 2002). It appears that the NF-κB transcription activity may have both pro- and anti-apoptotic effects. While CO interacts with NF-κB sub-genes A1 and c-IAP2 to promote anti-apoptotic activity, the NF-κB gene in itself can act as a pro-

inflammatory agent through proteasome pathways and upregulation of inflammatory mediators via its promoter gene (Soares et al. 2002). Important components of cellular apoptosis involve caspases (family of protease enzymes), *Fas* and *Bax* proteins (*Bcl-2* family), that have been shown to be inhibited by CO (Ryter & Choi 2016; Wang et al. 2011; Zhang et al. 2003).

1.5.3.4 Anti-Proliferative Effects

The anti-proliferative effects of CO were examined by Morita et al. (1995). They demonstrated that, under hypoxic conditions, HO-1 and subsequent CO production significantly increased, leading to the upregulation of cGMP in vascular smooth muscle cells. The addition of CO inhibitors and CO scavengers completely inhibited this response. The cGMP produced in the vascular smooth muscles did not involve NO (Morita et al. 1995).

The CO-cGMP pathway has been suggested to prevent vascular smooth muscle cell proliferation and intimal hyperplasia (Motterlini & Otterbein 2010; Ryter & Otterbein 2004). Otterbein et al. (2003) demonstrated CO-induced anti-proliferative effects in rodent models, dependent on the activation of both cGMP and p38 MAPK (Otterbein et al. 2003a).

1.5.3.5 Anti-Inflammatory Effects

CO has been shown to exhibit anti-inflammatory effects, both *in vitro* and *in vivo*. In an *in vitro* model of sepsis, CO inhibited TNF-α release, along with other inflammatory markers (Otterbein et al. 2000). Application of CO in an

orthotopic lung transplant prevented severe intra-alveolar haemorrhage and intravascular coagulation (Song et al. 2003). CO was also found to significantly improve renal function, decrease pro-inflammatory cytokine expression and renal tubular injury, as well as inhibit tubular apoptosis in a kidney transplant model (Abe et al. 2017). Furthermore, CO has been shown to inhibit NADPH oxidase- and mitochondria-dependent ROS, while also downregulating NF-κB activity, thereby reducing TNF-α-induced leukocyte-endothelial upregulation of E-selectin, ICAM-1, and VCAM-1 (Chhikara et al. 2009; Ferran et al. 1995; Ryter & Choi 2016; Soares et al. 2002).

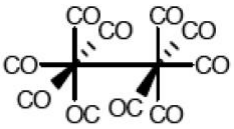
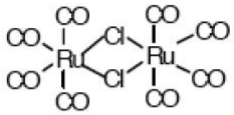
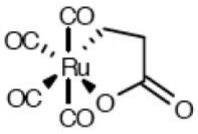
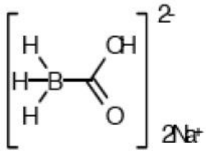
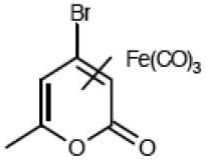
1.5.4 Carbon Monoxide-Releasing Molecules

To avoid the toxicity of CO inhalation, the development of carbon monoxide-releasing molecules (CO-RMs) was undertaken. CO-RMs are molecules capable of releasing CO on-demand, delivering it to the tissues without significant changes in COHb levels (Motterlini & Foresti 2014; Ryter & Choi 2016).

The first CO-RMs were synthesized by Motterlini et al. (2002) (Table 1.1). CORM-1 ($[Mn_2(CO)_{10}]$), the first CO-RM produced, contains manganese at its centre and releases CO rapidly (Motterlini et al. 2002). However, it needs photo-activation and, therefore, has limited use *in vivo*. CORM-2 contains ruthenium ($[Ru(CO_3Cl_2)]$) and requires organic solvents to be dissolved via ligand substitution making its clinical use also limited. CORM-3 ([Ru(CO)3Cl(glycinate)])

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

Adapted from Motterlini (2007).

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

is a water-soluble version of CORM-2, also containing ruthenium. CORM-3 is a rapid equimolar CO releaser (within 10 minutes of being dissolved in water) and, thus, has a potential for clinical use (Motterlini et al. 2002; Wu & Wang 2005).

At present, ruthenium does not appear to have any physiological function while being used as experimental anti-cancer drugs. Consequently, metal toxicity and DNA alteration remain a concern for human studies (Emsley 1989; Bergamo & Sava 2011). Alternative forms of CO-RMs that are, in theory, more physiologically friendly have been developed including CORM-A1 and CORM-401. CORM-A1 ([Na2H3BCO2]) contains a boron-centric carboxylic group rather than a transition metal, allowing pH- and temperature-dependent CO release rather than ligand substitution. CORM-401 ($[Mn(CO)_4S_2CNMe(CH_2CO_2H)]$), in contrast, contains a manganese centre and releases 3 moles of CO per mole of CORM-401. Both forms of CO-RM are slower releasers of CO compared to CORM-3 with a half-life of approximately 21 minutes (Almeida et al. 2016; Fayad-Kobeissi et al. 2016; Inoue et al. 2017; Motterlini et al. 2005).

1.5.4.1 CORM-3

Administration of CORM-3 has been shown to be beneficial in several animal models, ranging from aortic vasodilation (Foresti et al. 2004), cardiac inotropy (Musameh et al. 2009), anti-ischemic effects on renal tissue (Sandouka et al. 2006), acute liver failure (Yan et al. 2016), vasodilation in cirrhotic rats (Bolognesi et al. 2007), reduced inflammation in haemorrhagic stroke

(Yabluchanskiy et al. 2012), and inhibition of leukocyte-derived MPO activity in vascular endothelial injury (Patterson et al. 2014).

Sandouka et al. (2006) demonstrated improved reperfusion and glomerular filtration rate in I-R models of kidney transplant following treatment with CORM-3 (Sandouka et al. 2006). In a murine model of acute liver failure, CORM-3 significantly inhibited pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and increased IL-10 (an anti-inflammatory cytokine), while also inhibiting the expression of iNOS, Toll-like receptor-4, and NF-κB transcription activities (Yan et al. 2016). CORM-3 also displayed an anti-inflammatory effect in haemorrhagic stroke by inhibiting TNF-α (Yabluchanskiy et al. 2012). *In vitro* experiments on the heart also demonstrated significant cardioprotection in I-R models when the heart was re-perfused with CORM-3 (Clark et al. 2003). Additionally, in an *in vitro* human vascular endothelial cell (HUVEC) model of inflammation, CORM-3 significantly inhibited leukocyte-derived MPO activity (Patterson et al. 2014). MPO is a mediator of oxidative stress, important in the innate immune system. However, when excessive leukocytes are activated during severe inflammatory responses, stimulation of MPOs and subsequent ROS production contribute to vascular and organ dysfunction (Cepinskas et al. 2008; Patterson et al. 2014).

Recently, CORM-3 has been found to show promise as a possible pharmacological agent in the treatment of ACS. In a rat model, the administration of CORM-3 at fasciotomy led to improved microvascular perfusion, significantly decreased tissue injury, and diminished tissue leukocyte activation (Lawendy et al. 2014). In a preclinical porcine model of ACS, CORM-3 not only improved

muscle injury but also significantly inhibited inflammation at both local and systemic levels. This was characterized by complete inhibition of systemic TNF-α release as well as suppression in oxidative bursts of leukocytes (Bihari et al. 2018).

To transition the promising results into human studies, Bihari et al. (2019) developed an *in vitro* model of ACS, using HUVEC stimulated with serum isolated from ACS patients. Incubation of HUVEC with ACS serum led to an endothelial barrier breakdown, an increase in oxidative stress and apoptosis, as well as polymorphonuclear leukocyte activation. The application of CORM-3 offered significant protection against these detrimental effects (Bihari et al. 2019).

1.6 PURPOSE OF THE THESIS

If not treated promptly, ACS is a limb-threatening and often life-threatening condition. The current gold standard for treatment is surgical fasciotomy. While the pathophysiology of ACS is not entirely understood, it shares many similarities with I-R injury. Although it is generally accepted that irreversible tissue damage occurs around 6 to 8 hours, previous studies have demonstrated myonecrosis as early as 3 hours within the onset of ACS.

The effects of complete ischemia (where the onset is known, as all circulation is cleanly cut off) and subsequent reperfusion injury have been extensively studied. Unlike complete ischemia, however, ACS occurs in the face of patent vessels; distal pulses are present in the majority of ACS patients, indicating that the *macro*circulation within the involved compartments is still

intact, while the *micro*circulation becomes dysfunctional. The diagnostic challenges become even greater, as ACS is a clinical diagnosis, and multiple risk factors can delay timely diagnosis. The onset of ACS in itself is often difficult to identify.

Despite proposed non-operative alternatives in case reports or small case series, none have been proven to be an effective first-line treatment or a substitute for fasciotomy. Recently, the role of CO as a potential pharmacological therapeutic agent for the treatment of ACS has been explored. In preclinical studies, using both small (rat) and large (porcine) models of ACS, administration of CO, in the form of water-soluble CO donor, CORM-3, at fasciotomy has resulted in decreased microvascular perfusion deficits, diminished tissue injuries, blocked leukocyte activation, and inhibition of systemic TNF-α release (Bihari et al. 2018; Lawendy et al. 2014).

The purpose of this thesis was to evaluate the effects of CO in ACS without fasciotomy, by investigating its short- and longer-term effects on microvascular dysfunction, myonecrosis, and inflammatory response. For this experiment, CORM-3 was chosen as the CO donor, given its water solubility and rapid CO release. We hypothesized that the administration of CORM-3 would produce a lasting beneficial effect. The ultimate goal is to identify whether CO can be used as a stand-alone first-line treatment of ACS, or at least extend the surgical window. For the purpose of this thesis, the terms ‘elevated ICP’ and ‘ACS’ were used interchangeably. However, it is important to note that ACS is a clinical diagnosis and is only applicable to humans.

CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMAL CARE

The experimental protocol for the study was approved by the Animal Care Committee of the Canadian Council on Animal Care (CCAC) at the University of Western Ontario, London, Ontario, Canada. The daily animal care, handling, and housing conformed with the guidelines established by the CCAC. The rats were housed in pairs, in clear plastic cages, and under 12:12 light:dark cycle. They had access to food and water *ad libitum*.

2.2 ANIMAL DESCRIPTION

Twenty-nine male Wistar rats were used for this study. Their body weight ranged between 190g and 260g.

2.3 CARBON MONOXIDE

A water-soluble CO donor, carbon monoxide-releasing molecule-3 (CORM-3) (tricarbonylchloro-glycinate-ruthenium(II), [Ru(CO)3Cl-glycinate]; molecular weight 295gmol-1), was used in this experiment. It was synthesized in our lab using previously published method (Clark et al. 2003). A stock solution of 10mg/ml was prepared fresh, in isotonic saline, just before the injection. Each rat received 10mg/kg of CORM-3 intravenously, via the left lateral tail vein.

Inactive CORM-3 (iCORM-3) was used as an inert control. iCORM-3 was prepared by dissolving CORM-3 in normal saline 72 hours prior to injection; this

allowed it to release all CO from the stock solution prior to administration to the animals. The lack of CO in iCORM-3 had been previously confirmed spectrophotometrically by myoglobin conversion assay (Bihari et al. 2017).

2.4 EXPERIMENTAL SETUP

2.4.1 General Overview

ACS was generated in the right hind limb of rats by elevating ICP under general anaesthesia. Elevated ICP was maintained at Δp <30mmHg (ICP value of 40-65mmHg) for 2 hours. Experimental drug injections were administered; animals were then allowed to recover. Adequate pain control was provided to all rats using buprenorphine (Buprenex 0.05mg/kg IM q8h). During the recovery period, the rats were housed individually to prevent identification errors and erratic social behaviour.

Following the proper recovery time in accordance with the assigned experimental group (24, 48 or 72 hours), all rats were re-anaesthetised and intravital video microscopy (IVVM) was performed. At the conclusion of IVVM, animals were euthanized by cardiac puncture under the deepest plane of anaesthesia.

Sham animals underwent all procedures, but the ICP remained at baseline level of 0mmHg.

2.4.2 Anaesthesia

Anaesthesia was carried out using inhalational isoflurane. Induction of general anaesthesia was achieved using 5% isoflurane in 100% oxygen gas at a flow rate of 2L/min. Anaesthesia was maintained using 2% isoflurane at a flow rate of 0.5L/min and adjusted as needed to maintain adequate anaesthetic depth. A digital rectal thermometer probe was used to monitor core body temperature. A heating lamp was used to maintain body temperature at 37°C.

2.4.3 Acute Compartment Syndrome

The experimental model was previously described by Lawendy et al. (2011). Briefly, ICP elevation was achieved by continuous infusion of isotonic saline solution into the anterior compartment of the hindlimb using a 24-gauge angiocatheter (BD, Franklin Lakes, NJ). An electronic compartmental pressure monitoring system (Synthes USA, Paoli PA) was inserted into the posterior compartment through a 14-gauge angiocatheter (BD, Franklin Lakes, NJ). Both the anterior and posterior compartments became isobaric as the pressures rose, which allowed ICP monitoring from the posterior compartment. Continuous saline infusion was administered through a gravity-feed system where the isotonic saline bag was placed 120cm above the infusion site (Figure 2.1).

Elevated ICP was maintained between 40mmHg and 65 mmHg to achieve Δp between diastolic blood pressure and ICP of <30mmHg for two hours. It has been previously estimated that one hour of ischemia in rodents is equivalent

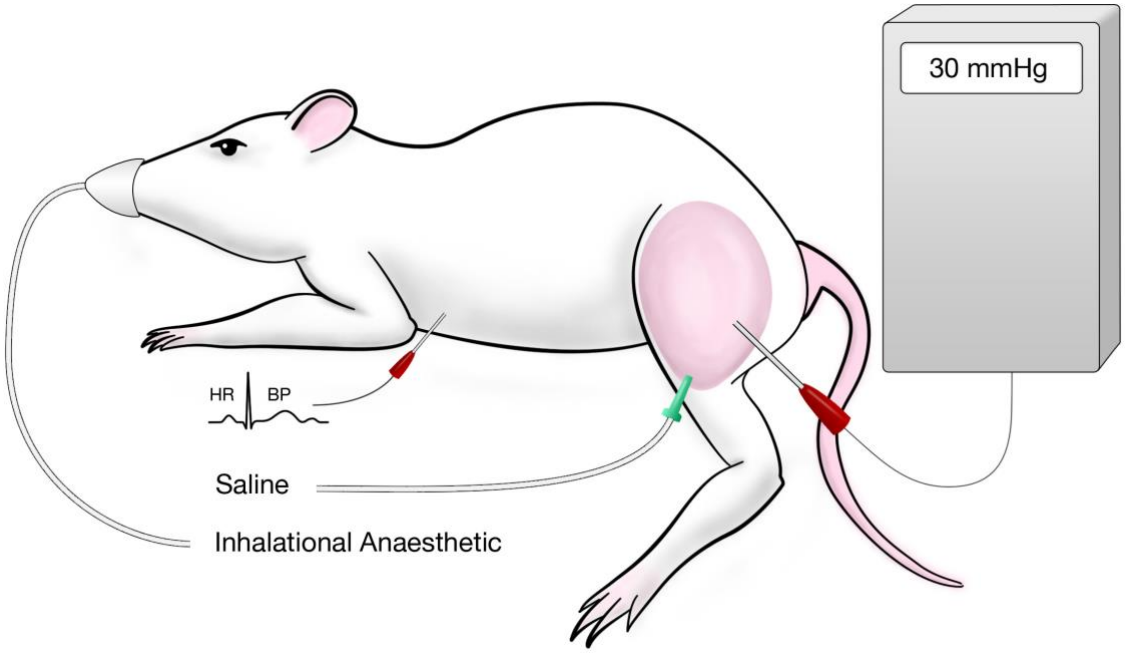


Figure 2.1 **Schematic illustration of rat ACS experimental setup.** Isotonic saline is continuously infused in the anterior compartment of the hind limb, maintaining elevated ICP between 40mmHg and 65mmHg. An angiocatheter connected to an electronic compartmental pressure monitoring system is inserted in the posterior compartment.

Reproduced with permission from Bihari (2017).

to about four hours in humans, given the metabolic differences (Hoppeler & Weibel 2005; Hulbert et al. 2007; Lawendy et al. 2011; Marquet et al. 2005); hence two hours of elevated ICP should be the equivalent of about eight hours in humans.

2.5 EXPERIMENTAL GROUPS

Rats were randomly assigned to one of four main groups: (1) sham, (2) ACS followed by 24 hour recovery, (3) ACS followed by 48 hour recovery, and (4) ACS followed by 72 hour recovery. Each group was further subdivided into animals that received CORM-3 or those receiving iCORM-3 injection. In this experiment, each rat received only a single dose of either CORM-3 or iCORM-3.

2.5.1 Sham

Six animals underwent all procedures, but the compartment pressure remained at baseline level of 0mmHg. Rats received an injection of CORM-3 (n=3) or iCORM-3 (n=3), followed by IVVM.

2.5.2 ACS with 24hr Recovery

Eight rats underwent ACS, followed by IV administration of CORM-3 (N=4) or iCORM-3 (N=4). They were then allowed to recover for 24 hours, as described in section 2.4.1. Following the recovery period, animals were re-anaesthetised and underwent IVVM.

2.5.3 ACS with 48hr Recovery

Seven animals underwent ACS, followed by CORM-3 (N=4) or iCORM-3 (N=3) injection. They were then allowed to recover for 48 hours. Following recovery period, all animals were re-anaesthetised and underwent IVVM.

2.5.4 ACS with 72hr Recovery

Eight rats underwent ACS, followed by CORM-3 (N=4) or iCORM-3 (N=4) injection. They were then allowed to recover for 72 hours. Following recovery period, all animals were re-anaesthetised and underwent IVVM.

2.6 INTRAVITAL VIDEO MICROSCOPY

2.6.1 Muscle Preparation

Under general anaesthesia, the extensor digitorum longus (EDL) muscle was prepared for IVVM using careful dissection and handling to avoid iatrogenic injuries. A lateral incision over the experimental hind limb was made. The underlying biceps femoris was exposed and incision through its fascial layer was undertaken, allowing retraction of the muscle. The tibialis anterior (TA), peroneus longus (PL), and lateral gastrocnemius (LG) muscles were then identified. Meticulous dissection was carried out to avoid traumatizing the saphenous vein and to maintain adequate hemostasis. Subsequently, the EDL was identified between TA and PL. The underside of TA and PL were gently brushed off and retracted without touching the fibers of EDL. Once exposed, the distal EDL

tendon identified over the ankle was ligated using 3-0 silk and severed as close as possible to the bony insertion site.

Following EDL preparation, the rat was then carefully moved onto the stage of IVVM microscope; the EDL muscle belly was partially reflected anteriorly into the saline bath containing vital dyes and stabilized using the 3-0 silk ligature (Potter et al. 1993).

2.6.2 Vital Dye Staining

Two fluorescent dyes, ethidium bromide (EB) (Sigma Aldrich, Mississauga, ON) and bisbenzimide (BB) (Sigma Aldrich, Mississauga, ON) in concentrations of 5μg/ml each were added to the saline bath in which the EDL was placed on the microscope slide. Both dyes bind DNA, hence label cell nuclei. Due to its size and hydrophilic nature, EB does not penetrate intact cell membrane; therefore, it was used to label injured or dead cells (i.e. cells with compromised cellular membrane). BB, a lipophilic dye, is able to enter all cells, thus labelling the nuclei of all cells (Daly et al. 1992; Potter et al. 1995). Cellular injury was expressed as the ratio of EB-labelled nuclei to BB-labelled nuclei (EB/BB).

