August 2015

Potential Therapeutic Role of Hydrogen Sulfide-Releasing Molecule GYY4137 in a Rat Model of Acute Compartment Syndrome.

Moustafa Haddara  
The University of Western Ontario

Supervisor  
Dr. Abdel-Rahman Lawendy  
The University of Western Ontario

Graduate Program in Surgery

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

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POTENTIAL THERAPEUTIC ROLE OF HYDROGEN SULFIDE-RELEASING MOLECULE GYY4137 IN A RAT MODEL OF ACUTE COMPARTMENT SYNDROME

(Thesis format: Monograph)

by

Moustafa Haddara

Graduate Program in Surgery

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Acute limb compartment syndrome (ACS) causes a unique form of limb ischaemia, which induces intense inflammatory response resulting in microcirculatory dysfunction, neutrophil activation and cell injury. Increased intracompartmental pressure is the hallmark of ACS. Decompression by fasciotomy is the gold standard treatment. While fasciotomy saves the limb from ischaemic threat, paradoxically, it causes further damage to the muscle by reperfusion injury. In addition, it does not address the inflammatory element purported to increase the tissue injury in ACS.

Recent evidence suggests that hydrogen sulfide (H$_2$S) can mitigate the damage associated with ischaemia-reperfusion injury. The purpose of this thesis was to examine the value of H$_2$S treatment in a rat model of ACS, using GYY4137 as H$_2$S-releasing molecule. We have demonstrated significant cytoprotective role of H$_2$S on the skeletal muscle following ACS.

These results suggest a potential therapeutic value of H$_2$S as an adjunctive to fasciotomy, for patients suffering ACS.

Keywords: acute compartment syndrome; fasciotomy; tissue injury; inflammation; hydrogen sulfide; carbon monoxide; GYY4137; CORM-3.
CO-AUTHORSHIP

While all of the co-authors listed below made important contributions to this work, I performed all the experiments, data collection, interpretation, and the statistical analysis. The manuscript was entirely written and prepared by me, with the assistance and critical review by the co-authors.

Dr. Abdel-Rahman Lawendy, MD, PhD, FRCSC, provided much 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, MSc, provided invaluable technical support, taught me all the necessary skills to perform this project, and offered guidance and support on data collection, analysis, and interpretation. She trained me on the rat model of compartment syndrome that had been developed by this lab. She also kindly assisted in the manuscript editing.
DEDICATION

I dedicate this work to the memory of my father, the first scholar I had ever met. He was a pivotal figure in the Egyptian academic culture, though humble and conscientious. He taught me to love the knowledge, not merely to learn, and encouraged me to delight in the adventures of intellectual curiosity.

To my mother, it shall be difficult to express my gratitude towards you. Heaven truly is at the feet of our mothers.

To my brothers and sisters: my life’s biggest strength and the beautiful memories of my childhood.

To my wife Sahar: through all of our life’s curvy roads, you have been my heavenly abode.

To Raneem and Mohammad, whom I’ll forever hold close to my heart.
ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and support of supervisors, professors, advisors, laboratory assistants, librarians, colleagues, family and friends.

I wish to express my sincere thanks to Dr. Abdel-Rahman Lawendy, my supervisor, not only for his mentorship and support during this entire process and beyond, but also because he believed that I can make the appropriate shift in my career towards academic studies after a long time in private clinical practice. His confidence in my progress was a huge encouragement for me. It was truly a privilege to work with him.

I am also grateful to Dr. David Sanders and Dr. Christopher Bailey for their advice and support, as members my advisory committee.

Special thanks to Mrs. Aurelia Bihari for all of her help and support. She taught me skills necessary for the completion of this project, like handling animals, anaesthesia, fine experimental techniques and off-line video analysis. I acknowledge her for this and for being a patient tutor through this project.

I would also like to acknowledge the other members of the Trauma Research Lab (Hussein Abdo, Dr. Erin Donohue, and Calvin Poon) for making my time at the lab an enjoyable one.
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1.1. COMPARTMENT SYNDROME

1.1.1. Overview

Acute compartment syndrome (ACS) is a unique form of ischemia affecting group of muscles and nerves enclosed within a relatively noncompliant fascial sheath (Matsen, Winquist, and Krugmire 1980; Sadasivan et al. 1997). The hallmark of ACS is elevation of the intracompartmental pressure (ICP), which results in progressive microvascular ischemia and, if left untreated, leads to muscle cell death, loss of motor function, or loss of limb (Matsen 1975; Rorabeck 1984). The severity of cellular deterioration observed in ACS appears to be more than that seen in ischemia alone (Heppenstall et al. 1988; Heppenstall et al. 1989).

ACS is a critical clinical episode that typically occurs after trauma, and represents a diagnostic and therapeutic challenge to orthopaedic surgeons. Emergency surgical decompression by fasciotomy remains the only proven treatment that mitigates the high pressure, thus saving the tissues from progressive damage (Matsen, Winquist, and Krugmire 1980; Shaw and Spencer 1995). However, fasciotomy is not an entirely benign procedure and is associated with substantial morbidity, as well as potential mortality (Shaw and Spencer 1995; McQueen and Court-Brown 1996). The awareness of ACS has increased significantly in the recent years due to serious complications that...
resulted in medicolegal issues (Taylor, Sullivan, and Mehta 2012; Shadgan et al. 2010; Giannoudis et al. 2002).

Solid evidence suggests that intense inflammatory response and release of reactive oxygen metabolites are critically involved in the development of the cellular damage in ACS (Perler, Tohmeh, and Bulkley 1990; Sadasivan et al. 1997; Kearns et al. 2004; McGarr 2009; Lawendy, Sanders, Bihari, and Badhwar 2011; Lawendy 2014a). Despite the high morbidity and litigation associated with ACS, there is no medical treatment available for routine use, as an adjunctive to surgical decompression to improve the prognosis. Conversely, with the current available knowledge of the pathophysiology of ACS and ischaemia reperfusion injury, there could be a chance to discover a therapeutic agent that may improve the tissue tolerance to ischaemia, or reduce its deleterious effects.

1.1.2. Sequelae Of ACS

The outcome of untreated ACS is catastrophic. Ischaemic insult to the nerve and muscle may lead to muscle contractures, joint stiffness and neurological deficits, resulting in severe functional disabilities. Amputation may be necessary in some cases, particularly if opportunistic infection flares up in the dead muscles (Styf 2003). The deleterious effects of ACS do not occur only locally in the affected limb, but may also happen systemically, resulting in remote organ involvement and severe metabolic disturbance (Heemskerk and Kitslaar 2003; Finkelstein, Hunter, and Hu 1996; Bocca et al. 2002; McQueen and Duckworth 2014; Whitesides and Heckman 1996; Malik et al. 2009). Acute renal
failure, and even death from the ‘crush syndrome’ are known results of massive muscle necrosis seen in ACS (Reis and Michaelson 1986). The complications are strongly related to the duration of ACS (Pearse 2002). Many researchers reported good clinical recovery if fasciotomy was performed within 6 hours (Rorabeck and Clarke 1978). However, irreversible damage has been reported in 37% of patients after only 3 hours of ACS in a large clinical study (Vaillancourt et al. 2004). The 3-hour window was found to be comparable with a tourniquet-induced ischemia in an animal experimental model (Heppenstall et al. 1986). It was reported that only 8% of patients regained useful function when surgery had been done after 12 hours of ACS (Sheridan and Matsen 1976).

1.1.3. Historical Perspective

Richard Volkmann was one of the first to realise that ACS resulted from an ischaemic insult to the muscles rather than just nerve paralysis, as it had been believed. He named the condition “ischaemic muscle paralysis and contracture” pointing to both, the known pathology and the common end result (Volkmann 1881; Volkmann 2007). This term was then adopted by several authors (Huntington 1907; Hildebrand 1906; Düben 1969; Botte et al. 1996). In 1908, the term “Volkmann’s ischemic paralysis and contracture” appeared in the literature (Sayre 1908), which described the pathology, the end-point, and accredited Volkmann for his scientific contribution. The new term became very popular with many authors (Thomas 1909) and was occasionally shortened to “Volkmann’s
paralysis” (Brooks 1922), or “Volkmann’s contracture” (Clarke 1946; Griffiths 1940).

By the middle of the last century, there had been increasing reports of cases of ACS, but with unusual presentations and different clinical scenarios, which was thought to be entities different from ACS. Therefore, special names were given to those conditions: e.g. “march gangrene” (Blandy and Fuller 1957) and exercise ischaemia” (Kirby 1970). Thus, the term “syndrome” started to appear in the literature reflecting the varied clinical picture, complexity of pathophysiology and systemic, as well as local consequences of ACS.

In the English literature, the term “anterior tibial syndrome” (Carter 1949) appeared in a study analysing previous publication of two cases that had suffered necrosis of the anterior compartment muscles without any fractures: one had developed following a repair of muscular hernia, while the second one had been a soldier who had developed bilateral anterior tibial muscle necrosis following marching (Sirbu 1944). Mortez suggested the term “compartment ischemia syndrome” (Mortez 1953). Kunkel and Lynn, the Saskatoon surgeons, simplified the term to “compartment syndrome” (Kunkel and Lynn 1958). Matsen then successfully put forth all the published literature, attempting to understand the principal mechanisms behind the clinical presentations, and outlining the diagnostic and therapeutic rationale. The result was the unified concept of ACS (Matsen 1975).
1.1.4. Clinical Anatomy

A compartment is a closed osseofascial anatomical space packed with muscles, nerves and vessels. The muscles in the extremities are arranged into groups, each enclosed within a fascial envelope (Ellis 2006). Each compartment has a limited capacity to accommodate a sudden increase in the volume of the contents, as happens when acute severe oedema or bleeding inside the compartment follow trauma (Matsen 1975). Knowledge of the surgical anatomy of the compartments is a paramount necessity for a safe and complete surgical decompression. In the leg, there are four distinct anatomical compartments: anterior, lateral, superficial posterior and deep posterior (Figure 1.1).

1.1.5. Aetiology

Fractures cause 69% of cases of ACS, with tibial shaft fractures being the most common of all (McQueen, Gaston, and Court-Brown 2000). Soft tissue trauma is the second most common (23.2%) (McQueen, Gaston, and Court-Brown 2000). Other causes of ACS include arterial injury, reperfusion injury, constrictive dressings and casts, bleeding disorders, injection injuries, burns, and infection (Donaldson, Haddad, and Khan 2014). The overall incidence of ACS following extremity trauma is 2.8% (Branco et al. 2011).

1.1.6. Epidemiology

ACS affects mainly young adults, but it has been reported in all ages, including children and neonates (Willis 2012; Ragland et al. 2005; Goubier, .)
Figure 1.1. Anatomical compartments of the leg, shown at the level of the mid calf.
The average age for males is 32 years, while that for females is 44 years (McQueen, Gaston, and Court-Brown 2000). It is estimated that 3.1 per 100,000 of the population suffer compartment syndrome per year, men representing 91% of cases (McQueen 2000). In the extremities, 75% of ACS affects leg compartments, 16% the forearm, 4% the hand, 3% the thigh, while only 1% is reported in the arm or the foot (Vaillancourt et al. 2004).

1.1.7. Diagnosis

The diagnosis of ACS is, essentially, a clinical skill; diagnosis can be confirmed by the measurement of intracompartmental pressure (Elliott and Johnstone 2003; Hargens et al. 1989; Matsen, Winquist, and Krugmire 1980). A high index of clinical suspicion helps in detecting early signs of ACS, before the irreversible damage occurs. ICP measurement is currently done using sophisticated digital pressure probes.

Clinical findings include a swollen, tense, and tender compartment. Sensory deficits and motor weakness can be detected early, while paralysis occurs late in the presentation. The classical symptom is pain out of proportion to what would normally be expected from the existing injury, and is characteristically accentuated by passive stretch of the afflicted muscles (Hargens and Mubarak 1998). The most striking sign is the presence of palpable distal arterial pulsation in most patients, contrary to cases of complete ischaemia (Whitesides and Heckman 1996).
1.1.8. **Intracompartmental Pressure (ICP)**

The pathognomonic factor in ACS is elevated ICP (Matsen 1975). The critical pressure threshold beyond which muscle cannot survive had been thoroughly investigated: an absolute compartment pressure of 30 mmHg had been suggested by some authors (Hargens et al. 1978; Mubarak et al. 1978; Mubarak, Pedowitz, and Hargens 1989; Hargens et al. 1981), while 45 mmHg had been suggested by others (Rorabeck and Clarke 1978). Some also suggested calculating the difference between the mean arterial pressure and compartmental pressure, calling it the differential pressure ($\Delta P$) (Heckman et al. 1993; Hartsock et al. 1998; Mars and Hadley 1998; Heppenstall et al. 1988). A $\Delta P$ of 30 mmHg was found to impair muscle metabolism significantly at normal conditions, while a $\Delta P$ of 40 mmHg was found to cause similar effect in an injured muscle (Heppenstall et al. 1989).

1.1.9. **Fasciotomy**

The treatment in the late 1800s and early 1900s mainly addressed the complications of ACS rather than termination or prevention of the disease (Rowlands 1905; Sayre 1908). It was not until the mid 1970s that the literature showed general consensus on fasciotomy as the standard treatment for ACS (Willhoite and Moll 1970; Sheridan and Matsen 1976).

Fasciotomy is a surgical procedure in which the fascial wall of the compartment is opened longitudinally, through a substantial surgical exposure, to fully decompress the contents (Mubarak and Owen 1977; Finkelstein, Hunter,
and Hu 1996) (Figure 1.2). Fasciotomy is the gold standard treatment of ACS and should be done as soon as the diagnosis is reached.

The most important predictor of outcome after ACS is time to fasciotomy. A significant progression of tissue injury and irreversible damage are very likely if elevated ICP persists for more than 6 hours (Rorabeck 1984; Sheridan and Matsen 1976; Elliott and Johnstone 2003). In clinical practice, this critical period is called a 'surgical window'.

Although fasciotomy is a limb saving procedure, it comes with its own complications. Even when carried out in a timely manner, the sequelae of ACS cannot be fully avoided in every case (Echtermeyer and Horst 1997; Giannoudis et al. 2002). Thus, there is a great need to find an adjunctive therapy, or alternative treatment to surgery, that can reduce complications, prolong the surgical window and improve the overall clinical outcome of the ACS.

1.2. PATHOPHYSIOLOGY OF ACS

Pathophysiology of ACS appears to be very complex. Although there have been many research studies, explanations, and theories, it is not yet fully understood. The hallmark of compartment syndrome is the elevated ICP, with oedema as an essential cause.
Figure 1.2. Fasciotomy of the leg compartments. For a successful fasciotomy, all affected compartments should be decompressed by complete opening of the fascial envelope, including the deep ones, through a skin incision along the length of the compartment, taking care not to injure nerves and vessels. Following fasciotomy, all wounds should be left open to allow expansion of the oedematous muscles. Wound care protocols should be initiated, until delayed closure.