2.6.3 Microscopy

Microscopy was used to assess microvascular perfusion, tissue injury, and leukocyte behaviour. Microvascular perfusion was assessed by recording five transilluminated adjacent fields of view containing complete capillary beds using

20x objective (final magnification 700x) for 60 seconds each. An additional 10 seconds was recorded from the same fields of view under epifluorescence illumination using the proper filters for BB (excitation wavelength = 343nm, emission wavelength = 483 nm) and EB (excitation wavelength = 482 nm, emission wavelength = 610nm) in order to assess tissue injury.

Leukocyte behaviour was assessed by transillumination in five randomly chosen post-capillary venules, with diameter of at least 20μm using 40x objective (final magnification 1400x) for 45 seconds each.

All videos were saved into the computer using Adobe Premiere software for offline video analysis.

2.7 OFFLINE VIDEO ANALYSIS

2.7.1 Microvascular Perfusion

Three equidistant parallel lines were drawn perpendicular to the capillary axis on the video monitor. Microvascular perfusion was quantified by counting the number of continuously-perfused capillaries (CPC), intermittently-perfused capillaries (IPC), and non-perfused capillaries (NPC) crossing each line, based on red blood cell movement (Lawendy et al. 2014). The CPC, IPC, and NPC were expressed as a percentage of the total number of capillaries.

2.7.2 Tissue Injury Analysis

The ratio of EB-labelled cells to BB-labelled cells (EB/BB) was used to evaluate the extent of tissue injury (Potter et al. 1995).

2.7.3 Analysis of Leukocytes

Leukocyte behaviour was analyzed by counting the number of rolling and adherent leukocytes per 30 seconds in each venule. An adherent leukocyte was defined as a cell remaining stationary for at least 30 seconds, while a leukocyte was considered rolling if it remained in close contact with the vessel wall during its entire movement (Granger et al. 1989). The ImageJ software (NIH, Bethesda, MD) was used to measure the venular area. The results were expressed as the number of leukocytes per 30 seconds per 1000μm^2 (Lawendy et al. 2011).

2.8 STATISTICAL ANALYSIS

All data were expressed as a mean ± standard error of the mean (SEM) and analysed using two-way analysis of variance (ANOVA) with Tukey multiple comparison post-hoc test. Data analysis was performed using Prism version 7.0c for Mac OS X (GraphPad Software Inc., San Diego, CA). The statistical significance was set at $p<0.05$. Minimum sample size calculation, based on leukocyte adhesion, was performed using StatMate (GraphPad Software Inc., San Diego, CA) with power set at 85%. We estimated that 48 rats were required to show a significant difference at an 85% confidence level. However, due to the COVID19 pandemic and circumstances out of our control, we were unable to perform analysis on the desired number of rats.

CHAPTER 3: RESULTS

3.1 MICROVASCULAR PERFUSION

3.1.1 Continuously-Perfused Capillaries

ICP elevation led to a significant decrease in CPC, from 79±9% in the sham group, to 39±5%, 44±10%, 35±4% in the 24-, 48-, and 72-hour post-ACS groups, respectively (two-way ANOVA, $p<0.0001$). Administration of CO donor, CORM-3, resulted in significant increase in the number of CPC to 57±3%, 61±3%, 53±7% at 24, 48, and 72 hours post-ACS, respectively versus 81±4% in sham ($p=0.0042$) (Figure 3.1). CORM-3 had no effect on the number of CPC in sham animals.

3.1.2 Intermittently-Perfused Capillaries

In response to ACS, the number of IPC increased, from 10±6% in the sham group, to 27±10%, 26±5%, 30±5% at 24, 48, and 72 hours post-ICP elevation, respectively (two-way ANOVA, $p=0.0365$). Application of CORM-3 resulted in 28±4%, 18±4%, 22±6% IPC at 24, 48, and 72 hours post-ACS, respectively versus 9±3% in sham ($p=0.3474$, n.s.) (Figure 3.1). CORM-3 injection had no effect on the number of IPC in sham rats.

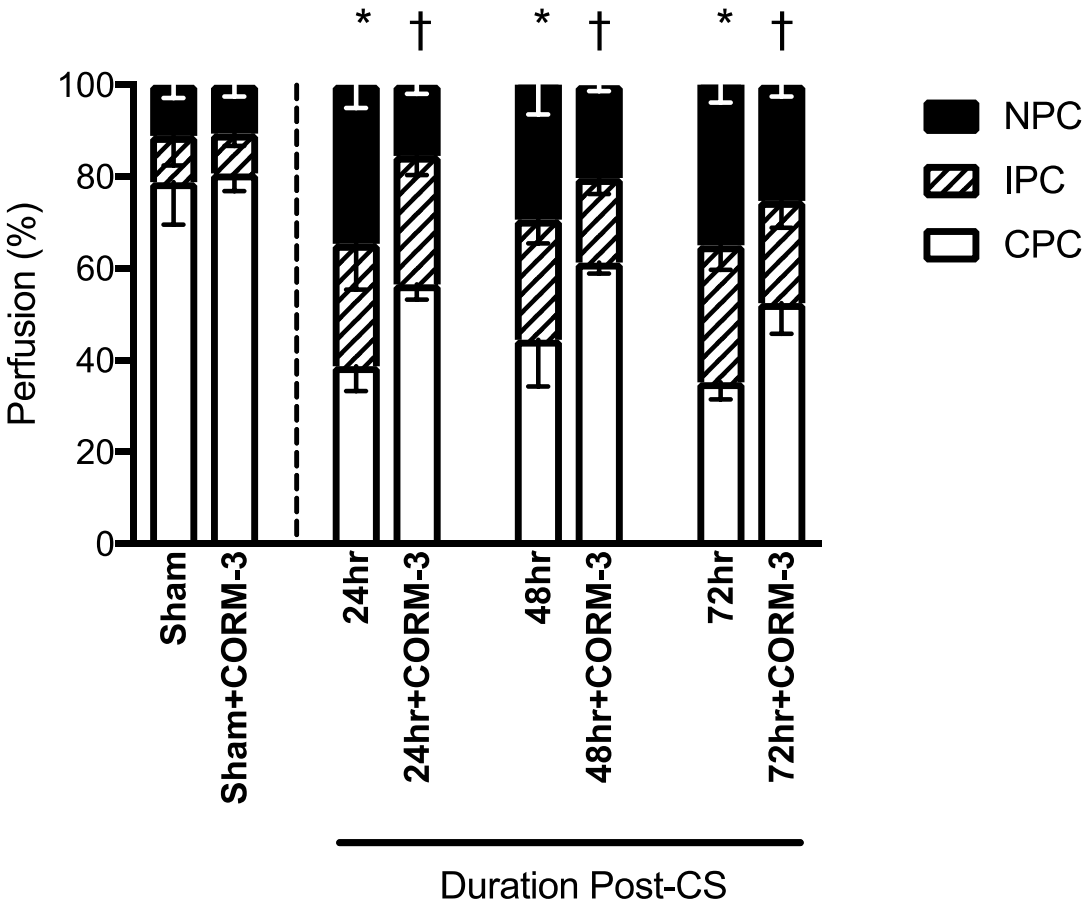


Figure 3.1 The effect of CORM-3 on skeletal muscle perfusion following ACS. ACS resulted in a significant microvascular dysfunction; administration of CO donor, CORM-3, partially restored the perfusion. CPC: continuously perfused capillaries; IPC: intermittently perfused capillaries; NPC: non-perfused capillaries (*$p<0.001$ from sham; †$p<0.05$ from ACS).

3.1.3 Non-Perfused Capillaries

A significant increase in the number of NPC was observed, from 11±3% in the sham group, to 35±5%, 29±7%, 35±4% at 24, 48, and 72 hours post-ICP elevation, respectively (two-way ANOVA, p=0.0006). Administration of CORM-3 led to a significant decrease in the number of NPC in all time-matched groups (16±2%, 20±1%, 25±3% at 24, 48, and 72 hours, respectively versus 11±3% in sham, p=0.0012) (Figure 3.1). CORM-3 did not produce any effect on the number of NPC in sham rats.

3.2 TISSUE INJURY

Significant tissue injury in ACS rats was observed: the EB/BB ratio of 0.03±0.003 in the sham group rose to 0.31±0.08, 0.41±0.08, 0.40±0.1 at 24, 48, and 72 hours post-ICP elevation, respectively (two-way ANOVA, p=0.0004). Administration of iCORM-3 had no effect on tissue injury in any of the ACS animals, while CORM-3 significantly reduced the EB/BB ratio to 0.15±0.05, 0.17±0.05, 0.20±0.04 at 24, 48, and 72 hours post-ICP elevation, respectively (two-way ANOVA, p=0.0013). Additionally, no significant difference was found between the sham group and animals that received CORM-3 (Figure 3.2).

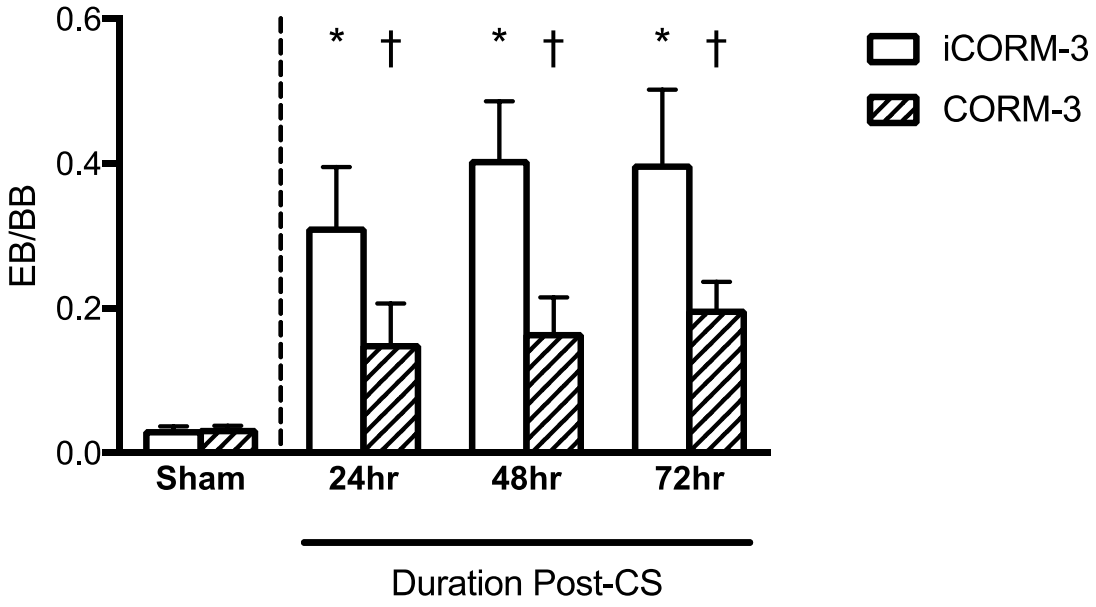


Figure 3.2 **The effect of CORM-3 on skeletal muscle tissue injury following ACS**. ACS resulted in a significant increase in tissue injury; administration of CO donor, CORM-3, significantly reduced tissue injury caused by ICP elevation (*$p<0.05$ from sham; †$p<0.05$ from ACS).

3.3 LEUKOCYTE BEHAVIOUR

3.3.1 Leukocyte Rolling

Significant increase in leukocyte rolling following ICP elevation was observed at 24 hours: from 4±2 leukocytes/30sec/1000μm^2 in the sham group, to 19±5 leukocytes/30sec/1000μm^2 in the ACS group (p=0.0007). At 48 and 72 hours post-ACS, leukocyte rolling was 9±1 and 10±2 leukocytes/30sec/1000μm^2, respectively; values which were not significantly different from the sham group (p=0.3603 and p=0.2749, respectively). Administration of CORM-3 resulted in a significant reduction in rolling leukocytes at 24 hours: 5±1 leukocytes/30sec/1000μm^2 versus 2±0 leukocytes/30sec/1000μm^2 in sham (p=0.0013). In the 48- and 72-hour groups, leukocyte rolling was 10±3 and 10±3 leukocytes/30sec/1000μm^2, respectively, which were not significantly different from iCORM-3 groups (p=0.9998 and p=0.9999, respectively) (Figure 3.3).

3.3.2 Leukocyte Adhesion

ICP elevation led to a significant increase in adherent leukocytes, from 1±1 in the sham to 5.6±0.8, 8.2±2.4, 6.7±1.0 leukocytes/30sec/1000μm^2 at 24, 48, and 72 hours following ACS, respectively (two-way ANOVA, p=0.0059). Administration of CORM-3 significantly reduced leukocyte adhesion to 2±1, 1±0, 3±1 leukocytes/30sec/1000μm^2 at 24, 48, and 72 hours post-ICP elevation, respectively (two-way ANOVA, p=0.0001) (Figure 3.4).

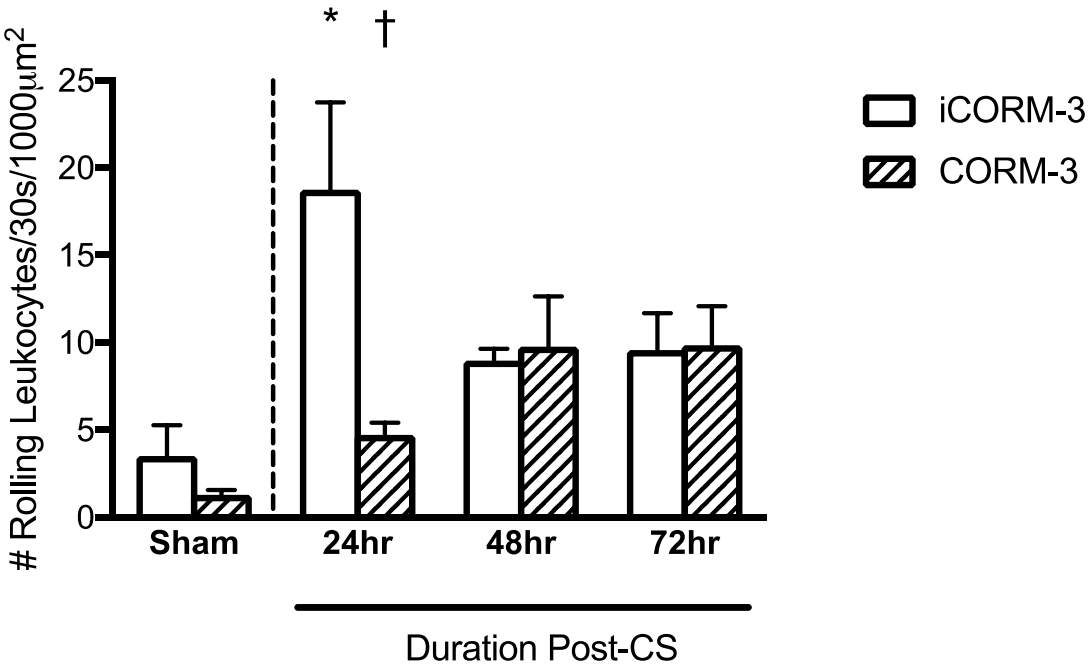


Figure 3.3 **The effect of CORM-3 on leukocyte activation following ACS: leukocyte rolling**. ACS led to a significant increase in leukocyte rolling; administration of CO donor, CORM-3, attenuated this response at 24hr post-ACS (*$p<0.05$ from sham; †$p<0.05$ from ACS).

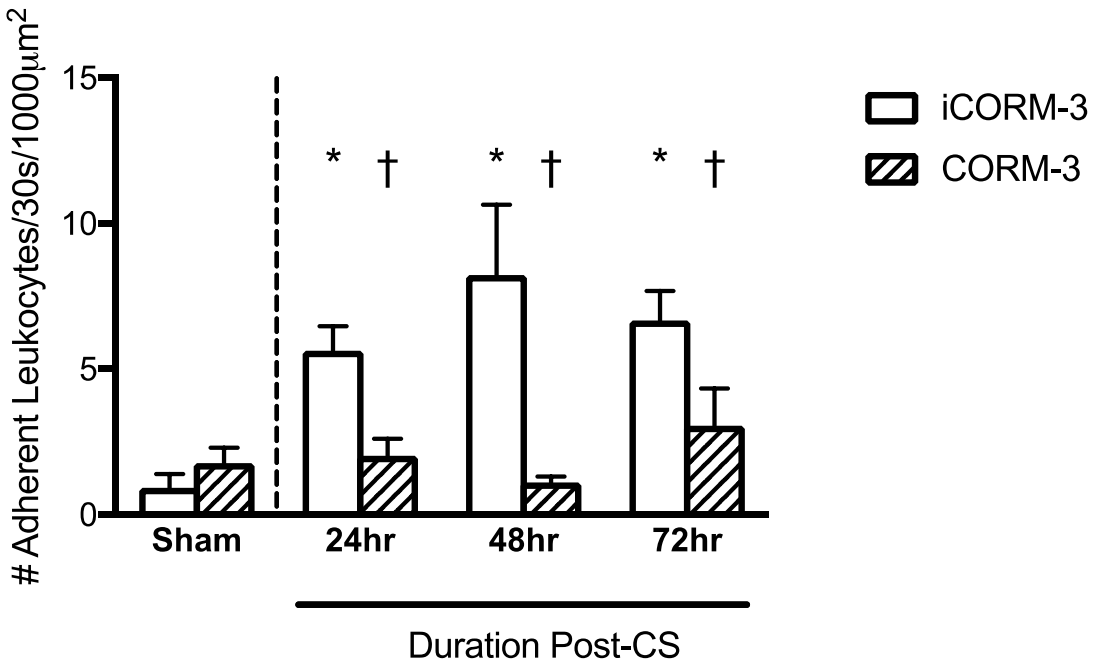


Figure 3.4 **The effect of CORM-3 on leukocyte activation following ACS: leukocyte adhesion**. ACS led to a significant increase in leukocyte adhesion; administration of CO donor, CORM-3 attenuated this response at all time points (*$p<0.05$ from sham; †$p<0.05$ from ACS).

CHAPTER 4: DISCUSSION

4.1 OVERVIEW OF RESULTS

ACS is a surgical emergency for which the gold standard treatment is open fasciotomy. However, ACS remains a clinical diagnosis with multiple risk factors that can lead to delayed or missed diagnosis. Consequently, delayed management of ACS can result in loss of limb function, systemic complications, and even death (Mortensen et al. 2020; Olson & Glasgow 2005; Pearse et al. 2002; Via et al. 2015).

Given the challenges of timely diagnosis and surgical intervention, many alternatives have been previously proposed. Yet, none have been proven as a stand-alone first line treatment. Furthermore, fasciotomies have their own set of issues and may also result in significant debilitating complications (Fitzgerald et al. 2000; McMillan et al. 2019; Schmidt 2017). Therefore, there is a need to develop alternative therapeutic interventions in the treatment of ACS, or at least, to prolong the surgical window.

Over the last decade, CO has shown therapeutic potential in animal models of ACS when administered upon fasciotomy. However, its effects in ACS without fasciotomy are unknown. Therefore, the aim of this experimental project was to assess the use of CO, liberated from water-soluble CORM-3, in ACS *without* surgical intervention.