AC, anterior compartment; LC, lateral compartment; DPC, deep posterior compartment; SPC, superficial posterior compartment.
1.2.1. Elevated ICP and Ischaemia

While the precise mechanism of muscle ischemia in ACS is elusive, it is widely accepted that it occurs mainly at the level of the microcirculation (Hartsock et al. 1998). Theories on the pathophysiology of ACS tried to explain how the elevated ICP would lead to ischaemia. Most of these considered only the mechanical force of elevated hydrostatic pressure as the cause of damage. The theories postulated that elevated ICP may result in ischaemia through an induction of arterial insufficiency, venous stasis, collapse of the capillaries, or a combination of these mechanisms (Murphy 1914; Brooks 1922; Jepson 1926; Griffiths 1940; Burton and Yamada 1951; Kinmonth 1952; Eichler and Lipscomb 1967). Once ICP reaches a critical level above the perfusion pressure, it will lead to severe hypo-perfusion (Hartsock et al. 1998; Hargens and Mubarak 1998).

1.2.2. Oedema and The Vicious Circle Concept

Oedema commonly occurs following trauma, but it is particularly severe and rapidly progressive in ACS. While it is not known why oedema occasionally goes beyond the limits in certain cases, it is well-known that it leads to increased ICP to a level that triggers the occurrence of ACS.

The self-perpetuating cycle is a plausible concept that could explain the relation between oedema and ICP. An increase in oedema would cause an increase in ICP, which would further increase the oedema and so on, leading to the establishment of ACS (Matsen and Clawson 1975) (Figure 1.3).
Figure 1.3. **The vicious circle concept.** Increased compartment pressure leads to an increase in tissue oedema, which, in turn, leads to increased compartmental pressure and so on. The process would be self perpetuating. *Adapted from (Matsen and Clawson 1975).*
1.2.3. Inflammation

Echtermeyer questioned if the pathophysiology of ACS could only be explained by the high ICP (Echtermeyer and Horst 1997). Experimental studies had suggested that cellular hypoxia during the phase of elevated ICP would induce a cascade of inflammatory reactions resulting in microcirculatory changes and tissue damage (Perler, Tohmeh, and Bulkley 1990; Sadasivan et al. 1997). Recently, inflammation was shown to play a major role in the induction and augmentation of cellular damage seen in ACS (Manjoo et al. 2010; Wang, Baynosa, and Zamboni 2011a; Gillani et al. 2012; Blaisdell 2002; Lawendy 2014a). Leukocytes (predominantly neutrophils) coupled with the production of pro-inflammatory mediators were found significantly contributing to the cellular damage (Lawendy, Sanders, Bihari, and Badhwar 2011; Lawendy, Sanders, Bihari, Parry, et al. 2011).

It was not only local inflammation that had been observed in ACS, but also a systemic inflammatory response (Harvey et al. 2012; Lawendy et al. 2011; McGarr 2009; Lawendy 2014a). Our laboratory had previously shown that elevation of ICP led to a progressive sustained elevation in the serum tumor necrosis factor alpha (TNF-α), which further continued to increase, even at 45 minutes after fasciotomy (Lawendy, Sanders, Bihari, Parry, et al. 2011; Lawendy et al. 2011; Lawendy et al. 2014). Lawendy et al also reported 7-fold increase in hepatocellular injury in an animal model of limb compartment syndrome (Lawendy 2014a, chap. 4).
1.3. ISCHAEMIA AND COMPARTMENT SYNDROME

1.3.1. Compartment Syndrome versus Complete Ischaemia

While tissue ischaemia is the common denominator of ACS and complete ischaemia, the elevated ICP creates a different pathophysiological state. ACS develops and progresses while arterial pulsation is still present, reflecting a unique ischaemic insult compared to complete ischaemia.

Heppenstall et al compared the pathological effects of complete ischaemia and compartment syndrome in a canine model (Heppenstall et al. 1986). They elevated ICP for 3 hours, followed by 2 hours of reperfusion. The structural and metabolic deterioration of the skeletal muscle was then assessed. ACS was induced by autologous plasma infusion in the anterior compartment, while acute ischaemia was induced by a pneumatic tourniquet. The researchers reported a marked ultrastructural deterioration of skeletal muscle in ACS group compared to that observed in complete ischemia. They also demonstrated striking dissimilarities in the metabolic response between the two groups. The two most notable observations in the ACS group were the rapid depletion of adenosine triphosphate (ATP) levels and the marked intracellular acidosis compared to the tourniquet group. In the complete ischemia group, ATP did not change at all throughout the experiment. Surprisingly, neither the acidosis nor the ATP levels recovered at 2 hours post-fasciotomy in the ACS group, while pH levels immediately recovered in the complete ischemia group following the release of tourniquet. The authors stressed the fact that in cases of ACS, there is always some degree of perfusion present (Heppenstall et al. 1986).
1.3.2. Cellular Effects of Ischaemia

Oxygen is fundamental to human physiology. While it is essential for mitochondrial ATP generation, ATP production is associated with the risk of oxidative damage; thus, oxygen concentrations must be tightly regulated (Semenza 1999).

Oxygen deficiency during ischaemia results in the reduction of ATP production and acidosis due to accumulation of lactic acid from glycolysis. Sodium-potassium pumps are ATP dependent; therefore, during ischaemia the intracellular sodium and calcium are increased. This attracts water into the intracellular environment, causing cellular oedema.

Ischaemia also promotes the production of pro-inflammatory substances, like leukocyte adhesion molecules, TNF-α and thromboxane A2. Additionally, ischaemia suppresses some ‘protective’ substances, like constitutive nitric oxide synthase (cNOS) and prostacyclin (Carden and Granger 2000).

If ischaemia persists beyond the critical point of tissue tolerance, cellular necrosis becomes inevitable. While reperfusion is the only way to minimize ischaemic necrosis, it initiates tissue injury beyond that caused by the ischaemic insult alone.

1.3.3. Limb Ischaemia-Reperfusion (IR) Injury

Restoration of blood flow (reperfusion) to ischemic tissue is vital, but sudden reperfusion after a prolonged period of ischaemia is also deleterious (Lejay et al. 2014). The tissue damage caused by reperfusion was found to be
progressively proportionate to the time of reperfusion (Carmo-Araújo et al. 2007), and was estimated to be more than that caused by ischemia alone in the rat skeletal muscle (Loerakker et al. 2011). The main factors responsible for local and systemic damage caused by IR appear to be the reactive oxygen species (ROS) and activated neutrophils (Gillani et al. 2012).

Sudden supply of hypoxic tissue with oxygen leads to rapid formation of large quantities of ROS in the mitochondria, which exacerbates the mitochondrial dysfunction that had already started during ischaemia, by damaging the mitochondrial membrane. While mitochondrial function is to necessitate preservation of membrane potential (Wang et al. 2011), the IR damage leads to loss of this, leading to cessation of ATP production and producing cell necrosis. It also results in matrix swelling and eventual rupture of the mitochondrial membrane, releasing cytochrome c into the cytosol. This initiates apoptosis (Wang, Baynosa, and Zamboni 2011b).

1.3.4. Low-Flow Ischemia

It has been known that the distal pulsation is intact in most cases of compartment syndrome. This may be interpreted as the ischaemia in ACS is not complete. Heppenstall et al stressed the fact that there was always some degree of perfusion in cases of ACS (Heppenstall et al. 1986). Lawendy et al have termed it ‘low-flow ischaemia’ (Lawendy 2014a).

Conrad et al compared complete and incomplete ischaemia in a murine model. They reported marked early increase of a pro-inflammatory cytokine KC,
which is analogous to human Interleukin-8 (IL-8), in the partial ischaemia group (Conrad et al. 2005). Its early presence in partial ischaemia elucidated difference in pathophysiological sequences compared to complete ischaemia (Ghasemi et al. 2011). Lawendy et al also demonstrated significant early increase in the pro-inflammatory TNF-α during the course of ACS, in contrast to complete ischaemia (Lawendy 2014a).

1.3.5. ACS and IR Injury

The severity of cellular damage reported in ACS appears to be more than that seen in complete ischaemia alone (Heppenstall et al. 1988; Heppenstall et al. 1989). This fact created a general impression that partial ischaemia is more damaging than complete ischaemia. However, recent studies reported that complete ischaemia causes more muscle damage than that caused by partial ischemia, contrary to what was generally believed (Conrad et al. 2005; Conrad et al. 2006). This new insight does not explain the extensive damage in ACS compared to complete ischaemia based on blood flow rate. Thus, there must be another mechanism.

There are several pathological similarities between IR and ACS-induced striated muscle injury (Heppenstall et al. 1986; Sadasivan et al. 1997; Tollens, Janzing, and Broos 1998). The understanding that the reperfusion injury is more damaging than ischaemia itself may be the key to how to explain this puzzling fact (Loerakker et al. 2011). This compelled researchers to consider ACS as one of the clinical situations associated with skeletal muscle IR injury (Gillani et al.)
2012), in which the reperfusion resulted in marked oxidative stress and an intense inflammatory response, thereby maximizing the local damage already caused by ischaemia, Additionally, it also induced a systemic inflammatory response (Carden and Granger 2000; Gim et al. 2007; Gillani et al. 2012).

It should be remembered that ACS also results in a much greater level of metabolic strain and cellular deterioration than IR injury alone (Stephen R Kearns et al. 2010). Therefore, both ischaemia and IR may be concomitantly occurring in ACS.

1.3.6. Fasciotomy and Reperfusion Injury

Upon release of elevated ICP, Sadasivan et al reported no-reflow phenomenon, similar to that of IR injury (Sadasivan et al. 1997). Surgical decompression by fasciotomy promptly aborts the self-perpetuating cycle of oedema/elevated ICP by normalising the tissue pressure, thus saving the muscle by regaining blood supply. However, this leads to a sudden reperfusion of previously hypoxic tissues with oxygen-rich blood, producing an intense IR injury, mainly by the liberated ROS. This effect is called a ‘second hit’ (Sellei, Hildebrand, and Pape 2014) which, in conjunction with the activated neutrophils, leads to an increase in the microvascular dysfunction and the net tissue damage (Gillani et al. 2012).

Reperfusion injury normally occurs only after allowing normal blood flow to resume (for example, rearterization), which, theoretically, is what fasciotomy does in ACS. Still, this does not explain the early changes and damages seen
immediately following normalization of compartment pressure after 2 hours of experimental ACS (Sadasivan et al. 1997; Lawendy, Sanders, Bihari, Parry, et al. 2011).

1.4. PATHOLOGICAL FINDINGS IN ACS

Apart from the myonecrosis, reported in ACS by most authors (Volkmann 1881; Brooks 1922; Mabee and Bostwick 1993; Sheridan, Matsen, and Krugmire 1977; Zimmerman and Shen 2013), recent researchers were able to visualise the in vivo changes occurring within the muscle immediately following fasciotomy in animal models.

1.4.1. Microvascular Dysfunction

Microcirculation is the part of blood circulatory system that provides skeletal muscles with oxygen and various nutrients, and removes the noxious waste products of metabolism (Hudlicka 2011). The vitality of the muscle, or any living tissue, depends on adequate perfusion through the capillary circulation. In normal physiological conditions, most capillaries are continuously perfused (CPC), with few intermittently perfused (IPC) or non-perfused (NPC) capillaries.

Microcirculation is considered a ‘battlefield’ in inflammation and reperfusion (Wang, Baynosa, and Zamboni 2011a). Using the modern intravital video microscopy (IVVM) technology, researchers in our laboratory were able to elucidate the microvascular dysfunction associated with ACS, using a rat model (Lawendy, Sanders, Bihari, Parry, et al. 2011; Manjoo et al. 2010; McGarr 2009).
They reported significant reduction in the CPC, coupled with a significant increase in the NPC; in other words, there was a failure to restore the normal physiological micro flow. The microvascular dysfunction noticed in ACS was, to a great extent, similar to that reported in reperfusion injury following complete limb ischaemia, where the vascular bed behaves contrary to what is expected following re-establishment of the blood flow (Potter et al. 1993; Menger and Messmer 1993; Lam et al. 1994; Blaisdell 2002; Galiuto and Crea 2006; Maksimenko and Turashev 2012). A significant increase in the number of NPC causes an overall microvascular blood flow failure. This characteristic scenario is known as the ‘no-reflow phenomenon’ (Nanobashvili et al. 2003; Reffelmann and Kloner 2006). The main players responsible for the microvascular dysfunction appear to be ROS and activated leukocytes (Sadasivan et al. 1997; Gillani et al. 2012; Blaisdell 2002).

It worth mentioning that there are two distinct types of no-reflow: structural or otherwise irreversible, and functional (Galiuto and Crea 2006). The severity is related to the pathogenesis and duration of ischaemic insult (Reffelmann and Kloner 2006; Galiuto and Crea 2006).

1.4.2. Tissue Injury

Cellular injury is one of the earliest recognized sequelae of ACS. Studies have shown that tissue damage could be reverted by therapeutic modalities, implying that some damage may have been reversible (Lawendy et al. 2014; Manjoo et al. 2010). In vivo experiments in leukocyte-deplete rats have
demonstrated 50% reduction in cellular injury after 90 minutes of induced ACS (Lawendy 2014b), highlighting the important role of activated leukocytes in producing the cell damage.

Recent studies have shown that cell damage in IR injury is not just necrotic but, to a great extent, apoptotic, and appears to be induced by the effect of TNF-α (Sherwood and Toliver-Kinsky 2004). Apoptosis, in contrast to necrosis, is a programmed cell death process that can be triggered by several factors, but needs the caspases enzymatic pathway for apoptosis execution (de Nigris et al. 2003; Haddad 2004; Riedl and Shi 2004; Nikoletopoulou et al. 2013).

1.4.3. Leukocyte Activation

Activated leukocytes tend to roll and adhere to the endothelium prior to transmigration through the vessel wall (Ley 2008). Significant increase in leukocyte rolling and adhesion was reported in the post capillary venules of the skeletal muscle in a rat model of ACS (Lawendy et al. 2014; Manjoo et al. 2010; Lawendy 2014a; McGarr 2009). Leukocyte activation (rolling and adhesion) is controlled by differential expression of various adhesion molecules.

1.5. MECHANISM OF INFLAMMATION IN ACS

The literature reports of studies exploring the mechanism(s) of inflammation and tissue damage in ACS are scarce. However, mechanisms and pathways elucidated in IR injury can provide valuable insight on the possible
damaging and/or protective factors in ACS. There are enough similarities between IR injury and ACS to assume that both may be sharing some of these.

1.5.1. Oxidative Stress

1.5.1.1. Overview

At normal conditions, aerobic metabolism within the mitochondria produces very active and potentially damaging ROS. These are potent oxidizing agents capable of cellular membrane lipid peroxidation and activation of neutrophils (Granger 1988). ROS are normally controlled by the natural antioxidant activity within the system. Oxidative stress is a state of imbalance between the pro-oxidants and antioxidants, in favour of the pro-oxidant (Ma, Qi, and Chen 2008; Ogura and Shimosawa 2014; Bar-Or et al. 2015). Several ROS may be involved in IR injury and ACS: superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (*OH), hypochlorous acid (HOCl), and nitric oxide-derived peroxynitrite (Perler, Tohmeh, and Bulkley 1990; Hancock, Desikan, and Neill 2001; C. Li and Jackson 2002).