4.1.1 The Effect of ACS

Although the true underlying pathophysiology of ACS remains elusive, there is mounting evidence that ischemic insult may be occurring concurrently with reperfusion injury, rather than a pure ischemic event followed by reperfusion syndrome. Reperfusion following ischemia causes oxidative stress and mediates oxygen-derived ROS, leading to cellular damage through lipid peroxidation, leukocyte-endothelial activation, upregulation of inflammatory mediators such as TNF-α and IL-1β working synergistically along with leukotrienes, thromboxane A_2, prostaglandins, and platelet-activating factors, thus causing further microvascular dysfunction, changes in vascular permeability, cell death, tissue necrosis, soft tissue swelling, increased ICP, and potential systemic inflammatory responses (Cowled & Fitridge 2011; Gourgiotis et al. 2007; Grace 1994; Lawendy et al. 2014; Lutz et al. 2010; Soares et al. 2002; von Keudell et al. 2015).

4.1.1.1 Microvascular Changes

Hindlimb ICP elevation resulted in a significant increase in non-perfused and intermittently-perfused capillaries, whereas the number of continuously-perfused capillaries significantly decreased, leading to a low-flow ischemic state. These findings are consistent with previous studies of ACS assessing CPC, IPC, and NPC changes using IVVM in rat models, where microcirculatory deficits were found to occur by shifting continuous perfusion towards altered and/or absent flow (Bihari et al. 2017; Lawendy et al. 2011, 2014, 2015; Manjoo et al. 2010). A

previous canine study had also characterized ACS as an incomplete and maldistributed microvascular blood flow rather than complete ischemia (Sadasivan et al. 1997).

The results corroborate with previous studies of I-R, showing early significant microcirculatory changes. Menger et al. (1992) used a hamster striated muscle I-R model, showing significant capillary hemodynamic changes at 30 minutes and 2 hours following reperfusion, but very little changes between 2 hours and 24 hours (Menger et al. 1992). In rat gracilis muscle I-R, Olivas et al. (2001) had demonstrated progressive microvascular dysfunction mainly within the first 7 hours of reperfusion. However, the magnitude of hypoperfusion saw very little change between 7 and 48 hours after reperfusion (Olivas et al. 2001). This suggests rapid and non-reversed microvascular dysfunction when left untreated, which was reflected in our study. However, given that ischemia occurs concurrently with reperfusion in ACS, the magnitude of perfusion deficits and timing of its deleterious effects likely differ from a pure I-R injury.

The no-reflow phenomenon has been used to describe the failure of capillary reperfusion and has been described in previous cardiac (Schofer et al. 1985), renal (Summers & Jamison 1971), epigastric free flap (May et al. 1978), and skeletal muscle (Blaisdell 2002) models of I-R. However, its underlying mechanism remains controversial. Debate still exist whether it is a consequence of ischemic muscle and cell death leading to non-perfused vessels, or whether the reperfusion injury ultimately shunts vascular perfusion. The aetiologies of the no-reflow phenomenon have been separated into two categories: structural and

functional. Structural no-reflow implies vasoconstriction, microembolization, endothelial damage, and extravascular compression (interstitial edema) while the functional variant is a result of leukocyte, erythrocyte, and platelet aggregation, observed especially in the post-capillary venules (Blaisdell 2002; Durante & Camici 2015; Maksimenko & Turashev 2012; Niccoli et al. 2010).

The no-reflow phenomenon can be appreciated in our study with the significant amount of NPC. In ACS, increased fluid extravasation from the vascular bed increases interstitial pressures leading to ICP elevation; this causes adjacent capillary lumen compression, progressive non-perfusion and ischemia (Gute et al. 1998; Lawendy et al. 2011). However, microcirculatory deficits are not only dependent on passive vasoconstriction secondary to increased interstitial pressure, but also on pro-inflammatory responses from reperfusion leading to vascular endothelial damage and tissue necrosis.

4.1.1.2 Tissue Injury

In low-flow ischemia, since there is still a degree of perfusion, reperfusion injury occurs concomitantly with ischemia rather than a no-flow ischemic event followed by reperfusion. Thus, parenchymal damage is likely caused by the perfusion deficits leading to hypoxemia, coupled with the ensuing inflammatory response (Bihari et al. 2017; Forbes et al. 1996; Gute et al. 1998; Lawendy et al. 2011, 2014, 2015). Although controversial, it appears that reperfusion following partial ischemia or elevated ICP produces more severe tissue damage than after complete ischemia (Heppenstall et al. 1986; Perry et al. 1984; Roberts et al.

1985). However, in a murine I-R experiment, Conrad (2005) demonstrated significantly reduced tissue viability at 24 hours post-reperfusion following complete ischemia, despite having greater early inflammatory response from partial ischemia (Conrad et al. 2005).

In our study, the EB/BB ratio significantly increased, indicating extensive tissue damage from elevated ICP at all examined time points. The EB/BB ratio appears to have plateaued at the 48-hour mark, showing slightly higher value than what was found in the 24-hour group, but minimal difference compared to the 72-hour time point. Comparatively, an I-R rat study performed by Olivas et al. (2001) demonstrated muscle necrosis worsening over the first 8 hours, followed by minimal changes to the damaged tissues between 8 and 48 hours of reperfusion (Olivas et al. 2001).

Reperfusion injury is a major contributor to the pro-inflammatory response. In a study by Bihari et al. (2017), no-flow ischemia followed by reperfusion demonstrated significant increase in EB/BB staining during the 90-minute reperfusion interval. However, after 2 hours of ischemia and prior to reperfusion, it was still at nearly the same level as in the sham group, indicating that the maximum amount of tissue necrosis occurred during reperfusion, and not during ischemia alone. The results also correlated with the systemic TNF-α levels, where TNF-α sharply increased at the time of reperfusion (Bihari et al. 2017). Thus, the pro-inflammatory response and subsequent tissue damage re-enforces the idea of underlying reperfusion injury in ACS with concurrent progressive ischemia.

The assumption that ischemic insult and reperfusion injury are occurring simultaneously in ACS is based on previous studies demonstrating perpetual and recurrent local I-R events, free radical generation, and endothelial damages, especially in peripheral vascular diseases (PVD). Both ambulation and exercise, but not while at rest, can induce progressive reperfusion syndrome from chronic hypoperfusion in patients with PVD. Its clinical symptom is known as intermittent claudication and can eventually progress to critical limb ischemia (Ciuffetti et al. 1991; Dopheide et al. 2013; Hickman et al. 1994). Similar mechanisms have been observed in the progression of non-healing chronic wounds (Mustoe et al. 2006) and diabetic foot ulcers (Alavi et al. 2014).

In our study, ICP elevation caused significant cellular injury and tissue damage. Historically, cellular necrosis was thought to be a form of unregulated cell death, as a consequence of disease process. However, there is increasing evidence that cellular necrosis is, in fact, mediated by defined (but not fully understood) molecular pathways, and is termed *necroptosis.* Although necroptosis shares upstream inflammatory mediators with apoptosis (eg. TNF-α), their downstream pathways are biologically distinct (Linkermann & Green 2014; Van Cruchten & Van den Broeck 2002; Vanlangenakker et al. 2011a,b). In I-R injuries, it appears that both necroptosis and apoptosis are involved in cell death. A combination of both apoptotic and necrotic cells has been found in I-R studies of rat gracilis muscles (Wang et al. 2008), murine gastrocnemius muscles (Tran et al. 2012), as well as other organs, including the brain (Degterev et al. 2005), heart (Oerlemans et al. 2012), kidney (Linkermann et al. 2013), and lungs

(Fischer et al. 2000). Previous studies of ACS have shown significant systemic pro-inflammatory cytokine TNF-α elevation (Bihari et al. 2018; Lawendy et al. 2014, 2016). Although it is impossible to differentiate cell death by necroptosis and apoptosis in this study, both pathways were likely involved, given that they are both potentiated by TNF-α.

Leukocytes are a major contributor to tissue damage and muscle necrosis. Tissue damage appears to be mediated by inflammatory cytokines, such as TNF-α and/or IL-1β (Donohoe et al. 2018; Lawendy et al. 2011). Sadasivan et al. (1997) had previously demonstrated significant reduction in microvascular injury and neutrophil infiltration in ACS under neutropenic conditions (Sadasivan et al. 1997). In a rat model of ACS, Lawendy et al. (2015) compared ACS in leukopenic and normal rats. Leukopenia led to the suppression of leukocyte recruitment and no significant muscle tissue injury was observed. Meanwhile, leukopenia had no effect on perfusion deficits (Lawendy et al. 2015). This demonstrated that in ACS, ischemic insult also occurs distinctively from inflammatory-mediated damage. In our study, elevated ICP also led to leukocyte activation and was associated with significant tissue injury.

4.1.1.3 Leukocyte Activation

Leukocyte activation in response to elevated ICP showed significantly higher number of rolling leukocytes at 24 hours, but not at 48 or 72 hours. In fact, the number of rolling leukocytes trended towards normal in the latter 2 groups (still elevated compared to sham, but not significantly). Meanwhile, significant

increase in leukocyte adhesion in post-capillary venules was observed at all time points, with peak levels occurring at 48 hours post-ICP elevation.

The results corroborate with those of previous experiments examining the relationship between leukocytes and vascular endothelial inflammation. In a rat model of endotoxin-induced uveitis (EIU), Miyamoto et al. (1996) demonstrated peak numbers of rolling leukocytes between 12 and 24 hours (Miyamoto et al. 1996). Another experiment had shown peak rolling leukocyte at 24 hours and returned to normal by 72 hours (Baatz et al. 1995). In a rat endotoxin-induced intestinal inflammation study, the peak number of rolling leukocytes was found between 6 and 12 hours, and subsequently trended towards control-group levels from 24 to 72 hours while the number of adherent leukocytes peaked at 24 hours before trending back down (Watanabe et al. 2018). A report of rat hindlimb allograft rejection found a significant increase in rolling leukocytes up to 24 hours post-transplantation. The number of rolling leukocytes continued to increase by 72 hours, however, not significantly. Meanwhile, leukocyte adhesion continued to increase significantly between 24 and 72 hours (Ozer et al. 1999).