1.5.1.2. ROS in IR Injury

ROS play an integral role in the progression of reperfusion injury, by inducing cell necrosis, apoptosis and activation of leukocytes (Hancock, Desikan, and Neill 2001; P. H. Chan 2001; C. Li and Jackson 2002; Wang et al. 2006; Arató et al. 2008; Wang, Baynosa, and Zamboni 2011b). NADPH oxidase and xanthine oxidase are perhaps two of the most important enzymes involved in the
generation of ROS (Hancock, Desikan, and Neill 2001), whereas superoxide dismutase (SOD) and glutathione peroxidase appear as two of very important defensive mechanisms to ameliorate the harmful effects of ROS.

1.5.1.2.1. ROS in ACS

ROS also appear to be important players contributing to the cellular damage in ACS. Ricci et al were able to markedly reduce the muscle damage of post-ischaemic ACS by administration of free radical scavengers in a canine model (Ricci et al. 1989). Likewise, Perler et al were able to inhibit the formation of compartment syndrome by ablation of free radical-mediated reperfusion injury (Perler, Tohmeh, and Bulkley 1990).

1.5.2. Neutrophil Activation

During reperfusion, activated leukocytes exhibit rolling behaviour, followed by firm adhesion to the endothelium of the post capillary venules. This creates favourable microenvironment permitting transmigration to the interstitial space (Weiss 1989). Upon activation, neutrophils release the contents of their intracellular granules containing ROS, cytokines, chemokines and proteolytic enzymes that mediate the injurious effects in reperfusion injury (Welbourn et al. 1991; Hampton, Kettle, and Winterbourn 1998; Arnhold 2004; Klebanoff 2005; Robinson 2009).
1.5.3. Endothelial Dysfunction

Endothelial dysfunction is one of the characteristics of IR injury. Upon exposure to ischaemia or reperfusion, the endothelium gets activated, creating a pro-inflammatory and pro-thrombotic surface, accompanied with loss of local vasodilation mechanisms. These changes collectively are termed endothelial dysfunction (Girn et al. 2007). The resulting vasoconstriction of the muscle microcirculation creates the known capillary no-reflow phenomenon (Wang et al. 2005; de With et al. 2005). Reperfusion-induced vasoconstriction has been attributed to several factors, but recently was found to be caused by deficiency of endothelial nitric oxide synthase (eNOS) (but not the inducible nitric oxide synthase iNOS) (Wang, Baynosa, and Zamboni 2011b).

1.5.4. Cytokines

Cytokines are a group of signalling molecules that play an important role in modulation of the immune response (Cannon 2000; Zhang and An 2007). Almost all nucleated cells are capable of synthesizing cytokines and, in turn, respond to them (Dinarello 2000). Cytokines can regulate the expression of genes at the transcriptional level through selective alterations of expression of various transcription factors, thereby inducing the production of other cytokines and cell surface receptors. Additionally, they can also down-regulate themselves by a negative feedback loop (Grivennikov et al. 2006).
1.5.4.1. Pro-Inflammatory Cytokines

Pro-inflammatory cytokines are involved in the upregulation of inflammatory mediators. TNF-α and Interleukin-1 (IL-1) are probably the most studied cytokines in IR injury. Both are produced at the site of local inflammation and their net effect is considered synergistic (Dinarello 2000). Interestingly, both exert similar roles but through different pathways and different receptors, showing a certain degree of redundancy (Dinarello 2000). TNF-α and IL-1 play important roles in downstream cytokine/chemokine-triggered leukocyte activation, production of endothelial adhesion molecules, stimulation of phagocytosis, and induction of apoptosis (Khan 2008). They also upregulate iNOS and induce transcription of cyclooxygenase-2 (COX-2), leading to an increase in prostaglandin-2 (PGE-2) synthesis (Zhang and An 2007; Dinarello 2000).

In ACS, a sustained elevation in TNF-α has been demonstrated during the period of elevated ICP, with a further, more pronounced increase following decompression (Lawendy et al. 2014).

1.5.4.2. Anti-Inflammatory Cytokines

These are immunoregulatory molecules that limit the potentially injurious inflammatory reaction due to the induction of the proinflammatory cytokines, if it becomes persistent or excessive (Opal and DePalo 2000). Their physiologic and pathologic roles are increasingly recognized in determining the outcome of disease by timely restoration of the proper balance. Molecules like IL-4, IL-10, IL-
13, and transforming growth factor-β (TGF-β) can suppress the production of IL-1, TNF-α, and vascular adhesion molecules; therefore, they are considered anti-inflammatory cytokines (Dinarello 2000).

1.5.4.3. **Chemokines**

Chemokines are cytokines that possess chemotactic properties attracting the circulating leukocytes towards the site of inflammation or injury (Sherwood and Toliver-Kinsky 2004). IL-8 is an example of a potent chemokine that specifically attracts neutrophils. Additionally, it is capable of stimulating their adherence to the endothelium of post capillary venule (Kobayashi 2006; Bickel 1993). Neutrophil chemoattractant protein (KC) is the murine equivalent of human IL-8 (Conrad et al. 2005).

1.5.5. **Transcription Factors**

Transcription factors (TF) are proteins that control the copying of genetic material from DNA to the messenger RNA (Tjian 1996). This is done by the TF binding to a specific DNA region adjacent to the target gene. TF may increase or decrease the gene expression by activating or blocking RNA polymerase, the enzyme responsible for converting the DNA to messenger RNA (Schwabe, Rybakova, and Bruggeman 2012).

Nuclear factor kappa-B (NF-κB) is a TF induced in response to a wide range of stimuli during IR injury, particularly oxidative stress and proinflammatory
cytokines (Nichols 2004; Lille et al. 2001). Activator protein-1 (AP-1) is another TF induced in a similar fashion (Lefler et al. 2002). Activation of NF-κB and AP-1 in IR results in the upregulation of expression of various inflammatory molecules, including cytokines, adhesion molecules, and iNOS (Lefler et al. 2002; Nichols 2004).

1.5.6. Xanthine Oxidase

Xanthine oxidase (XO) is an enzyme known to generate superoxide and hydrogen peroxide (J. K. Smith, Carden, and Korthuis 1989). Interactions with these two ROS can yield a range of cytotoxic agents, including hydroxyl radicals (Granger, Höllwarth, and Parks 1986; B. J. Zimmerman, Grisham, and Granger 1990). XO-derived ROS are known to activate NF-κB leading to an upregulation of various inflammatory molecules.

XO is produced by many tissues when subjected to IR insult, and is released in the systemic circulation (Tan et al. 1995). The circulating XO binds to the surface of endothelial cells and continues to produce oxidants. This, in turn, can trigger endothelial dysfunction, thereby contribute to the local damage, and cause remote organ injury (Pacher, Nivorozhkin, and Szabó 2006).

NO has been shown to act as an endogenous suppressor of XO activity; thus, reduced level of the NO-mediated suppression of XO may result in increased superoxide generation (Ichimori et al. 1999; Pacher, Nivorozhkin, and Szabó 2006).
1.5.7. Caspases

Caspases are a family of endoproteases that play a critical role in controlling inflammation and cell death. Activation of apoptotic caspases results in the generation of a cascade of signalling events that allows a controlled demolition of cellular components (McIlwain, Berger, and Mak 2013). This is how some caspases were given the nickname of ‘executioner’ proteins (Riedl and Shi 2004). On the other hand, activation of inflammatory caspases results in the stimulation of innate immune responses, and the subsequent production of active proinflammatory cytokines (McIlwain, Berger, and Mak 2013).

Recent evidence from experimental models of IR has shown that apoptosis doubled necrosis in skeletal muscle after 4 hours of ischemia and 24 hours of reperfusion (Wang et al. 2008).

1.5.8. Heme Oxygenase

Heme oxygenase (HO) is an enzyme that degrades heme to biliverdin, iron and carbon monoxide (CO). There are two isoforms: the inducible HO-1 and constitutively-expressed HO-2. HO-1 functions as a ‘sensor’ of cellular stress, thus increasing cellular adaptation to stress and limiting tissue damage (Motterlini and Foresti 2014).

The HO system has been shown to play important cytoprotective functions through the effects of its by-products. CO has been reported to diminish the severity of microvascular dysfunction and inflammation caused by ACS
(Lawendy et al 2014), while biliverdin and iron were found to be potent antioxidants (Ryter et al 2006).

1.6. POTENTIAL NON-OPERATIVE TREATMENTS FOR ACS

Apart from emergency surgical decompression and standard supportive treatment to prevent other systemic sequelae during the recovery period, few other treatment options exist for routine use in ACS (S. A. Olson and Glasgow 2005).

Adjunctive therapy is used together with the primary treatment to increase the chance of a cure, or to increase the efficacy of the primary treatment. In ACS, the primary treatment is the surgical decompression, and any medical or physical treatment given in conjunction with fasciotomy is considered adjunctive treatment.

Since the cellular damage in ACS appears to be a result of intense ischaemia-induced inflammatory reaction coupled with oxidative stress, therapeutic modalities specifically targeting these factors may be beneficial in ACS as adjunctive treatment.

1.6.1. Hyperbaric Oxygen

Hyperbaric oxygen therapy showed promising results as an adjunct to fasciotomy, by decreasing complications from ACS. It has been shown to have the ability to reach ischaemic tissues suffering low perfusion during elevated ICP
state. Previously, it was found to be effective in reducing amputation rate and improving wound healing (Greensmith 2004; Strauss 2012). It has also been reported to reduce swelling, improve fasciotomy wound healing and fight infection (Weiland 2007). There is no large scale clinical study available on hyperbaric oxygen therapy in ACS, possibly because the very specialised equipment needed for it is not available in most trauma centres.

1.6.2. Antioxidants

Inhibition of ROS by strong specific antioxidants has proven to be successful experimentally in ablation of free radical-mediated reperfusion injury. Perler et al demonstrated that by scavenging the superoxide radical at reperfusion with SOD or blocking the hydroxyl radical formation with deferoxamine, a significant amelioration of the rise in compartment pressure could be achieved (Perler, Tohmeh, and Bulkley 1990). Vitamin C is also a powerful general antioxidant that concentrates preferentially in leukocytes. It has been tried experimentally in an animal model of ACS, and has been reported to attenuate the muscle injury (Kearns et al. 2004).

1.6.3. Non-Steroidal Anti-inflammatory Drugs

Anti-inflammatory drugs, in theory, may have therapeutic effect in ACS. Indomethacin, a strong anti-inflammatory drug, demonstrated protective effects in an experimental animal model of ACS (Manjoo et al. 2010).
1.6.4. N-Acetylcysteine

N-acetylcysteine (NAC) has been shown to reduce the cellular damage due to production of free radicals from neutrophils, in an experimental animal model. It has been suggested that its activity may be due to the combined antioxidant and glutathione-replenishing activities (Kearns et al. 2010).

1.6.5. Gasotransmitters

All cells in the living organisms are constantly receiving information about their surrounding environment necessary for the control and regulation of their activities, mediated by signalling molecules. Examples of these include hormones, neurotransmitters, pheromones, and cytokines. A class of small gaseous molecules have recently been discovered to function as signalling molecules; hence, they were termed ‘gasotransmitters’. There are three known gasotransmitters synthesized by mammalian cells: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S) (Farrugia and Szurszewski 2014). There appears to be a considerable interplay among the members of the gasotransmitter family (Ling Li, Hsu, and Moore 2009; Whiteman and Moore 2009). While signalling molecules usually require specialised receptors to evoke a response, gasotransmitters do not (Moody and Calvert 2011). Instead, they can freely permeate the plasma membrane and pass the message directly to an intracellular target (Rui Wang 2003). The use of these transmitters as a pharmacological agent in IR injury has gained a lot of attention in the last two decades (Moody and Calvert 2011).
1.6.5.1. **Nitric Oxide (NO)**

NO was the very first gasotransmitter to be recognized. Its signalling role in the cardiovascular function as the endothelial-derived relaxing factor (EDRF) had been discovered by the 1998 Nobel Prize-winning work of Furchgott, Murad and Ignarro (The Nobel Foundation 2015). Since its discovery, NO has emerged as a fundamental signalling molecule responsible for the regulation of several cellular functions, as well as a potent mediator of cellular damage in a wide range of conditions (Pacher, Beckman, and Liaudet 2007).

NO is synthesized from L-arginine by the enzyme NOS (Michel and Feron 1997). It was identified as a strong vascular smooth muscle relaxant (Furchgott and Zawadzki 1980). Endothelial NOS (eNOS) is the isoform that plays a main role in vasorelaxation (Lamas et al. 1992). NO produced inside endothelial cells diffuses directly from the endothelium to the smooth muscle layer within the vascular wall, where it exerts its relaxing action (Dudzinski et al. 2006).

NO has been reported to protect against ischaemic injury in the heart, brain, liver, and other tissues, when administered as a therapeutic agent (Siegfried et al. 1992; Hataishi et al. 2006).

1.6.5.2. **Carbon Monoxide (CO)**

CO is a well-known toxic gas, recently identified as another gasotransmitter produced in humans (Marks et al. 1991). CO is produced endogenously through the activity of HO (Ryter and Otterbein 2004; Knauert et
Synthesis of CO has been shown to occur in almost all tissues (Maines 1997).

CO, like NO, is also a known vasodilator, with direct effect on vascular smooth muscle (Kharitonov et al. 1995) and the central nervous system (Wiesel et al. 2001). It has been demonstrated to be involved in cell proliferation, inflammation, neurotransmission, mitochondrial biogenesis, autophagy and apoptosis (Brouard et al. 2000; Suliman et al. 2007; Knauert et al. 2013).

Cytoprotective effects of CO have been reported in several IR conditions, as well as in an animal model of ACS (Hangaishi et al. 2000; Yet et al. 2001; Tulis et al. 2001; Sato et al. 2001; Nakao et al. 2005; Ott et al. 2005; Scott et al. 2009; Patel et al. 2012; Lawendy et al. 2014). It appears to act through different cytoprotective pathways, although the precise molecular interactions have not yet been fully elucidated (Knauert et al. 2013).

Recent studies have demonstrated strong anti-inflammatory properties of CO. One of the proposed pathways appears to be related to the activation of HO expression, which itself can decrease inflammation, and thrombosis (Patel et al. 2012). CO also appears to inhibit the activity of NADPH oxidase, thus reducing the production of ROS. Finally, it is also a potent vasodilator, providing benefit by improving the microcirculation in IR injury (Motterlini and Otterbein 2010).

Previously, our laboratory demonstrated that carbon monoxide, liberated from carbon monoxide-releasing molecule-3 (CORM-3), was able to mitigate the microvascular dysfunction and cellular injury in a rat model of ACS (Lawendy et al. 2014).
1.7. HYDROGEN SULFIDE

Hydrogen sulfide gas (H$_2$S) has long been known for its bad smell and toxic nature (Reiffenstein, Hulbert, and Roth 1992). Recently, it had been discovered that mammalian body can generate H$_2$S endogenously, at cellular level, in many tissues and vital organs (Stipanuk and Beck 1982; Goodwin et al. 1989; Hosoki, Matsuki, and Kimura 1997). The role of endogenously produced H$_2$S has been suggested as an important signalling molecule, with invaluable cytoprotective functions (Abe and Kimura 1996; Snijder et al. 2013; Kimura 2014a; Hancock and Whiteman 2014).