The early peak in rolling leukocytes, combined with later increase in leukocyte adhesion can be explained by the leukocyte-endothelial activation sequence. The selectin receptors found on leukocytes (L-selectin), endothelium (E-selectin) and both the endothelium and platelets (P-selectin) act as mediators of leukocyte rolling behaviour. In I-R injury, inflammatory cytokines, complement factors, and ROS activate selectins to initiate rolling leukocytes. P-selectins found in pre-formed pools in Weibel-Palade bodies in the endothelium are quickly

released within 30 minutes. Meanwhile, E-selectin is induced under transcriptional control, and will take several hours to reach its peak. Additionally, shedding of the constitutively expressed L-selectin is inversely proportional to leukocyte activation and works synergistically with P-selectins to stimulate rolling leukocytes. Loss of L-selectins is also associated with activation of MAC-1, important for later ICAM expression (Cowled & Fitridge 2011; Kurose et al. 1994; Ley et al. 2007; Panes & Granger 1998; Weibel & Palade 1964).

E-selectin appears to return to baseline levels by 24 hours from onset of inflammatory stimulus, while P-selectin has been found to be upregulated for up to 24 hours and expressed for up to 48-72 hours following leukocyte cascade activation (Cowled & Fitridge 2011; Hallahan et al. 1996; Panes & Granger 1998). In rat models, P-selectin was found to be maximally expressed up to 24 hours following cerebral artery reperfusion (Suzuki et al. 1997) and P-selectin mRNA expression was upregulated up to 24 hours in retinal I-R (Nishijima et al. 2004). In a pig model of inflammation, peak expression of E-selectin was found within the first few hours while returning to near baseline levels at 24 hours (Binns et al. 1996). Additionally, all 3 selectins interact with leukocytes via P-selectin glycoprotein ligand-1 (PSGL-1). While P- and L-selectins seem to share similar binding configuration to PSGL-1, it differs with E-selectin (Somers 2000, Ley 2003). PSGL-1 binding with E-selectin appears to be transient compared to P-selectins (Ley & Kansas 2004; Vestweber & Blanks 1999).

To initiate arrest and adhesion, CD11/CD18 neutrophilic αβ-integrin complexes bind onto the endothelial ICAM-1 and ICAM-2 ligands, whereas

neutrophilic VLA-4 interacts with MADCAM-1 and VCAM-1 ligands. While VCAM-1 and ICAM-1 peak expressions have been highly variable, ranging from 6 hours to 36 hours, these ligands are, nonetheless, highly expressed up to 48 hours post-activation (Cowled & Fitridge 2011; Eppihimer & Granger 1997; Hallahan et al. 1996; Langer & Chavakis 2009; Panes & Granger 1998). Therefore, the higher expression of selectins within the first 24 hours and early weaning help explain the initial surge in rolling leukocytes while adhesion endothelial factors usually have increased upregulation up to 48 hours post-activation, if not longer.

Inflammatory cytokines and chemokines play a key role in leukocyte activation. In a rat model of ACS, Donohoe et al. (2018) identified 16 detectable systemic cytokines/chemokines, 6 of which were significantly upregulated following ICP elevation. These included pro-inflammatory cytokines TNF-α, IL-1β, along with chemokines growth-related oncogene-α (GROα), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α). They also found increased levels of anti-inflammatory cytokine IL-10. Subsequently, administrating TNF-α neutralizing antibodies (NA) alone or TNF-α NA concurrently with IL-1β NA resulted in a significant decrease of leukocyte rolling, whereas giving IL-1β NA alone had no effects. Meanwhile TNF-α NA alone or combined with IL-1β NA produced significant reduction in leukocyte adhesion, while IL-1β NA alone only produced partial improvements (Donohoe et al. 2018). Although GROα, MCP-1, and MIP-1α were not tested against NA, they remain important mediators of pro-inflammatory response and leukocyte activities, whereas IL-10 is an anti-inflammatory cytokine (Bechara et al. 2007;

Deshmane et al. 2009; García-Bonilla et al. 2011; Maurer & von Stebut 2004; Menten et al. 2002; Ouyang & O'Garra 2019). Thus, identifying the exact roles and temporal activation of these cytokines/chemokines on rolling leukocytes would help elucidate the differences seen between the earlier and later time points in our study.

4.1.2 Therapeutic Effects of CORM-3-Derived CO

CO has been found to exhibit potent anti-inflammatory, anti-proliferative, anti-apoptotic, and vasodilatory effects. However, given the toxicity of inhaled CO, CO-RMs were synthesized in order to control CO delivery without elevating COHb levels (Guo et al. 2004; Lawendy et al. 2014; Motterlini & Otterbein 2010; Ryter & Choi 2016).

4.1.2.1 Effects of CORM-3 on Microvascular Perfusion

Systemic application of CORM-3 led to partial restoration of perfusion following ICP elevation: there was a significant increase in CPC and decrease in NPC. Microvascular perfusion remained relatively constant and unchanged between the 24-, 48-, and 72-hour groups. Although significant microvascular improvements were observed following treatment with CORM-3, the perfusion deficit still remained significant compared to the sham group. The results of this study are comparable with those of ACS treated with CORM-3 at fasciotomy: CORM-3 significantly restored the number of CPC and reduced the number of NPC in rat (Lawendy et al. 2014) and porcine models of ACS (Bihari et al. 2018),

as well as in pure I-R injury (Bihari et al. 2017). However, these studies carried out microvascular analysis between 45 minutes and 3 hours following elevation of ICP, whereas we assessed longer-term results.

CO has been shown to mediate vasodilation via sGC. In the endothelial cGMP-dependent pathway, CO is in fact a weaker agonist than NO (Kajimura et al. 2010). However, CO appears to mediate NO-independent cGMP in the vascular smooth muscle cells, preventing proliferation and intimal hyperplasia. CO also mediates cGMP-independent pathways by mediating calcium-potentiated potassium membrane channels to induce vasodilation (Jaggar et al. 2002; Motterlini & Otterbein 2010; Ryter & Choi 2016; Ryter & Otterbein 2004; Suematsu et al. 1995; Wang et al. 1997).

NO also plays an important role in vasoregulation. Vasoconstriction is not only a result of passive changes from increased interstitial pressure and edema, but also due to a vasomotor component and autonomic dysfunction (Durante & Camici 2015; Nanobashvili et al. 2003). Pericytes in the microvascular wall act as contractile cells, activated by vasoactive hormones. Capillary dilatation from pericyte activity appears to be mediated by the NO/cGMP pathway (Bettaga et al. 2015; Hall et al. 2014; Kelley et al. 1988; Nanobashvili et al. 2003; Sakagami et al. 2001). CORM-3 has been shown to stimulate NOS in the endothelium and activate cGMP pathway, while inhibiting NOS in the acetylcholine-induced endothelium-dependent relaxation (Alshehri et al. 2013). In our study, CORM-3 resulted in improved microvascular perfusion, decreasing the no-reflow

phenomenon, which could be explained, at least in part, by interplay of vasodilatory effects of CO and NO.

4.1.2.2 Effects of CORM-3 on Tissue Injury

In our experiments, tissue injury was significantly reduced following administration of CORM-3. EB/BB ratio in our study ranged between 0.14-0.2, values that are slightly higher than those found previously, where CORM-3 was given at fasciotomy or upon reperfusion (Bihari et al. 2017; Lawendy et al. 2014). However, those results reflect tissue injury at very early time points (45 to 90 minutes) compared to 24, 48, and 72 hours in our current study.

Our results are consistent with those of others who had previously demonstrated that CORM-3 was capable of lessening cellular damage and increasing tissue survival, including decreased cardiac infarct size and muscle damage (Clark et al. 2003; Guo et al. 2004; Varadi et al. 2007), improved renal graft survival and mitigating parenchymal damage (Sandouka et al. 2006; Vera et al. 2005), and demonstrated antiapoptotic effects in lung I-R injuries (Zhang et al. 2003). While the exact mechanisms of cellular protection are most likely related to the vasodilatory and anti-inflammatory properties, there is another possibility: inhibition of apoptosis. CO appears to target multiple layers of the pro-apoptotic pathways, including inhibition of cytochrome *C* oxidase and NADPH oxidase-derived ROS (Boczkowski et al. 2006; Taillé et al. 2005; Zuckerbraun et al. 2007), upregulation of NF-kB-dependent anti-apoptotic genes (A1 and c-IAP2) via P38 MAPK activation and downregulation of ERK1/2 (Boczkowski et al. 2006;

Brouard et al. 2002; Ryter et al. 2007; Soares et al. 2002; Zhang et al. 2003; Zuckerbraun et al. 2007).

Leukocytes, particularly neutrophils, also appear to play a significant role in generating the observed endothelial damage. Activated neutrophils secrete MPO, leading to the formation of ROS and destruction of cellular membrane (Dejonckheere et al. 2011; Eltzschig & Eckle 2011; Hobeika et al. 2008; Lutz et al. 2010; Panes & Granger 1998). Application of CORM-3 was found to modulate polymorphonuclear (PMN)-induced ROS production, as well as mitigate cell surface levels of elastase in several *in vitro* models, with subsequent reduction in PMN rolling and adhesion along endothelial cells, thus decreasing endothelial dysfunction (Bihari et al. 2019; Mizuguchi et al. 2009; Patterson et al. 2014). It can be surmised that, although CO is not a free radical scavenger, it may have the ability to suppress the oxidative stress indirectly, by modulating the activity of redox enzymes, thereby minimizing the extent of endothelial injury.

4.1.2.3 Effects of CO and CORM-3 on Leukocyte Activity

In our study, leukocyte-endothelial activation appeared to be inhibited by CORM-3, as demonstrated by a significant reduction of adherent leukocytes at 24-, 48-, and 72-hour time points. Surprisingly, rolling leukocytes were markedly reduced by CORM-3 only at 24 hour, but not at later times.

Multiple studies have demonstrated anti-inflammatory properties of CO and its inhibitory effects on leukocyte activity. For example, animals with acute pancreatitis or sepsis treated with CO donor, CORM-2, demonstrated significant

reduction of ICAM-1 and VCAM-1 adhesion molecules, inhibition of inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as downregulation of NF-kB expression (Liu et al. 2019a,b). In intestinal and hepatic I-R models, CORM-2 diminished expression of TNF-α, NF-kB activity, leukocyte and endothelial adhesion molecules, as well as neutrophil infiltration (Katada et al. 2010; Wei et al. 2010). CO was found to inhibit a variety of inflammatory cytokines, including MCP-1, in a murine renal I-R (Ruan et al. 2014), while increasing anti-inflammatory cytokine IL-10 in a rat model of pancreatitis (Chen et al. 2010) and a murine model of acute liver failure (Yan et al. 2016). Various *in vitro* and *in vivo* experiments have shown that application of CORM-3 led to inhibition of TNF-α-mediated activation, translating into a decrease in VCAM-1, E-selectin, integrin complexes, as well as caspases (pro-apoptotic) activity (Bergstraesser et al. 2012; Bihari et al. 2019; Motterlini 2007; Song et al. 2009; Vadori et al. 2009). Additionally, CORM-3 has been shown to have cytoprotective effects in cardiac and renal I-R through upregulation of mitochondrial ATP-dependent potassium channels and calcium-potentiated potassium channels, downregulation of NADPH oxidase-generating superoxide free radical, suppression of NF-kB activity, and inhibition of E-selectin and ICAM-1 (Caumartin et al. 2011; Clark et al. 2003; Guo et al. 2004).

CO appears to suppress Toll-like receptor activity and NF-kB gene transduction pathways involved in inflammatory response. By inhibiting NF-kB activity, TNF-α-mediated upregulation of adhesion molecules (such as E-selectin, ICAM-1 and VCAM-1) is suppressed (Chhikara et al. 2009; Choi et al. 2017; Soares et al. 2002). CO has also shown potent inhibitory effects on

lipopolysaccharide-induced upregulation of TNF-α and IL-1β (Otterbein et al. 2000). Previous studies have demonstrated, *in vitro*, suppression of leukocyte-endothelial interaction of platelet-activating factor, CD11b integrin surface levels, cell surface elastase (proteolytic enzyme), MMP activity and neutrophil-derived MPO activity; substantially decreasing adhesion and transmigration of leukocytes in inflammation-stimulated environments (Bihari et al. 2019; Inoue et al. 2017; Megías et al. 2007; Mizuguchi et al. 2009; Patterson et al. 2014; Urquhart et al. 2007; Zhuang et al. 2017).

While CORM-3 appears to have a significant effect on leukocyte adhesion and transmigration, its effects on leukocyte rolling remains questioned. In our experiment, significant increase in rolling leukocytes occurred only at 24 hours, but not at 48 or 72 hours post-ICP elevation. This can be explained, at least in part, by selectin modulation and their differential PSGL-1 binding (Ley 2003; Somers et al. 2000; Vestweber & Blanks 1999). Previous studies of CO-RMs have shown suppression of the E-selectin receptors in renal rat I-R (Caumartin et al. 2011) and *in vitro* HUVEC models (Song et al. 2009). However, CORM-3's effect on P-selectin remains largely unknown. A study of lipopolysaccharide-stimulated platelet model using blood from healthy volunteers demonstrated a decrease in platelet P-selectin expression following CORM-2 treatment (Liu et al. 2016). Given that CORM-3 attenuation of rolling leukocyte behaviour only occurred at 24 hours, its suppression is likely through the inhibitory effects of CO on inflammatory cytokines. However, CORM-3's significant therapeutic effect on adherent leukocytes in all 3 groups suggests that CO also has a direct cytokine-

independent effect on adhesion molecules, such as immunoglobulin superfamily (eg. ICAM-1, VCAM-1) and αβ-integrin complexes (eg. LFA-1, MAC-1) (Bihari et al. 2017; Lawendy et al. 2014). Finally, uncovering temporal activation and effects of other inflammatory cytokines/chemokines on leukocytes, as well as their response to CORM-3 will help further elucidate the leukocyte activity observed in this study.

4.2 STUDY LIMITATIONS

Despite the encouraging results, there are several limitations to our study. First, the duration of elevated ICP was based on previous studies, as well as on physiological and mathematical assumptions (Hoppeler & Weibel 2005; Lawendy et al. 2011, 2014; Radermacher & Haouzi 2013). Therefore, it was assumed that 2 hours of elevated ICP in rats corresponded to 8 hours in humans. However, future studies are required to correlate ACS equivalence in rats and humans.

Second, only one injection of CORM-3 was administered, using a single route. Therefore, whether there is an additive or dynamic effect of multiple injections and/or injection sites, as well as the optimal rate of CO release in the treatment of ACS remain unclear.

In this experiment, no functional testing (i.e. assessing rat hindlimb function) was performed. Thus, no clinical correlations could be made. Finally, while CORM-3 does not seem to elevate COHb levels in animal models, it has yet to be tested in humans. How CORM-3 injections translate in humans during ACS is unknown.

4.3 FUTURE DIRECTIONS

This study remains an early experimental study of CORM-3 without fasciotomy. Further studies are required to elucidate its role as a potential first-line treatment in ACS or as an adjunct to prolong the surgical window.

1) Analyze the effects of CORM-3 during shorter intervals, e.g. at 6- and 12-hours post-elevation of ICP, to identify whether there are more prominent effects in the earlier stages.
2) Compare outcomes using different injection doses and frequencies, as well as alternative routes of administration of CORM-3.
3) Perform functional limb assessments to determine the clinical outcomes of CORM-3.
4) Further studies are needed to clarify CORM-3's mechanism of action and its effects on tissue regeneration.
5) In previous animal studies of ACS, despite undergoing fasciotomies, inflammatory markers remained elevated systemically, often observed as a *second hit phenomenon*. Clinically, acute renal failure, rhabdomyolysis, systemic shock, and multiorgan dysfunction syndrome can occur despite fasciotomies. Although CORM-3 has shown its ability to suppress TNF-α and other inflammatory cytokines, it would be important to determine whether inhibition of systemic inflammatory cytokines can be sustained long-term when treated without fasciotomy (Bihari et al. 2017; Donaldson et al. 2014; Lawendy et al. 2014, 2016; Mabvuure et al. 2012).

4.4 CONCLUSIONS

Multiple alternative therapies to surgical fasciotomy have been proposed in the treatment of ACS. However, none of them have been validated as a potential first-line treatment. While the exact underlying mechanism of CORM-3 still needs further elucidation, it is likely that the CO released has multiple therapeutic molecular and cellular targets during ACS (Ryter & Otterbein 2004).

In this study, CORM-3 without fasciotomy has demonstrated improved microvascular perfusion, diminished tissue injury, and mitigated leukocyte activation. Based on the current literature, it is plausible to assume that CORM-3 played a major role in mediating vasodilation, reducing pro-inflammatory cytokines/chemokines and formation of ROS, thereby inhibiting leukocyte-endothelial adhesion molecules. This study is a novel experiment demonstrating sustained therapeutic effects of CORM-3 without fasciotomy in the treatment of ACS, indicating its potential role as a pharmacological agent in the management of ACS.

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APPENDICES

APPENDIX I: FASCIOTOMY TECHNIQUES

Currently, the only gold standard in the treatment of ACS remains surgical fasciotomy. The goal is to provide immediate pressure relief and decreased compartmental pressures. By incising the skin and rigid fascia, this increases the volume of the muscle compartments, thereby reducing the pressure and restoring microvascular perfusion (Schmidt 2017). During fasciotomy, the muscles are assessed for colour, consistency, contractility, and perfusion. Any necrotic tissue should be debrided (Lawendy & Sanders 2010; von Keudell et al. 2015).

The lower leg and forearm are the most commonly affected locations in ACS (Elliott & Johnstone 2003). In the lower leg, the most frequently described techniques are the single-incision (lateral incision) technique or two-incision technique (lateral and medial incisions). A single incision with concomitant fibulectomy has been previously described (Kelly & Whitesides 1967). However, this technique has fallen out of favour given that both the two-incision and single incision without fibulectomy techniques provide adequate release without the morbidity of fibular removal, while this technique also places the peroneal vessels and superficial peroneal nerve at risk (Ebraheim et al. 2016; Lawendy & Sanders 2010). In the forearm, the volar and dorsal approaches are used to decompress the muscle compartments.

I.1 LOWER LEG FASCIOTOMY

Although better visualisation of the soft tissues and neurovascular structures favours the two-incision technique, both approaches have yielded satisfactory results and demonstrated adequate 4-compartment release (Forsh & Wolinsky 2013; von Keudell et al. 2015).

I.1.1 Single-Incision Fasciotomy

The patient is positioned supine on the operative table. A tourniquet is applied to the thigh but not insufflated, and the limb is freely prepped and draped. A bump is also placed under the hip. A single lateral incision along the fibula is made longitudinally extending from the fibular head and ends just proximally to the lateral malleolus. Anterior and posterior skin flaps are created; the anterior and lateral compartments are identified, and, using dissecting scissors, longitudinal fasciotomies are performed. It is essential to identify and protect the superficial peroneal nerve in the distal third of the leg piercing out of the fascia from the lateral compartment. Using the posterior flap created and while elevating the structures in the lateral compartment, the superficial posterior compartment is exposed, and the fascia is incised. Subsequently, the soleus ridge is identified, and the soleus muscle is detached from the fibula in order to expose the deep posterior compartment and decompress it. Subperiosteal dissection is performed around the posterior side of the fibula as well to decompress the deep posterior compartment (Forsh & Wolinsky 2013; Gourgiotis et al. 2007; Lawendy & Sanders 2010).