1.7.1. History of H$_2$S

Bacterial activity has long been known to result in the production of hydrogen sulfide in the colon, producing gases with offensive smell (Suarez et al. 1997), and, occasionally, in the oral cavity, resulting in halitosis (Suarez et al. 2000). In contrast, the capacity of mammalian tissues to endogenously produce H$_2$S became known only within the last century (Sluiter 1930; Meister, Fraser, and Tice 1954; Koj, Frendo, and Janik 1967). Surprisingly, H$_2$S was considered as a by-product, rather than a physiologically active molecule, and thus has been largely ignored.

In the 1980s, the endogenous production of H$_2$S started to grab the attention, after an in vivo rat study demonstrating mammalian tissue production under physiological conditions (Stipanuk and Beck 1982). Stipanuk and Beck
measured the concentration of H$_2$S in different tissues and found it to be the highest in the liver, followed by the kidney; they also proved its presence in the heart, brain and skeletal muscle (Stipanuk and Beck 1982). While their work did not uncover the biological value of H$_2$S, it was enough to hypothesize that there must be a physiological role for this compound.

After analysing different biological processes in the brain, Abe and Kimura suggested that the endogenous H$_2$S acts as a neuromodulator (Abe and Kimura 1996). This observation was succeeded by several studies demonstrating the presence and biological value of H$_2$S, in many organs and tissues. Soon after, H$_2$S was reintroduced to the medical literature as a third gasotransmitter.

Canadian scientists at the University of Saskatchewan then demonstrated that H$_2$S significantly decreased blood pressure of rats, most likely through its relaxant effect on the vascular smooth muscle, and as an opener of K$_{ATP}$ channels (Zhao et al. 2001). Such observations quickly made H$_2$S a hot topic for active research, and highly raised its therapeutic expectations.

1.7.2. Description

H$_2$S is an inorganic gas. It is colourless, flammable and heavier than air. The gas emits odour of rotten eggs at very low concentration, and is poisonous at high concentrations (Hughes, Centelles, and Moore 2009; Wedmann et al. 2014).
1.7.3. Solution Chemistry of H$_2$S

H$_2$S can freely penetrate the cell membranes of all tissues, without using any specific transporting system. It is soluble in water and plasma, and readily dissociates into hydrogen (H$^+$) and hydrosulfide (HS$^-$) ions (Fiorucci et al. 2006). Under normal physiological conditions, two thirds of H$_2$S are dissociated (Kashfi and Olson 2013; Li and Moore 2008) as follows:

\[ H_2S \rightleftharpoons HS^- + H^+ \]

H$_2$S can reduce other compounds by simply giving away its H$^+$; this property gives H$_2$S the ability to directly reduce ROS, which would provide great value in cell protection. It is this property that gives it one of the most important proposed biological roles (Nagy et al. 2014).

1.7.4. Toxicity

The concentration of H$_2$S in unpolluted air is very low, only 0.03 – 0.1µg/m$^3$. The human nose is capable of detecting H$_2$S when its concentration reaches 11µg/m$^3$. However, when the concentration reaches 140mg/m$^3$, the olfactory mucosa adapts, subjecting the person to inhalational toxicity. A few breaths at a concentration of 700mg/m$^3$ can be fatal (Chou et al. 2006). The cellular toxicity of H$_2$S is attributed to its capacity to inhibit cytochrome c oxidase, the terminal enzyme of oxidative phosphorylation, producing cellular hypoxia (Koj, Frendo, and Janik 1967; Epithelium et al. 2002).
1.7.5. Endogenous Production of $H_2S$

$H_2S$ biosynthesis can occur via enzymatic and non-enzymatic pathways (Kolluru et al. 2013).

1.7.5.1. Enzymatic Pathway

In mammals, $H_2S$ is synthesized from the sulfur-containing amino acids, cysteine and homocysteine, through the activity of four enzymes of three enzymatic pathways (Kashfi and Olson 2013; Kuksis, Smith, and Ferguson 2014) (Figure 1.4). Two pathways, believed to produce most of the endogenous $H_2S$, are active in the cytosol: the cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). Both use pyridoxal 5’-phosphate (vitamin B6) as a cofactor (Kolluru et al. 2013; Hughes, Centelles, and Moore 2009; Kimura 2014b).

The third pathway is mainly localized in the mitochondrial matrix, and occurs in a two-step reaction utilising 3-mercaptopyrurate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT) enzymes (Kodela, Chattopadhyay, and Kashfi 2012; Kimura 2014b). All three pathways are distributed across many tissues, and are often jointly present in many organs. CBS, highly expressed in the brain, produces $H_2S$ from cysteine (Abe and Kimura 1996). CSE is expressed in the liver, pancreas, aorta, ileum, portal vein, and uterus (Kolluru et al. 2013; Kuksis, Smith, and Ferguson 2014), and also produces $H_2S$ from cysteine. The 3-MST is active in the brain and vascular system.
Figure 1.4. *Enzymatic pathways of hydrogen sulfide production.*

L-Cysteine can be converted to H$_2$S via two P5P-dependent pathways. H$_2$S synthesis via CSE also generates NH$_3$ and pyruvate; synthesis of H$_2$S via CBS generates L-serine. The P5P-independent pathway (occurring primarily in mitochondria) is a 2-step process: CAT converts L-cysteine to the intermediary, 3-mercaptopyruvate, (depends on α-ketoglutarate). 3-mercaptopyruvate is then converted 3-MST into H$_2$S.

*CSE,* cystathionine γ-lyase; *CBS,* cystathionine β-synthase; *P5P,* pyroxidal-5′-phosphate; *CAT,* cysteine (aspartate) aminotransferase; *3-MST,* 3-mercaptopyruvate sulfurtransferase.

*Adapted from* (Chan and Wallace 2013), *with permission.*
1.7.5.2. *Non-Enzymatic Pathway*

The non-enzymatic production of H$_2$S is less understood, and there is no general agreement about its value in the production of endogenous H$_2$S. It occurs through direct reduction of elemental sulfur, and from dietary inorganic and organic sulfides (Papapetropoulos, Whiteman, and Cirino 2014; Kolluru *et al.* 2013). Plants rich in dietary sulfides include *Allium* family; these have characteristic taste and smell (Munday 2012; Kuo, Chien, and Ho 1990).

*Allium* vegetables (garlic, onion and shallots) have long been felt beneficial to health as an antioxidant. Recent evidence suggests that a number of beneficial effects of garlic may be derived from H$_2$S production (Sener, Sakarcan, and Yegen 2007; Benavides *et al.* 2007; Y. Zhang *et al.* 2008; Namazi 2008; Tsubura *et al.* 2011; Padiya *et al.* 2011). The beneficial effects of garlic in the prevention of IR injury have been reported in several organs (Namazi 2008). Garlic extract was also shown to vasodilate rat aorta via H$_2$S production (Benavides *et al.* 2007).

Allicin, diallyl disulfide and diallyl trisulfide in garlic extract are all considered H$_2$S-donor compounds (Benavides *et al.* 2007; Kaschula *et al.* 2011). Under anoxic conditions, red blood cells were shown to produce H$_2$S from the garlic extract within minutes (Benavides *et al.* 2007). Also, an increase in exhaled H$_2$S levels was reported after infusion of diallyl disulfide in rats, thus providing an *in vivo* support for H$_2$S production from garlic (Insko *et al.* 2009).
1.7.6. **Release and Storage**

Despite the well-known enzymatic production pathways of H$_2$S, the mechanisms of its release are not well understood. It is assumed that either H$_2$S is released as soon as it is produced, or it is kept at intracellular stores, to be released in response to a physiologic signal (Ishigami et al. 2009).

1.7.7. **Metabolic degradation**

Degradation of H$_2$S occurs in almost all tissues by mitochondrial oxidation; the process produces inactive sulphate, which is then excreted in urine.

1.7.8. **Plasma Level**

The normal, physiologic level of H$_2$S in plasma is controversial (Olson 2009). Some studies have reported values of 10 – 300µM, while others have concluded that circulating levels of H$_2$S are negligible (Sparatore et al. 2009); thus physiological H$_2$S may only act by means of autocrine or paracrine mechanisms (Whitfield et al. 2008).

1.7.9. **Physiologic Role**

H$_2$S appears to plays a wide and varied physiological role (Kolluru et al. 2013). It has been shown to regulate blood pressure (Yang et al. 2008), inflammation (Hu et al. 2007; Ling Li, Hsu, and Moore 2009; Dufton et al. 2012), and to act as a cell protector in oxidative stress (Kimura and Kimura 2004; Whiteman et al. 2005; Kimura et al. 2006; Wu et al. 2014). H$_2$S has also been
reported as a neuromodulator (Whiteman et al. 2004), smooth muscle relaxant (Kimura 2010), learning and memory enhancer (Kimura 2002), aid in insulin secretion (Ali et al. 2007; W. Yang et al. 2005), and may even be involved in longevity (Kimura 2010). It may play a physiological role in many organ systems, including cardiovascular (Bucci and Cirino 2011; Al-Magableh, Kemp-Harper, and Hart 2015), respiratory (Kubo et al. 2007), digestive (Farrugia and Szurszewski 2014), brain and nervous system (B. H. Tan, Wong, and Bian 2010), kidney (Lobb, Sonke, et al. 2014), liver (Mani et al. 2014), endocrine, reproductive (X.-Y. Zhu, Gu, and Ni 2011), and muscle (DU et al. 2013).

H₂S has been shown to be capable of exerting analgesic effects (Distrutti et al. 2006; Smith 2009; Lin et al. 2014; Donatti et al. 2014). Combining H₂S with NSAIDs has opened new horizons for a safer and more effective results (McCarberg and Cryer 2014; Baskar et al. 2008; Chattopadhyay et al. 2012; Fiorucci, Santucci, and Distrutti 2007; Fomenko et al. 2014).

One of the most interesting effects of H₂S is the induction of a hibernation-like state in mice, where the metabolic rate is dramatically reduced to less than 10% of its normal hypothermic state. This has been termed ‘suspended animation’ (Blackstone, Morrison, and Roth 2005). The effect appears with the administration of sub-toxic concentration of inhalational H₂S. The suspended animation state was shown to be highly protective against ischemic damage to cells by preserving mitochondria (Elrod et al. 2007; Blackstone and Roth 2007; Bos et al. 2009).
1.8. **H₂S AND IR INJURY**

The protective ability of H₂S to reduce the extent of cellular damage in response to IR insult has been well documented by many researchers (Jha *et al.* 2008; Calvert, Coetzee, and Lefer 2010; Henderson *et al.* 2010; King and Lefer 2011; Lobb *et al.* 2012; Zhu *et al.* 2012; Lobb, Zhu, *et al.* 2014; Lobb, Sonke, *et al.* 2014).

It appears that multiple pathways may be involved, as there is no known single mechanism that could explain all the cytoprotective effects of H₂S: it was not possible to formulate a hypothesis of ‘one size fits all’ (Bos *et al.* 2014).

1.8.1. **Mitochondrial Protection**

H₂S cytoprotective role appears to start at the level of mitochondria, through the inhibition of cytochrome c oxidase function. This would reduce respiration at mitochondrial level during IR, thus reducing the overproduction of ROS (Kimura *et al.* 2005; Wagner *et al.* 2009; Veeranki and Tyagi 2014). Mitochondrial protection may also be mediated by reducing membrane potential depolarization (Calvert, Coetzee, and Lefer 2010; Tang *et al.* 2013).

1.8.2. **Antioxidant Properties**

The anti-oxidant properties of H₂S have been demonstrated by many researchers. H₂S has been shown to diminish the production of ROS when administered at therapeutic doses (Calvert, Coetzee, and Lefer 2010; Chen *et al.* 2006; Nicholson and Calvert 2010). The effect appeared to be mediated either by
directly scavenging ROS, or through indirect means, by upregulating intracellular antioxidants.

$H_2S$ has been shown to induce the production of GSH, known for its potent antioxidant role (Kimura, Goto, and Kimura 2010; Bos et al. 2014). Alternatively, Sun et al observed reduction in the levels of ROS through inhibition of mitochondrial cytochrome $c$ oxidase, and increasing activation of SOD (Sun et al. 2012). $H_2S$ also reported to reduce the oxidative stress by directly scavenging free radicals like superoxide and hydrogen peroxide (Geng et al. 2004), as well as the cytotoxic lipid oxidation products (Schreier et al. 2010; Calvert, Coetzee, and Lefer 2010), and the highly reactive peroxynitrite (Whiteman et al. 2004; Pacher, Beckman, and Liaudet 2007).

### 1.8.3. Anti-Inflammatory Effects

While the exact role of $H_2S$ in inflammation is not known, it has been a subject of several recent studies (Li and Moore 2008; R. Wang 2012). Whereas some have demonstrated clear anti-inflammatory effects of $H_2S$, others also reported that it may be pro-inflammatory. Most of the evidence, however, points towards the anti-inflammatory properties of $H_2S$ (Chan and Wallace 2013).

The results of Fiorucci et al have shown that exogenous application of $H_2S$ was capable of inhibiting leukocyte adherence to the vascular endothelium by suppressing the expression of intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-1 (LFA-1) (Fiorucci et al. 2005). $H_2S$ has also been shown to reduce production of pro-inflammatory cytokines and
chemokines, and assist the shift of macrophages to an anti-inflammatory phenotype (Wallace, Ferraz, and Muscara 2012; Dufton et al. 2012). In a rat model of colitis, H$_2$S was found to reduce the levels of mucosal TNF-α (Bai, Ouyang, and Hu 2005). Inhibition of liver TNF-α has also been reported (Fiorucci et al. 2006). Moreover, H$_2$S was shown to suppress NF-κB activation in the colon of patients suffering from ulcerative colitis (Bai, Ouyang, and Hu 2005). Suppressing NF-κB would, in turn, suppress the production of other inflammatory substances including cytokines, adhesion molecules, and iNOS (Lefler et al. 2002; Nichols 2004). H$_2$S also appears to reduce the progression of inflammation by enhancing the apoptotic death of polymorphonuclear leukocytes, thus limiting the neutrophil-mediated tissue injury (Mariggiò et al. 1998; Zanardo et al. 2006).

Finally, H$_2$S may exert some of its anti-inflammatory effects through upregulation of HO, which is known to reduce inflammation and exhibits cytoprotection by producing CO and bilirubin (Oh et al. 2006; Qingyou et al. 2004; Ryter, Alam, and Choi 2006).

1.8.4. Vasorelaxation

H$_2$S has been shown to exhibit potent vasodilator activity, both in vitro and in vivo (Bhatia 2005; Jackson-Weaver 2012; Stubbert et al. 2014). The effect appears to be through opening vascular smooth muscle K$_{ATP}$ channels (Bhatia 2005; Mustafa et al. 2011), and the inhibition of vascular tone (Jackson-Weaver 2012). The vasorelaxation can be by both nitric oxide-dependent and independent means (Altaany et al. 2014; Holwerda, Karumanchi, and Lely 2015).
Like nitric oxide, the function of H\(_2\)S is now considered as that of yet another major EDRF-like molecule, perhaps an endothelium-derived hyperpolarising factor (EDHF) (Mustafa et al. 2011; Tang et al. 2013).

1.9. **H\(_2\)S IN EXPERIMENTATION**

1.9.1. **Stimulation of Endogenous Production**

H\(_2\)S is naturally produced in mammalian tissues; perhaps, for therapeutic or experimental examinations, one can attempt to boost its endogenous production instead of applying it exogenously. Garlic or allyl disulfide were used in several experiments as H\(_2\)S-boosting substances, particularly in IR injury within the heart, kidney or the brain (Sener, Sakarcan, and Yegen 2007; Benavides et al. 2007). NAC and L-cysteine also function as precursors for the endogenous H\(_2\)S synthesis (Caliendo et al. 2010).

1.9.2. **Inhibition of Endogenous Production**

Inhibition of endogenous production of H\(_2\)S has been useful in studies examining its physiological role. Currently, pharmacological inhibitors of the CSE enzyme are available, and genetically targeted CSE inhibition in mice is another available option (G. Yang et al. 2008; Sha et al. 2014).

1.9.3. **Exogenous Application of H\(_2\)S**

There are several options available for the administration of H\(_2\)S in experimental settings. Apart from H\(_2\)S gas inhalation, H\(_2\)S-donor compounds can
be used. These may come in the form of sulfide salts, synthetic moieties (slow-releasing H₂S donors) or hybrids of H₂S donors.

One major drawback in using sulfide salts (e.g. NaSH or Na₂S) is the very rapid release and subsequent rapid decrease in H₂S levels upon systemic administration; this makes it difficult to achieve controlled, stable and therapeutic levels in vitro or in vivo (Bos et al. 2014).

On the contrary, slow-releasing H₂S donors (the synthetic moieties), were developed to achieve stable release of H₂S. These have been shown to release H₂S in a slower and steady level following their administration (Ling Li et al. 2007; Ling Li et al. 2008; Y. Zhao, Wang, and Xian 2011; Marutani et al. 2012; Predmore et al. 2012). GYY4137 (morpholin-4-ium-4-methoxyphenyl(morpholino) phosphinodithioate) is a water-soluble, slow-releasing H₂S donor (Ling Li et al. 2008). One millimole of GYY4137 releases approximately 40 micromoles of H₂S within the first 10 min of activation, and then steadily continues to release H₂S over a period of 3 hours. Systemic application of 133µmol/kg, either intraperitoneally (IP) or intravenously (IV), in rats was reported to increase plasma concentration of H₂S from 32 µmol/l to 80µmol/l within 30 min; H₂S remained elevated, at 50µmol/l, for further 3 hours (Kashfi and Olson 2013). In one in vitro study using human breast adenocarcinoma, a constant release of H₂S for up to 7 days was noticed following addition of GYY4137 to cell culture media. They postulated that GYY4137 may accumulate inside cancer cells and thereby release larger amounts of H₂S intracellularly (Lee et al. 2011).
Hybrids of H$_2$S donors are novel anti-inflammatory agents where the traditional NSAIDs are chemically modified to release H$_2$S. Thus, these combine the cyclooxygenase (COX)-inhibitory activity with COX-independent anti-inflammatory actions (Sidhapuriwala et al. 2007; Baskar et al. 2008; Zhang et al. 2012; Chattopadhyay et al. 2013; Lee et al. 2013).

1.10. STUDY MODELS FOR ACS

1.10.1. Human Volunteers

Studying ACS on humans is quite difficult, as the condition is potentially dangerous and permanent disabilities cannot be excluded. Moreover, it would be impossible to do certain in vivo studies on human the way it is done on laboratory animals like the intravital video microscopy (IVVM). The literature on ACS is scarce of experimental studies done on living human volunteers. We are aware of only two published experiments; the first was performed using cylindrical air splints applied to the legs of three enthusiastic investigators namely Frederick A. Matsen III, Keith A Mayo, and Richard B. Krugmire, JR. (Matsen et al. 1977), while the second was performed on 10 volunteers by wearing anti-shock trousers (Willy et al. 2001).

1.10.2. Animal Use

Using animals for experimental studies on ACS was reported in the 1800s when Volkmann published his insight on the pathology of ACS pointing out to the data he got from animal studies (Volkmann 1881). Dogs, pigs, rabbits, mice and
rats are the common animals utilized by different researchers over the past century (Jepson 1926; Bardenheuer 1910; Heppenstall et al. 1986). Research studies on animals are just tools that may indicate a potential therapeutic benefit of any drug. Recognising and addressing the limitations of pre-clinical animal studies is a paramount for a successful translation of therapy from the bench to the bedside (Lotfi et al. 2013).

1.10.2.1. ACS Rat Model

Lawendy and his group have developed a clinically relevant model for ACS (Lawendy, Sanders, Bihari, Parry, et al. 2011). The model is relatively simple, in which normal saline infusion is used to elevate the ICP of the anterior compartment. It has been validated in several studies (Lawendy, Sanders, Bihari, Parry, et al. 2011; Lawendy et al. 2011; Lawendy, Sanders, Bihari, and Badhwar 2011; Lawendy 2014a; Lawendy et al. 2015).

Rats are good models of human disease with easy to monitor physiology (Iannaccone and Jacob 2009; Clause 1998). They are also easy to house and handle, relatively inexpensive, and readily available. The suitable size of the rat, compared to the mouse, allows easy surgical fasciotomy and dissection of the skeletal muscle. The rat size also is quite suitable to be moved on the microscope for direct real-time visualization of the microcirculatory dysfunctions using the IVVM technology.
1.11. THESIS RATIONALE

The ultimate goal of this study was to test a potential pharmacological therapy for ACS: a therapeutic option that could be used as an adjunctive treatment to the standard surgical decompression, capable of reducing the extent of tissue damage resulting from the ACS-induced ischaemic insult, or the subsequent reperfusion injury.

1.11.1 Hypotheses

1.11.1.1. The Effect of \( H_2S \) on ACS

The main objective of the thesis was to assess the therapeutic potentials of \( H_2S \) in acute compartment syndrome. Based on previous studies in transplantation research or IR injury, it was hypothesized that \( H_2S \) would also have a cytoprotective effect on the skeletal muscle in ACS.

1.11.1.2. Combined CO/H\( _2S \) Effect

The second objective of this thesis was to explore the possible therapeutic effect of combining CO and \( H_2S \), as a possible adjunctive treatment of ACS.

It has been shown previously, in a rat model of ACS, that CORM-3-derived CO could be beneficial in minimising the ACS-induced microvascular perfusion derangements, tissue injury and inflammation (Lawendy et al. 2014). However, to our knowledge, there have been no studies examining the combined effects of CO and \( H_2S \), whether in IR injury or transplantation surgery (Siriussawakul, Chen, and Lang 2012).
Both H$_2$S and CO are lately-discovered gaseous signalling molecules. Their cytoprotective roles in IR injury has been documented in various organs and tissues (Ryter, Alam, and Choi 2006; Nicholson and Calvert 2010; Calvert, Coetzee, and Lefer 2010; Mancardi et al. 2009; Ozaki, Kimura, and Murase 2012). Therefore, we hypothesised that combination of H$_2$S and CO may have added protective benefit in ACS treatment, perhaps providing a synergistic therapeutic effect. In this study, we undertook to evaluate the cytoprotective potential of H$_2$S in ACS in reference to the established results of CORM-3.
CHAPTER 2. MATERIALS AND METHODS

2.1. ANIMAL CARE

All experimental protocols conducted in this study were approved by the Animal Use Subcommittee at the University of Western Ontario, London, Canada. The daily animal care, handling and housing were performed in accordance with the guidelines set out by the Canadian Council on Animal Care. All rats used for the experiments were housed in pairs, in clear plastic cages, under 12:12 light:dark cycle, and had access to food and water ad libitum.

2.2. ANIMAL DESCRIPTION

Twenty-five male Wistar rats, body weight 170–290 g, were used in the study.

2.3. EXPERIMENTAL TREATMENT DRUGS

Two treatment drugs were used in these experiments, plus an inert vehicle: hydrogen sulfide (H₂S) and carbon monoxide (CO).

2.3.1. Hydrogen Sulfide

Hydrogen sulfide-releasing molecule, GYY4137 (Cayman Chemicals, Ann Arbor, Michigan, USA), was used at a dose of 50 mg/kg (Li et al. 2009). It was diluted in normal saline just prior to injection, and administered intraperitoneally to the designated group. Normal saline served as an inert vehicle (control).
2.3.2. Carbon Monoxide

Carbon monoxide-releasing molecule-3 (CORM-3) was used at a dose of 10 mg/kg (Lawendy et al. 2014). CORM-3 was synthesized in our lab, in accordance with the previously published method (Motterlini & Otterbein 2010), and was diluted it in normal saline just prior to injection to be administered intraperitoneally to the designated groups.

Inactive CORM-3 (iCORM-3) served as an inert vehicle control; iCORM-3 was prepared in the same manner as CORM-3, but was allowed to release all CO from the solution prior to being administered to the animals.

2.4. EXPERIMENTAL SETUP

2.4.1. General Overview

Acute compartment syndrome (ACS) was experimentally generated in the rat hind limb under general anaesthesia. Elevated intracompartmental pressure was maintained at 30 - 40 mmHg for 2 hours, followed by fasciotomy and 30 minutes of reperfusion time. Intravital video microscopy (IVVM) was then performed. Experimental drug Injections were administered to all animals, as per the appropriate group, just before fasciotomy. Sham animals underwent all procedures, but the compartment pressure was maintained at a baseline level of 0mmHg.
2.4.2. Anaesthesia

Inhalational isoflurane was used to anaesthetize all animals. Induction of general anaesthesia was carried out by 5% isoflurane in a 1:1 mixture of oxygen and nitrogen gas, at a flow rate of 2L/min. Anaesthesia was maintained at 2% isoflurane, at a flow rate of 0.5L/min (Flecknell 1996), and adjusted as required to maintain the adequate anaesthetic depth. Left carotid artery was cannulated for the continuous monitoring of blood pressure, fluid replacement and arterial blood sampling. Rectal digital thermometer probe was used to continuously monitor the core body temperature; a constant core temperature of 37°C was maintained by the means of heating lamp.

2.4.3. Acute Compartment Syndrome

ACS was induced as previously described (Lawendy et al. 2011). Briefly, sterile isotonic saline solution (Baxter, Deerfield, IL) was continuously infused into the anterior compartment or the rat hind limb, elevating ICP via a 25-gauge needle (BD, Franklin Lakes, NJ). ICP was measured by an electronic compartmental pressure monitoring system (Synthes, West Chester, PA), placed in the anterior compartment via a 14-gauge angiocatheter (BD). Saline infusion was carried out by gravity-feed system, with the saline bag elevated 120 cm above the working bench level. ICP was maintained at an average of 30 - 40 mmHg for 2 hours (Figure 2.1).
Figure 2.1. **Experimental Setup in ACS.** Saline is infused into the anterior compartment of the rat hind limb, elevating ICP to 30mmHg. ICP is continuously monitored by the use of electronic compartment pressure monitor, also inserted into the anterior compartment.
2.4.4. Fasciotomy

Following 120 minutes of elevated ICP, fasciotomy and exposure of the Extensor Digitorum Longus muscle (EDL) were undertaken, as previously described (Tyml and Budreau 1991; Lawendy et al. 2011). Briefly, the skin over the lateral aspect of the leg was carefully incised, exposing the anterior and posterior compartments. The underlying biceps femoris muscle was partially incised to fully expose the tibialis anterior (TA), peroneus longus (PL) and lateral gastrocnemius muscles. Fasciotomy of the anterior compartment was carefully performed, taking care to avoid injury to the underlying muscles. EDL muscle was exposed by gentle separation of TA and PL, without physically touching its fibers. EDL tendon, identified at the ankle level, was tied using 3-0 silk ligature and cut as close as possible to the insertion. Following EDL preparation, animal was moved onto the stage of IVVM microscope, and the, EDL belly was partially reflected anteriorly into saline bath (Potter et al. 1993).

2.5 EXPERIMENTAL GROUPS

Rats were randomly assigned to one of the five experimental groups: (1) Sham, (2) ACS, (3) H₂S, (4) CO, (5) Combo.

2.5.1 Sham

All animals in this group (n=6) underwent all procedures, but the ICP was maintained at a baseline level of 0mmHg.
2.5.2 ACS

Animals in this group (n=5) underwent ACS, followed by inert vehicle injection at fasciotomy.

2.5.3 H$_2$S

Rats (n=4) were subjected to ACS, and received GYY4137 injection at fasciotomy. GYY4137 served as a pharmacological means of hydrogen sulfide delivery.

2.5.4 CO

Animals (n=4) were subjected to ACS, followed by CORM-3 administration at fasciotomy. CORM-3 served as a pharmacological means of CO delivery, as previously described (Lawendy et al. 2014).

2.5.5 Combo

Animals in the combo group (n=6) were subjected to ACS, and received a combined treatment of both GYY4137 and CORM-3 at fasciotomy.

2.6 INTRAVITAL VIDEO MICROSCOPY

Rats under anaesthesia were carefully moved from the surgical bench to the stage of IVVM microscope. Extreme care was given to the dissected EDL, avoiding its being traumatised, twisted, stretched, or even touched by anything.
The muscle was then reflected onto a glass slide, into saline bath containing vital dyes. The EDL was then stabilized to the microscope stage using the attached silk ligatures, and covered with glass coverslip.

### 2.6.1 Vital Dye Staining

Two fluorescent vital dyes were used at a concentration of 5µg/mL each: ethidium bromide (EB) (Sigma Aldrich, Mississauga, ON), and bisbenzimide (BB) (Sigma Aldrich, Mississauga, ON). BB stains the nuclei of all cells, as it is a small enough molecule to readily permeate across the cell membrane. EB stains only the nuclei of injured/damaged cells, as its larger size requires compromised cellular membrane (Potter et al. 1995). Cellular injury was expressed as the ratio of ethidium bromide-labelled nuclei to bisbenzimide labelled nuclei (EB/BB).

### 2.6.2 Microscopy

Microvascular perfusion and leukocyte behaviour within the post-capillary venules were recorded by transillumination in five adjacent fields of view with 20x and 40x objectives, respectively (final magnification of 700x and 1400x). Under 20x objective, each field of view contained a complete capillary bed, and was recorded for 60 seconds. Additional 10 seconds from the same fields of view were then recorded under the appropriate epifluorescence illumination, with the application of appropriate filters for BB (excitation wavelength=343nm, emission wavelength=483 nm) and EB (excitation wavelength=482 nm; emission wavelength=610nm).
Under the 40x objective, leukocytes were recorded within five randomly chosen post-capillary venules, with the approximate diameter of at least 20µm, for 45 seconds. All videos were captured into the computer using Adobe Premiere software for offline video analysis.

2.7 OFFLINE VIDEO ANALYSIS

2.7.1 Microvascular Perfusion

Microvascular perfusion was quantified by counting the number of capillaries crossing three equidistant parallel lines drawn on the computer monitor, perpendicular to the capillary axis (Lawendy et al. 2014). Each capillary was categorized into one of three functional states: continuously-perfused (CPC), intermittently-perfused (IPC) and non-perfused (NPC). The results were expressed as percentage of total capillary count in the capillary bed, as per their functional state (De Backer et al. 2007).

2.7.2 Injury Analysis

Tissue injury was expressed as a ratio of EB-stained to BB-stained cells (EB/BB) (Potter et al. 1995).