I.1.2 Two-Incision Fasciotomy

The patient is set up the same way as in the single-incision technique. A 20-25cm longitudinal incision is performed midway between the fibula and tibial crest (Figure I.1). Anterior and posterior skin flaps are created, and the lateral intermuscular septum is identified. Fasciotomies of the anterior and lateral compartments in line with the tibialis anterior and fibular shaft, respectively, are performed. Care is taken to identify the superficial peroneal nerve piercing through the fascia of the lateral compartment in the distal third of the leg. Attention is then turned towards the medial aspect of the lower leg. A medially based longitudinal incision is made 2cm posterior to the medial aspect of the tibia. The skin is retracted posteriorly, the saphenous nerve and vein are identified and protected, the septum dividing the superficial and posterior compartments is identified and released, and the fascia overlying the gastrocnemius-soleus complex is released. An incision overlying the flexor digitorum longus is then made to access and decompress the deep posterior compartment. When the soleus bridge is extended more than halfway down the tibia, its extended origin is also released in order to access the deep posterior compartment. (Forsh & Wolinsky 2013; Gourgiotis et al. 2007; Lawendy & Sanders 2010). For both techniques, the wound is left open with packing or loose skin closure for later primary closure or skin grafting. The leg is splinted with the foot plantigrade (Lawendy & Sanders 2010; von Keudell et al. 2015).

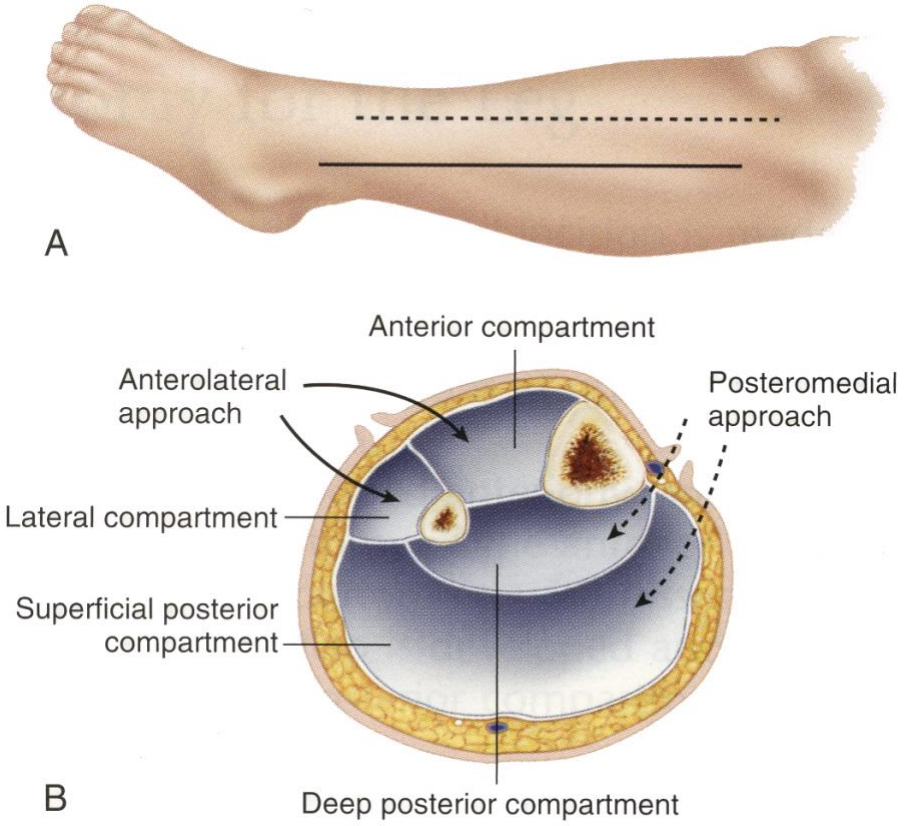

Figure I.1. **Two-incision fasciotomy**. The lateral incision is centred halfway between the fibular shaft and the crest of the tibia. Lateral intermuscular septum is exposed. The anterior compartment is released in line with the tibialis anterior while the lateral compartment is released in line with fibular shaft. A medial longitudinal incision 2cm posterior to the posterior margin of the tibia is created; gastrocnemius-soleus complex fascia is released followed by deep posterior compartment release via fascial incision over the flexor digitorum longus.

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I.2 FOREARM FASCIOTOMY

I.2.1 Volar Approach

The volar incision is performed using either a radial or ulnar curvilinear incision. Regardless of the choice of incision, the volar incision must begin proximal to the elbow flexion crease and terminate past the wrist crease (Figure I.2). Incisions at the elbow and wrist flexion crease are made at an oblique angle to avoid contracture. Proximally, the lacertus fibrosis is released and the brachial artery is explored. The mobile wad, flexor carpi ulnaris and its neurovascular bundle, flexor digitorum superficialis, and median nerve are decompressed, explored, and retracted. The fascia and epimysium overlying the flexor digitorum profundus are then incised. Distally, the carpal tunnel is always released during forearm fasciotomy. A distal volar ulnar approach to explore Guyon's canal may be used when the ulnar nerve is affected (Forsh & Wolinsky 2013; Lawendy & Sanders 2010).

I.2.2 Dorsal Approach

Typically, a volar approach alone is sufficiently adequate to decompress the forearm. Nonetheless, posterior compartment pressures must be assessed. If needed, a dorsal incision extending from 2cm distal to the lateral epicondyle to the centre of the wrist at the level of the radioulnar joint is made for the posterior forearm. Subsequently, the fascia overlying the extensor retinaculum and the mobile wad is decompressed, and the muscles of the mobile wad are individually

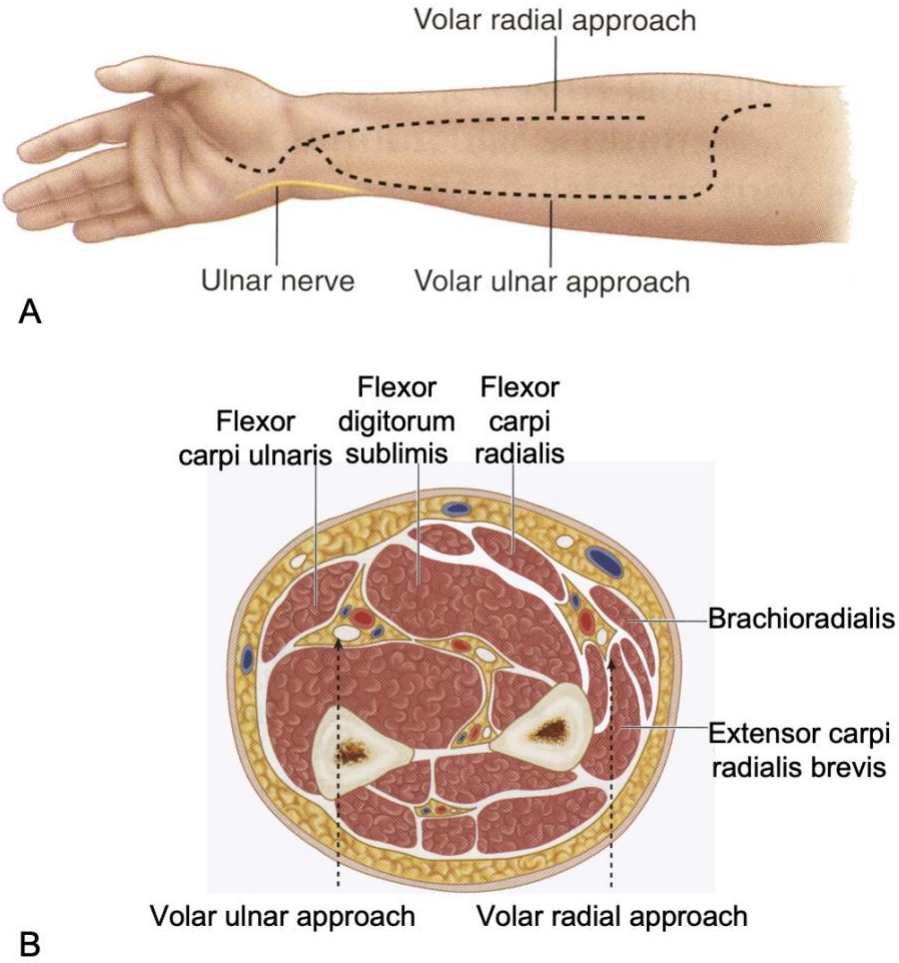

Figure I.2 **Fasciotomy of the forearm**. A volar curvilinear incision is performed, the superficial volar compartment is then released by identifying the mobile wad, flexor digitorum superficialis, flexor carpi ulnaris, and median nerve. Then, the flexor digitorum profundus in the deep compartment is exposed by incising the fascia longitudinally. Proximally, the lacertus fibrosis is released while distally, the carpal tunnel is decompressed.

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released. The incision(s) are kept open with moist gauze for later primary closure or skin grafting, and the arm is splinted with the elbow below 90 degrees of flexion (Forsh & Wolinsky 2013; Lawendy & Sanders 2010).

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II.1 Operative Techniques: Orthopaedic Trauma Surgery 2010;

Compartment Syndrome: Evidence-Based Approaches, Elsevier, pp.679-702

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Pathophysiology of Reperfusion Injury, Barr Smith Press, pp. 331-350

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II.3 Nature Reviews Immunology 2007; 7(9):678-689

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APPENDIX III: ANIMAL PROTOCOL APPROVAL LETTER

Western

AUP Number: 2017-146
PI Name: Lawendy, Abdel
AUP Title: Direct and Remote Organ Injury Following Compartment Syndrome
Approval Date: 04/01/2018

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2017-146:1: entitled " Direct and Remote Organ Injury Following Compartment Syndrome "
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
   http://www.uwo.ca/univsec/policies_procedures/research.html

   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
   e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to
hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

VITA

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Related Work Experience:	Research Assistant 2019 – 2020

Publications:

Wang P, Matache B, Suh N, Grewal R. Treatment of Stages IIIA and IIIB in Kienbock's Disease: A Systematic Review. *Journal of Wrist Surgery,* accepted July 14, 2020.

Wang P, Lawendy AR, Schemitsch E, Bihari A. Possible therapeutic application of CORM-3-derived carbon monoxide in acute limb compartment syndrome. *Orthopaedic Trauma Association*, Annual meeting, October 2020.