2.7.3 Analysis of Leukocytes

Leukocyte behaviour was classified as the number of rolling or adherent leukocytes per 30 seconds in each capillary venule. An adherent leukocyte was defined as a cell that remained stationary for a minimum of 30 seconds (Granger...
et al. 1989). The area of venules was measured using ImageJ software (NIH, Bethesda, MD). The results were expressed as number of leukocyte/30s/1000µm² (Lawendy et al. 2011).

2.8 TNF-α ELISA

Serum from animals was used to quantify systemic TNF-α levels at the following points: baseline (pre-ACS), 1 hour into ACS, 2 hours into ACS (just prior to injection and fasciotomy), 10 minutes post-fasciotomy, 20 minutes post-fasciotomy, 30 minutes post-fasciotomy and 45 minutes post-fasciotomy. Serum was obtained by allowing the whole blood to coagulate at room temperature for 30 minutes, followed by centrifugation at 1500xg for 20 minutes at 4°C. The supernatants (serum) were collected and stored at -80°C.

Levels of TNF-α were assessed using enzyme-linked immunosorbent assay (ELISA) kit (Pierce Biotechnology, c/o Thermo Scientific, Rockford, IL), as per manufacturer’s instructions. The assay was sensitive to less than 5pg/ml.

All samples were run in duplicate. Standard curve was obtained by performing serial dilutions of reconstituted lyophilized TNF-α standard; 2,500pg/ml, 833pg/ml, 500pg/ml, 278pg/ml, 93pg/ml, 31pg/ml and 0pg/ml were used to obtain the standard curve. Serum samples were diluted at 1:1 ratio with sample diluent buffer. All plate incubations were carried out at room temperature. Fifty µl of each sample or standard were added to the appropriate designated wells on a 96-well plate, and incubated for 1 hour. After three washes, 50µl biotinylated TNF-α antibody was added to each well and incubated for 1 hour.
Following three washes, 100µl streptavidin-HRP reagent was added to each well and incubated for 30 minutes. After the final three washes, 100µl tetramethyl benzidine (TMB) substrate was added to each well and the plate was incubated for 10 minutes in the dark. 100µl stop solution was used to stop the reaction. The absorbance was read on microplate reader (model 680, BioRad) at 450nm. The results were calculated by 4-point logistic curve fitting software against TNF-α standard.

2.9 STATISTICAL ANALYSIS

All data was analyzed using analysis of variance (ANOVA), with Newman-Keuls multiple comparison post-hoc test where appropriate. Two-way ANOVA was used to analyse the systemic TNF-α levels (pre- and post-treatment), while one-way ANOVA was used to analyse the rest of the results. Statistical significance was defined as p<0.05. Data analysis was performed using Prism version 4.0c for Mac (GraphPad Software Inc., San Diego, CA).
CHAPTER 3. RESULTS

3.1. MICROVASCULAR PERFUSION

3.1.1. Continuously-Perfused Capillaries (CPC)

Elevation of ICP resulted in a significant decrease in the CPC, from 75±2.6% in the sham to 48±5% in the ACS group (p<0.01). Hydrogen sulfide treatment (H₂S group) led to a significant increase in CPC from ACS, to 66.4±13.3% (p<0.05). The CPC in both the CO and Combo groups also significantly increased, compared to ACS group (62.7±4.8%, p<0.05 and 69.8±11%, p<0.01, respectively). There was no significant difference in the CPC between H₂S, CO and Combo groups compared to sham (Figure 3.1).

3.1.2. Intermittently-Perfused Capillaries (IPC)

There were no significant differences in IPC in any of the groups, although there was a trend towards an increase in the ACS group.

3.1.3. Non-Perfused Capillaries (NPC)

Elevation of ICP led to a significant increase in NPC, from 10.6±1.3% in sham to 29.9%±5.3% in ACS group (p<0.001). Hydrogen sulfide treatment (H₂S group) led to a significant decrease in NPC to 16.5±2.7% (p<0.05) when compared to ACS group. Both CO and Combo treatments resulted in a significant reduction in NPC, to 16.4±1.1% (p<0.05) and 18±1.9% (p<0.01), respectively, compared to ACS group (Figure 3.1). There were no significant differences in NPC between H₂S, CO, Combo and sham groups (Figure 3.1).
Figure 3.1  The Effect of H₂S on Skeletal Muscle Perfusion Following ACS.

ACS resulted in a significant microvascular dysfunction; H₂S partially restored the perfusion, similar to that of CO or combination of H₂S and CO (Combo). CPC, continuously perfused capillaries; IPC, intermittently perfused capillaries; NPC, non-perfused capillaries (*p<0.001 from sham; †p<0.05 from ACS).
3.2. TISSUE INJURY

Elevation of ICP caused a significant increase in tissue injury, from 0.09±0.02 in sham to 0.33±0.02 in ACS group (p<0.001). Hydrogen sulfide treatment (H₂S group) resulted in a significant decrease to 0.14±0.07 (p<0.001), compared to ACS group. Both CO and Combo groups also displayed a significant reduction in the tissue injury (0.16±0.07, p<0.01 and 0.13±0.01, p<0.001, respectively), compared to ACS group (Figure 3.2). There were no significant differences in the tissue injury between H₂S, CO, Combo or sham groups (Figure 3.2).

3.3. LEUKOCYTE BEHAVIOUR

3.3.1. Leukocyte Rolling

Elevation of ICP resulted in a significant increase in leukocyte rolling, from 1.33±0.29 leukocytes/30s/1000µm² in sham to 4.55±1.49 leukocytes/30s/1000µm² in ACS group (p<0.05). Hydrogen sulfide treatment (H₂S group) led to a significant reduction in leukocyte rolling to 1.82±0.51 leukocytes/30s/1000µm² (p<0.05), compared to ACS group. Both CO and Combo treatments also led to a significant decrease in leukocyte rolling (0.82±0.12 leukocytes/30s/1000µm² and 1.17±0.3 leukocytes/30s/1000µm²; p<0.05, respectively) when compared to ACS group. There were no significant differences in leukocyte rolling between H₂S, CO, Combo, and sham groups (Figure 3.3).
Figure 3.2  The Effect of H$_2$S on Skeletal Muscle Tissue Injury Following ACS. ACS resulted in a significant increase in tissue injury; H$_2$S significantly diminished the ACS-induced injury, similar to that of CO or combination of H$_2$S and CO (Combo) (*p<0.001 from sham; †p<0.01 from ACS).
Figure 3.3. The Effect of H$_2$S on Leukocyte Activation Following ACS:

**Leukocyte Rolling.** ACS led to a significant increase in leukocyte rolling; H$_2$S attenuated this response, similar to that of CO or combination of H$_2$S and CO (Combo) (*p<0.05 from sham; †p<0.05 from ACS).
3.3.2. Leukocyte Adhesion

Elevation of ICP resulted in a significant increase in leukocyte adhesion, from $0.83 \pm 0.16$ leukocytes/30s/1000µm$^2$ in sham to $4.49 \pm 0.79$ leukocytes/30s/1000µm$^2$ in ACS group ($p<0.001$). Hydrogen sulfide treatment (H$_2$S group) led to a significant decrease to $0.89 \pm 0.20$ leukocytes/30s/1000 µm$^2$ ($p<0.001$), compared to ACS group. Both CO and Combo groups also displayed significantly lower leukocyte adherence compared to ACS group ($1.29 \pm 0.25$ leukocytes/30s/1000 µm$^2$ and $0.67 \pm 0.16$ leukocytes/30s/1000 µm$^2$, $p<0.001$, respectively). There were no significant differences in leukocyte adhesion between H$_2$S, CO, Combo, and sham groups (Figure 3.4).

3.4 SYSTEMIC TNF-α LEVELS

Elevation of ICP resulted in a continuous, sustained increase in systemic levels of cytokine TNF-α, from the baseline of $1.3 \pm 1.5$pg/ml to $558.8 \pm 202.6$pg/ml and $1222.9 \pm 591.5$pg/ml at 1hr and 2hr ACS, respectively (Figure 3.5). Following fasciotomy, TNF-α levels continued to increase to $2834.38 \pm 730.1$pg/ml at 45 minutes post-fasciotomy. Hydrogen sulfide treatment (H$_2$S group) led to a significant reduction in systemic TNF-α levels to $893.7 \pm 450.4$pg/ml, $588.7 \pm 398.5$pg/ml, $454.7 \pm 253.4$pg/ml and $205.3 \pm 112.7$pg/ml at 10min, 20min, 30min and 45min post-fasciotomy, respectively ($p<0.05$ from ACS alone). Combo treatment resulted in an almost immediate significant reduction in systemic TNF-α levels to $31.8 \pm 26.84$pg/ml, $17.2 \pm 10.9$pg/ml, $8.9 \pm 10.8$pg/ml and $7.0 \pm 4.9$pg/ml at
Figure 3.4. The Effect of H₂S on Leukocyte Activation Following ACS:

Leukocyte Adhesion. ACS led to a significant increase in leukocyte adhesion; H₂S attenuated this response, similar to that of CO or combination of H₂S and CO (Combo) (*p<0.05 from sham; †p<0.05 from ACS).
Figure 3.5. Effect of H$_2$S on Systemic TNF-α levels in ACS. ACS led to significant increase in systemic TNF-a levels; H$_2$S injection at fasciotomy diminished this response, similar to that of combination of H$_2$S and CO (Combo) (*p<0.05 from baseline; †p<0.05 from ACS alone).
10min, 20min, 30min and 45min post-fasciotomy, respectively (p<0.001 from ACS alone). There was no statistical difference between H$_2$S and combo groups (Figure 3.5).
CHAPTER 4. DISCUSSION

4.1 GENERAL OVERVIEW OF COMPARTMENT SYNDROME

ACS is a known surgical emergency in which the viability of the compartment contents are endangered by the persistence of elevated ICP. The local and systemic outcome of untreated ACS may be catastrophic (Defraigne & Pincemail 1998; Bocca et al. 2002; Ritenour et al. 2008; Hershey 2013; Bonnaig et al. 2014). Fasciotomy remains the only gold standard treatment, and should be performed as soon as the diagnosis is reached; yet, it comes with its own complications, and the sequelae of ACS cannot be fully avoided (Echtermeyer & Horst 1997; Giannoudis et al. 2002).

The pathophysiology of ACS appears to be complex and still not yet fully understood. While there has been general agreement that the cellular damage occurs at the microcirculation level, there has been disagreement on the pathomechanisms of this damage (Ashton 1962; Matsen 1975; Whitesides et al. 1975; Ashton 1975; Matsen et al. 1979; Reneman et al. 1980; Heppenstall et al. 1988; Matsen & Rorabeck 1989; Perler et al. 1990; Hartsock et al. 1998).

4.1.1 ACS as a Low-Flow Ischaemia

Elevation of ICP in ACS does not appear to cause complete ischaemia (Heppenstall et al. 1986), but rather a low-flow ischaemia (Lawendy 2014). Even though, ACS causes more ultrastructural damage in skeletal muscle compared to complete ischaemia (Heppenstall et al. 1986; Heppenstall et al. 1988;
Heppenstall et al. 1989). While ACS and partial ischaemia share a unique temporal and qualitative patterns of cytokines expression compared to complete ischaemia (Conrad et al. 2006; Lawendy et al. 2014), partial ischaemia causes less muscle damage than complete ischaemia (Conrad et al. 2006). These puzzling observations raised the question if the damage in ACS could only be explained based on the magnitude of ischaemia resulting from high ICP (Echtermeyer and Horst 1997). On the other hand, several pathological and biochemical similarities have been found between ACS and IR injury including the induced microvascular dysfunctions in the skeletal muscle and early intense inflammatory response (Heppenstall et al. 1986; Sadasivan et al. 1997; Tollens et al. 1998; Loerakker et al. 2011; Lawendy, Sanders, et al. 2011; Lawendy et al. 2014; Zhao et al. 2014), and thus compelled researchers to consider ACS as one of the clinical situations associated with skeletal muscle IR injury (Gillani et al. 2012).

This new insight, considering both ischaemia and IR injury are occurring in ACS, could explain the mystifying fact that the observed damage in ACS is usually beyond that of ischaemic one. In orthodox thinking, the expected scenario would be an ischaemic insult during the phase of elevated ICP and IR injury after fasciotomy, as normalizing the ICP by fasciotomy allows the oxygenated blood to flow to the ischaemic muscles. Whereas this sounds a logical scenario, it does not explain the IR-like microvascular dysfunctional changes observed immediately following fasciotomy (Sadasivan et al. 1997; Lawendy, Sanders, et al. 2011) compared to 3 hours of reperfusion following...
complete ischaemia (Olivas et al. 2001). These aforementioned observations led us to assume that IR injury could be happening concomitantly during the course of elevated ICP and become maximized after fasciotomy. The mechanism of occurrence of IR injury during the phase of elevated ICP may need to be evaluated experimentally.

Hargens et al (1981) stated that it was unknown how much microvascular occlusion and reduced flow exist at a tissue pressure of 30 mmHg or greater after studying pressurized canine leg compartments using technetium-99m (Hargens et al. 1981). Hartsock et al (1998) have shown significant decreases in the number of perfused capillaries as tissue pressure became progressively elevated during in vivo video microscopy of the rat cremaster muscle (Hartsock et al. 1998). From these findings, it was clear that microcirculation does not cease completely or uniformly in the muscle as the ICP rises.

The low-flow ischaemia occurring in ACS should not be considered as a low-degree of global muscle ischemia, but rather a heterogeneous or mal-distributed blood flow pattern in the muscle, as described by Sadasivan et al (1997). Elevated ICP may be creating a differential state of microvascular dysfunctional where some micro areas within the muscle are suffering severe ischaemia, while others are better perfused. Endothelium and leukocytes would quickly become activated in the ischaemic milieu. The non-perfused and perfused areas may physiologically alternate by selective arteriolar response, as a protective mechanism against ischaemic necrosis, or due to changes in interstitial fluid distribution. Allowing blood to flow in the previously non-perfused
micro areas would then create a limited micro-reperfusion injury. Occurrence of multiple micro-reperfusions would release the pro-inflammatory cytokines and ROS that are known to cause capillary leak and tissue oedema, leading to an increase in tissue pressure, resulting in more micro areas of non-perfused muscle, thus initiating the known theory of vicious cycle.

While the sudden supply of acutely ischaemic tissue by oxygenated blood is the common known cause to induce IR injury, non-acute hypoperfusion situations also display a form of continuous and recurrent IR injuries. For example, in peripheral arterial disease, where there is a long standing hypoperfusion of the limb muscles, IR injuries with release of ROS have been reported on walking (Ciuffetti et al. 1991; Hickman et al. 1994; Dopheide et al. 2013; Hiatt et al. 2015). IR injury has even been blamed also for chronic non-healing wounds (Mustoe et al. 2006), and the pathogenesis and progression of diabetic ulcers (Alavi et al. 2014). These findings strengthen our assumption of concomitant occurrence of ischaemia and IR in ACS during elevated ICP phase.

Recently, experimental studies have shown that ACS induces a cascade of inflammatory reactions, resulting in microcirculatory changes and tissue damage (Perler, Tohmeh, and Bulkley 1990; Sadasivan et al. 1997; Manjoo et al. 2010; W. Z. Wang, Baynosa, and Zamboni 2011a; Gillani et al. 2012; Blaisdell 2002; Lawendy 2014; Lawendy et al. 2015). Several pro-inflammatory mediators and ROS are known to be released during this process, resulting in microcirculatory dysfunction, leukocyte activation and cell damage (Menger et al. 1992; Sadasivan et al. 1997; Tollens, Janzing, and Broos 1998; Lawendy,
McGarr, et al. 2011; Gillani et al. 2012). Although timely fasciotomy saves limbs from further ischaemic insult, it does not address the inflammatory element resulting from the ischaemia itself or from the IR injury. Moreover, the sudden reperfusion, induced by fasciotomy, probably causes the so called ‘second hit’ (Sellei et al. 2014), where ROS increase the microvascular dysfunction and maximizes the net tissue damage (Gillani et al. 2012).

4.1.2 The Search for the Magic Bullet

Although ACS contributes significantly to morbidity and mortality by inducing an array of ischaemic and intense inflammatory response, there has been little progress made towards development of translatable therapeutic interventions that might ameliorate ACS sequelae. A therapeutic intervention capable of changing the biochemical environment during the elevated ICP and/or following fasciotomy would be of significant benefit (Henderson et al. 2010).

Cellular damage in ACS appears to be a result of intense ischaemia-induced inflammatory reaction, coupled with oxidative stress; thus, targeting these factors may be beneficial. The ideal adjunctive therapy should be able to either prevent the progress of ACS (perhaps by enhancing ischemic tolerance of the tissue), or at least reduce the damage caused by oxidative stress and inflammation. The final outcome would be a reduction in the need for surgery, improvement in function, and fewer systemic complications. Additionally, the post-fasciotomy muscle oedema would be diminished, allowing for an easier delayed wound closure, without the need for skin grafting.
There is a marathon race toward discovering pharmacological agents capable of blocking or ameliorating the damages in ACS. Research in transplantation surgery and ischaemic heart disease has enriched the body of knowledge in the quest for the potential therapeutic agents in IR. Our laboratory has previously shown the efficacy of carbon monoxide (CO) and indomethacin in mitigating the damaging effects of ACS on skeletal muscle (Lawendy et al. 2014; Manjoo et al. 2010). This led us to the current work, the purpose of which was to evaluate the role of H\textsubscript{2}S in ACS.

4.2 EFFECTS OF ELEVATED ICP

We found that elevation of ICP resulted in a significant local effects on skeletal muscle, as demonstrated by the microvascular perfusion changes (Figure 3.1), tissue injury (Figure 3.2) and leukocyte activation (Figures 3.3. and 3.4). We also demonstrated systemic effects of elevated ICP as demonstrated by increased TNF-\textalpha level (Figure 4.5).

4.2.1. Perfusion Changes

Significant increase in the number of non-perfused capillaries appears to be a consistent finding in ACS (Lawendy, Sanders, et al. 2011; Manjoo et al. 2010; McGarr 2009). This observation resembles the no-reflow phenomenon associated with the IR injury.
Using the modern technology of IVVM, direct visualisation of the skeletal muscle microcirculation has demonstrated a failure to restore the normal physiological micro-flow following fasciotomy (Figure 3.1). Consistent with previous studies, this hypoperfusion was evidenced by significant increase of NPC coupled with significant decrease in CPC (Manjoo et al. 2010; Lawendy, Sanders, et al. 2011; Lawendy et al. 2014; Lawendy 2014; Lawendy et al. 2015).

Microcirculatory studies of IR described this failure of capillary perfusion as ‘no-reflow’. It had been demonstrated in several organs, such as the brain (Ames et al. 1968), skeletal muscle (Blaisdell 2002), kidney (Summers & Jamison 1971), and even skin (Chait et al. 1978). The occurrence of no-reflow would lead to focal ischemia and hypoxia, and despite normalization of compartment pressures, it would reduce recovery in muscle and increase the damage. The decrease in CPC was reported to correlate well with the increase in tissue necrosis in IR injury (Olivas et al. 2001).

The complexity of the pathogenesis of no-reflow is not completely understood (Durante & Camici 2015). No-reflow could be either a functional disturbance of microcirculation caused by vasoconstriction (Galiuto & Crea 2006; Maksimenko & Turashev 2012), or structural change caused by luminal blocking by aggregates of erythrocytes or leukocytes with platelets (Jaffe et al. 2008; Niccoli et al. 2008). While the functional no-reflow is a potentially reversible process, tissue injury may become irreversible after the 7-8 hour interval (Olivas et al. 2001).
Our data displayed considerable deterioration of the perfusion following ACS, as seen at 30 minutes post-fasciotomy (Figure 3.1). There was a significant increase in the non-perfused capillaries, and a significant reduction in continuously perfused capillaries, consistent with what had been reported in previous publications. Thus our data confirmed the hypoperfusion state/the no-reflow phenomenon seen after fasciotomy (Lawendy 2014; Lawendy, Sanders, et al. 2011). The decrease in CPC was found to correlate well with the increase in percentage of tissue damage (Figure 3.2), consistent with what previously found (Olivas et al. 2001).

Although there are great similarities noticed between the no-reflow phenomenon reported in ACS and that reported in IR injury following complete ischaemia, there are clear temporal and qualitative differences. While we reported significant decrease in CPC after only 30 minutes of fasciotomy, a similar significant decreases was reported after 3 hours of reperfusion after complete ischaemia (Olivas et al. 2001).

### 4.2.2 Tissue Injury

Our data demonstrated a significant cellular injury after 2 hours of elevated ICP (Figure 3.2). We reported 33% cellular injury, compared to 9% seen in sham. This was consistent with the previously reported cellular injury ratio (Manjoo et al. 2010; Lawendy, Sanders, et al. 2011; Lawendy et al. 2014).

These data are also consistent with previous studies demonstrating an increase in tissue necrosis due to IR but with different temporal and qualitative
patterns, as we reported significant tissue injury after 30 minutes of fasciotomy, while it required 7 hours of reperfusion to show significant tissue injury after 3 hours of complete ischaemia (Olivas et al. 2001).

Cellular death appears to be the net result of all destructive mechanisms involved in pathophysiology of ACS. Cell necrosis has long been considered to be the natural fate of ischaemic insult. Recent evidence from experimental models indicates that apoptosis is a major contributor to IR-induced cell death (Genescà et al. 2002; Zhang et al. 2008; Wang et al. 2013). However, there are several confounding factors that determine the extent and degree of the damage, like the duration of ischemia and the tissue type (Gillani et al. 2012). In IR studies, apoptosis has been shown to exceed necrosis in skeletal muscle (Wang et al. 2008), as well as the heart (McCully et al. 2004), liver (Rüdiger & Clavien 2002), kidney (Du et al. 2003), and brain (Vogt et al. 1998). Kajstura et al demonstrated that cardiomyocyte apoptosis began after 2 hours of ischemia in vivo (Kajstura et al. 1996). It was estimated that the ratio of apoptotic and necrotic cell death is 31:1 at 2 hours after rat coronary artery occlusion (Anversa et al. 1998). While in skeletal muscle the difference did not appear to be that extensive, it was estimated to be 2:1 after 4 hours of ischemia and 24 hours of reperfusion in an in vitro rat study (Wang et al. 2008).

Apoptosis is a programmed cell death triggered by stimuli from outside or within the cell, leading to the activation of caspases and subsequent cell death (Primeau et al. 2002). The apoptotic program is complex, involving pro-apoptotic and protective proteins. Controlling these factors can modify the outcome of the
response to an apoptotic stimulus (Scarabelli et al. 2006). The knowledge that IR could induce apoptosis in skeletal muscle is clinically relevant, since it could allow for a therapeutic design aimed at apoptosis inhibition. Caspases are the executioners of the apoptotic process (Fischer et al. 2003). TNF-α is one of the most important external stimuli capable of inducing cell apoptosis in response to IR (de Nigris et al. 2003; Haddad 2004; Wu et al. 2014). Mitochondria appear as the main source of internal apoptotic stimuli, due to apoptotic factors present in the mitochondrial intermembrane space (Saelens et al. 2004). Cytochrome c, a component of the electron transport chain, is generally the earliest and most critical initiating factor for mitochondrial-mediated apoptosis when released into the cytosol from the inner mitochondrial membrane (Jiang & Wang 2004).

While ischaemia itself may be an expected cause, inflammation and microvascular dysfunction are also strongly involved in the cellular damage seen in IR injury, and, to a great extent, in ACS. ROS in particular are considered the main factor responsible for cellular damage caused by both IR and ACS (Perler et al. 1990; Sadasivan et al. 1997; Hancock et al. 2001; Chan 2001; Li & Jackson 2002; Kearns et al. 2004; Wang et al. 2006; Arató et al. 2008; Kearns et al. 2010; Wang et al. 2011b; Gillani et al. 2012) Both ischemia and IR are known to induce defects in the electron transport chain, leading to increased production of ROS (Kalogeris et al. 2014). While there are several sources of ROS, xanthine oxidase enzyme and activated neutrophils are the most common source. ROS tend to oxidize biomolecules, such as membrane-bound polyunsaturated fatty acids and DNA, damaging their structural or functional roles (Chen et al. 2012).
In addition, they also activate cyclooxygenase and lipoxygenase pathways (Toyokuni 1999), which, in turn, are responsible for neutrophil activation. Furthermore, ROS impair endothelium-dependent vasodilator mechanisms, exacerbating the microcirculatory dysfunction. Thus, the destructive effects of ROS may lead to either necrosis or apoptosis. The intense inflammatory reaction also contributes to tissue death in several ways. While activated leukocytes are responsible for tissue injury by the capillary plugging and production of proteolytic enzymes, inflammatory mediators play a major additional role. Necroptosis, another type of cell death induced mainly by TNF-α and NF-κB signalling, is a form of regulated cell death not mediated by caspases (Sun & Wang 2014). Inflammation is known to contribute to this process (de Almagro & Vucic 2015).

Previously, several experimental therapies targeting ROS have demonstrated reduction of ACS-associated cell injury, reflecting the reversible nature of cell damage. Kearns et al (2004) successfully diminished the cell injury in experimental ACS using vitamin C, while Perler et al (1990) did so by the ablation of ROS. In a more recent study, a potent antioxidant, N-acetylcysteine, demonstrated significant reduction of cell injury due to ACS (Kearns et al. 2010).

4.2.3 Leukocyte Activation

Involvement of leukocytes in the pathophysiology of ACS has been one of the earliest observations where the differences between ACS and complete ischaemia were clearly highlighted.
Leukocytes, predominantly neutrophils, significantly contribute to the pathogenesis of IR injury (Korthuis et al. 1988; Carden et al. 1990; Schofield et al. 2013; Sabido et al. 1994). Similar role for neutrophils has been demonstrated in ACS (Sadasivan et al. 1997; Lawendy et al. 2011; Lawendy et al. 2015). Ischaemic insult leads to upregulation of chemical mediators, which, in turn, activate neutrophils. TNF-α, IL-1β, IL-6 and IL-8 are all major chemical mediators involved in the neutrophil activation process (Ball et al. 2013). Activated neutrophils further exacerbate the tissue damage by the release of ROS, proteolytic enzymes, and physical blockage of the capillaries thereby preventing reperfusion of the tissue (Schofield et al. 2013).

Endothelium is very sensitive to any change in the internal milieu of the blood vessel. In IR, the endothelium becomes activated and creates a proinflammatory and prothrombotic surface to which leukocytes and other blood components adhere, resulting in endothelial dysfunction (Girn et al. 2007). Integrins and selectins are adhesion molecules that are generated in the activation process of endothelium and leukocytes, leading to neutrophil recruitment via a multistep cascade. Activated leukocytes exhibit a characteristic sequence pattern within the post capillary venules, namely rolling, adhesion, and transendothelial migration (Schofield et al. 2013).

Using IVVM, we were able to provide direct evidence of significant neutrophil activation in ACS. Both rolling (Figure 3.3) and adherence (Figure 3.4) were significantly increased after 2 hours of elevated ICP and 30 minutes of reperfusion. These findings are in agreement with previous observations of many
other investigators (Sadasivan et al. 1997; Manjoo et al. 2010; Lawendy, Sanders, et al. 2011; Lawendy et al. 2014; Lawendy 2014; Lawendy et al. 2015)

4.2.4 TNF-α Levels

In this study, we observed a marked increase in the levels of circulating TNF-α in ACS-challenged animals. TNF-α escalated as ICP increased and continued to do so after fasciotomy. This observation had previously been reported by Lawendy et al (2014).

4.3 THE EFFECT OF EXPERIMENTAL DRUGS ON ACS

4.3.1 Hydrogen Sulfide

Recently, H₂S has demonstrated considerable protective role in IR injury. The cytoprotective role of H₂S has been well documented in different tissues and organs. While the mechanism(s) remain largely unknown, it is thought to be multifactorial: reduction of the oxidative stress directly, upregulation of antioxidant enzymes, promotion of vasorelaxation, and modulation of mitochondrial respiration (King & Lefer 2011). H₂S has also been shown to attenuate leukocyte-mediated inflammation and inhibit apoptosis (Henderson et al. 2011; Jackson-Weaver 2012).

In this study, we examined the potential protective effect of H₂S on skeletal muscle microcirculation in a rat model of ACS. While H₂S has been used experimentally as an inhalational gas and as a parenteral preparation via H₂S-
 donor molecules, we preferred the parenteral route. We avoided the use of
gaseous H₂S, not only because of its offensive smell and the inherent hazard of
being a highly toxic gas, but also because of the practical difficulties in
standardizing the dose, each subject would receive by inhalation. Additionally,
lung inflammatory reactions have been reported following inhalational H₂S
application (Trevisani et al. 2005), which may have altered our outcomes of the
inflammatory response due to ACS. Parenteral compounds are safer to store and
handle, easy to prepare, and permit weight-dependent calculation of
administered amount, thus ensuring consistency of dosing. Whereas both the
intraperitoneal and intravenous routes have almost identical pharmacokinetic
effects (Li et al. 2008), we opted for the intraperitoneal route because of ease of
administration.

Using our rat model of ACS, we were able to demonstrate the
cytoprotective value of H₂S. We found that H₂S markedly improved the
microvascular perfusion (as evidenced by a significant increase in the number of
continuously perfused capillaries and a reduction in the non-perfused capillaries)
(Figure 3.1), reduced the overall cell injury (Figure 3.2) and leukocyte activation
(Figures 3.3. and 3.4) within the post capillary venules.

4.3.1.1 H₂S and Microvascular Perfusion

Systemic administration of H₂S resulted in a noteworthy improvement in
the muscle perfusion (Figure 3.1). We found a strong inhibitory effect of H₂S on
the no-reflow phenomenon, with substantial increase in the number of
continuously perfused capillaries. The net effect of H$_2$S was the re-establishment of the microvascular perfusion to levels near those seen in the sham. This suggests that GYY4137-derived H$_2$S may have a great potential, not only in restoration/maintenance of adequate perfusion, but a subsequent protective role against further damage as well.

The mechanism(s) by which H$_2$S improved the microcirculation are not fully understood, but many pathways could play a role. Perhaps it works through re-establishing the redox balance between the pro-oxidants and anti-oxidants by directly scavenging ROS, enhancing the innate antioxidant mechanisms, and/or reducing the production of ROS themselves (Henderson et al. 2010; Bos et al. 2014).

The direct scavenging power of H$_2$S has not escaped a notice by many observers. It has been suggested that H$_2$S is capable of directly scavenging superoxide and hydrogen peroxide (Geng et al. 2004), cytotoxic lipid peroxidation products (Schreier et al. 2010; Calvert et al. 2010), the highly reactive peroxynitrite (Whiteman et al. 2004; Pacher et al. 2007), as well as hypochlorous acid (Whiteman et al. 2005; Laggner et al. 2007). The damaging power of ROS is due to their extreme reactivity, stemming from their chemical structure: these molecules have unpaired electrons. The readiness of H$_2$S to reduce other compounds by simply giving away a proton H$^+$ may explain its ability to directly reduce ROS, without the need of any enzymatic activity (Nagy et al. 2014).

H$_2$S appears to play a very important role in augmenting the body’s innate antioxidant response (Calvert et al. 2010; Nishida et al. 2012). It has been
reported to increase the synthesis of GSH (Jha et al. 2008), and to increase activation of SOD (Sun et al. 2012). Both SOD and GSH are well-known important defensive mechanisms against the harmful effects of ROS. SOD is the major scavenger of superoxide (Fang et al. 2009). H₂S has consistently been reported to increase the expression of SOD under oxidative stress (Kimura & Kimura 2004; Kimura et al. 2006; Wu et al. 2014). On the other hand, GSH is known for its potent antioxidant role by reducing hydrogen peroxide to water (Albrecht et al. 2011).

H₂S has been shown to reduce oxidative stress by diminishing the production of ROS when administered at therapeutic doses (Calvert et al. 2010; Chen et al. 2006; Nicholson & Calvert 2010). This effect appears to be mediated through inactivating ROS-producing enzymes. Sun et al. (2012) observed reduction in the levels of ROS through reversible inhibition of mitochondrial cytochrome c oxidase through partial inhibition of oxidative phosphorylation. In addition, H₂S has also been reported to inhibit the expression of NADPH oxidase (Tyagi et al. 2009; Muzaffar et al. 2008) and xanthine oxidase, most likely by activation of SOD (Bar-Or et al. 2015).

H₂S may have inhibited the no-reflow phenomenon by its potent vasodilator effect. It has been proposed that H₂S-mediated vasorelaxation is mediated through the opening of the mitochondrial K\textsubscript{ATP} channel (Zhao et al. 2001; Cheng et al. 2004), a property that may be the key factor in reducing the infarct size in cardiac muscles following IR injury (Zhao et al. 2001; Johansen et al. 2006; Osipov et al. 2009). Interestingly, the vasorelaxing power of H₂S
appears to be directly correlated to the severity of the local hypoxia (Koenitzer et al. 2007; Dombkowski et al. 2011).

While NO is considered to be the most potent endothelium-derived relaxing factor in the vasculature, H₂S is capable of increasing NO levels by upregulation of eNOS in ischemic tissue. Conversely, NO has been shown to upregulate H₂S production by increasing the expression of CSE. Thus, H₂S and NO appear to be mutually dependent in controlling vascular relaxation (Coletta et al. 2012).

4.3.1.2 H₂S and Cellular Injury

We have demonstrated that H₂S administration in ACS led to a significant reduction in the cellular injury (Figure 3.2). Our findings are consistent with those of others, who had demonstrated the cytoprotective potential of H₂S in a rat model of myocardial IR (Sivarajah et al. 2009; Yao et al. 2012; Wu et al. 2014), liver (Mani et al. 2014), and kidney (Lobb et al. 2012). Henderson et al. had shown that not only was H₂S protective in skeletal muscle IR, but this protection was long lasting, for up to 4 weeks after the initial IR insult (Henderson et al. 2010). This would suggest that the therapeutic effect of H₂S does not merely shift the cellular damage several hours ahead, but is truly protective to the tissue.

While the exact mechanism of cellular protection is most likely related to the effect on vasodilation and anti-oxidant properties, there is another possibility: inhibition of apoptosis through the direct effect of H₂S on mitochondria. H₂S has been reported to preserve the mitochondrial function and membrane integrity
upon administration at the commencement of reperfusion in the ischaemic heart (Elrod et al. 2007). Through its ability to inhibit of cytochrome c oxidase function, H₂S is capable of inhibiting the electron transport chain during early phases of IR, thus reducing the overproduction of ROS (Kimura et al. 2005; Wagner et al. 2009; Veeranki & Tyagi 2014). Mitochondrial protection may also be mediated by maintaining membrane potentials. As an active opener of mitochondrial Kₘₐₜₚ channels, H₂S would prevent mitochondrial hyperpolarization, calcium overload and deleterious opening of the mitochondrial permeability transition pore (Calvert et al. 2010; Tang et al. 2013).

Previously, it has been demonstrated that the administration of H₂S at reperfusion was capable of reduction of cardiomyocyte apoptosis, both in vitro and in vivo (Elrod et al. 2007). This effect is most likely mediated by the ability of H₂S to inhibit the activation of caspases (Rinaldi et al. 2006).

H₂S could also inhibit apoptosis through inhibition of TNF-α, which is a major extracellular stimulus to induce apoptosis. We demonstrated significant reduction in the systemic levels of TNF-α, in our experiment, following H₂S injection (Figure 3.5).

Given the fact that we administered H₂S upon fasciotomy, it would appear that the protective effect of H₂S was most likely due to its modulation of the severity of reperfusion injury that follows fasciotomy. This kind of therapeutic application is considered a ‘pharmacological postconditioning’. H₂S-mediated pharmacological postconditioning had been successfully tested in completely
ischaemic rat skeletal muscle, and shown to significantly limit IR-induced damage (Henderson et al. 2011).

4.3.1.3 $H_2S$ and Leukocyte Activation

In the current study, $H_2S$ demonstrated a strong anti-inflammatory role, evidenced by the inhibition of leukocyte activation. $H_2S$ administration resulted in a significant reduction in the number of both adherent and rolling leukocytes in the post-capillary venules (Figures 3.3 and 3.4). This anti-inflammatory property of $H_2S$ has an added value in the cytoprotective power of $H_2S$, knowing that leukocyte activation amplifies the extent of damage through its role in inflammatory response.

$H_2S$ appears to play an important role in inhibiting leukocyte activation in normal physiological conditions. For example, inhibition of $H_2S$ synthesis in healthy animals led to a rapid increase in leukocyte adherence to the vascular endothelium (Zanardo et al. 2006). Rats with a diet-induced vitamin B deficiency (a co-factor in $H_2S$ synthesis) exhibited significantly enhanced accumulation of leukocytes in the affected tissues (Flannigan et al. 2014). Heterozygous mice deficient in cystathionine-β-synthase (i.e. diminished synthesis of $H_2S$) also exhibit increased levels of leukocyte adherence to the vascular endothelium and an increase in vascular permeability (Kamath et al. 2006).

Just like in our study, administration of a therapeutic dose of $H_2S$ has been shown to produce a marked suppression of inflammatory response in
several other animal models, including that of endotoxic shock (Fiorucci et al. 2007; Li et al. 2007). While the mechanism of the effect of H₂S on leukocyte adhesion is yet to be elucidated, Ball et al. had demonstrated that H₂S suppressed leukocytes recruitment due to downregulation of L-selectin expression on activated human neutrophils (Ball et al. 2013). Additionally, H₂S has been shown to inhibit myeloperoxidase activity within neutrophils (Pálinkás et al. 2014), promote the shift of macrophages to an anti-inflammatory phenotype (Dufton et al. 2012), and induce apoptosis of neutrophils (Serhan et al. 2007). Finally, H₂S has also been shown to inhibit endothelial ICAM-1 expression, triggered by hyperglycaemia (Guan et al. 2013).

It has been demonstrated that exogenous administration of H₂S is capable of reducing the production of a number of proinflammatory cytokines, by suppressing NFκB activity (Sen et al. 2012). While H₂S appears to suppress the proinflammatory cytokines, it does not affect the expression of IL-10, which is anti-inflammatory (Dinarello 2000; Li et al. 2007). Recently, an interesting regulatory interaction between IL-10 and H₂S has been described. IL-10-deficient mice that spontaneously developed colitis were found to exhibit impaired H₂S synthesis in the colon. Administration of H₂S to these animals increased IL-10 expression and restored normal colonic H₂S synthesis (Gemici et al. 2015).

H₂S also appears to lead to an upregulation of HO, which is known to reduce inflammation and exhibit cytoprotection by producing CO and bilirubin (Oh et al. 2006; Qingyou et al. 2004; Ryter et al. 2006).
4.3.1.3 \textit{H}_2\textit{S} and \textit{TNF-\alpha}

We reported dramatic reduction of \textit{TNF-\alpha} levels following injection of \textit{H}_2\textit{S}, reaching baseline values in 45 minutes (Figure 3.5). Inhibition of \textit{TNF-\alpha} (one of the most potent pro-inflammatory cytokines) reflects the compelling role of \textit{H}_2\textit{S} as an anti-inflammatory agent, not only protecting tissues locally at the site of ischaemia, but also in reducing the systemic sequelae of ACS.

Interestingly, the \textit{TNF-\alpha} level pattern was mirrored by leukocyte activation in the ACS-challenged muscle, as demonstrated by changes in adherent and rolling leukocytes within the post capillary venules (Figures 3.3 and 3.4).

4.3.2 Combination of Hydrogen Sulfide and Carbon Monoxide

Both \textit{H}_2\textit{S} and \textit{CO} are gaseous signalling molecules with documented cytoprotective roles in IR injury (Ryter \textit{et al.} 2006; Nicholson & Calvert 2010; Calvert \textit{et al.} 2010; Mancardi \textit{et al.} 2009; Ozaki \textit{et al.} 2012). Our data demonstrated that the improvement in the microvascular perfusion and tissue injury, as well as inhibition of leukocyte activation and \textit{TNF-\alpha}, induced by GYY4137-derived \textit{H}_2\textit{S}, was very comparable to that induced by CORM-3-derived \textit{CO}, as reported by Lawendy \textit{et al} (2014).

Though we hypothesised that combination of \textit{H}_2\textit{S} and \textit{CO} may have an added protective benefit in ACS, the combined effect of \textit{H}_2\textit{S} and \textit{CO}, in our experiments, was not significantly different from that of either of them, to our surprise.
There could be several possibilities explaining the failure of combination treatment. First, perhaps the improvements we had observed could represent the maximum improvement that could ever be achieved by any drug. Thus, combining two or more agents would not be of value. For example, it is possible that capillary damage produced structural no-reflow phenomenon, thus it would not respond to any treatment (Galiuto & Crea 2006).

Another possibility is that both H$_2$S and CO may be exhibiting their protective effects by influencing one common pathway, albeit from different starting points. For example, H$_2$S may exert some of its anti-inflammatory effects through upregulation of HO, while CO is the final product of this pathway (Oh et al. 2006; Qingyou et al. 2004; Ryter et al. 2006). HO-derived CO has been shown to control vascular tone in NO-dependent manner (Ishikawa et al. 2005); H$_2$S has been found to increase NOS expression in endothelial cells (Bir et al. 2012). The actions of both H$_2$S and CO converge at cGMP, although H$_2$S does not directly activate soluble guanylate cyclase (Coletta et al. 2012; Schallner et al. 2013).

Finally, there is a possibility that a much more complex interplay between the two molecules occurs. Some effects may be synergistic, while and others antagonistic; the final outcome would be dictated by which effect would be more pronounced. For example, CBS has been proposed to be a specific sensor for CO (Omura 2005). Thus, CO would have the potential to inhibit CBS activity and, therefore, the generation of endogenous H$_2$S (Taoka & Banerjee 2001).
4.4 STUDY LIMITATIONS

While our results demonstrated significant potential role of \( \text{H}_2\text{S} \) as a novel pharmacological therapeutic agent in ACS, we also recognize that there were certain limitations to our study. We used a small animal model of simulated acute compartment syndrome, and although there are some similarities between rat and human physiology, there are also significant differences between the two species (Radermacher & Haouzi 2013). Thus, the observed data may not necessarily reflect the human physiologic responses to an extremity ACS.

In our experiment, we chose to induce ACS for two hours, based on our previous publication demonstrating the beneficial effects of CO (Lawendy et al. 2014). The equivalence of two hours of ACS in rats is questionable when compared to humans. Many studies exist that compare the physiological processes among living organisms (basal metabolic rate, life span, body mass and other parameters), and how they mathematically relate to one another. Different equations applicable to all living organisms are available for comparative physiology (Marquet et al. 2005; Hulbert et al. 2007; Hoppeler & Weibel 2005; Radermacher & Haouzi 2013). Based on these, it was extrapolated that one hour of ischaemia in a rat muscle would be the equivalent of about 4 hours in humans (McGarr 2009; Lawendy 2014), which means that the 2 hours we used in the rat model could be equal to 8 hours of ACS in humans. While the design of our experiment was not geared to prove this theory, it would be interesting to validate it in future studies.
Another limitation is the type of tissue examined. Our study was designed to determine the potential of H$_2$S in attenuation of the ACS-associated damaging effects in the striated muscle. Although our data suggests skeletal muscle protection in terms of reduced cell damage, reduced leukocyte activation and improved microcirculation, the study design did not address the protective effects on the nerves or the antinociceptive effects of H$_2$S. Success in reducing muscle damage does not necessarily mean associated nerve protection, as it was noticed previously that ischaemic preconditioning may prevent skeletal muscle tissue injury, but not nerve lesion upon tourniquet-induced ischaemia (Schoen et al. 2007)

While we demonstrated the immediate protective effects of H$_2$S, we did not study the overall long-term changes, sequelae, and recovery of muscle function. Thus, any functional clinical outcomes cannot be directly inferred from our experiments.

Finally, while we assessed one dose of H$_2$S delivered intraperitoneally just before fasciotomy, we did not investigate the dose–response relationship. Knowing that timing of the medication may change the results, additional studies would be required to determine the optimum time to administer H$_2$S, and to examine the possibility that more than one dose may be needed.

4.5 FUTURE DIRECTIONS

In the future, there are several possible experiments that could further elucidate the role of hydrogen sulfide in ACS.
1. **H$_2$S dose-time optimization:** Considering that H$_2$S was recently shown to serve as a “fuel” for mitochondria during anoxia (Chan & Wallace 2013; Szabo et al. 2014), this opens a new horizon for trials during the state of elevated ICP, as this mechanism may ameliorate the ischaemic insult itself. Adding to its known cytoprotective role, H$_2$S may be able to reduce the demand for fasciotomy.

2. The next logical step for this line of work is to evaluate functional outcomes of the treatment in recovery studies. Gait analysis testing a few days following the ACS, and even microscopic examination of skeletal muscle should provide the necessary functional as well as pathological data to support the use of H$_2$S in clinical trials.

3. Evaluation of the effects of H$_2$S on Large animal model would be of more clinical relevance than the rat model, and thus would be the next logical step forwards clinical trials.

4.6 **SUMMARY AND CONCLUSIONS**

Although ACS remains a significant source of morbidity and mortality, there has been little progress made toward the development of an adjunctive therapeutic intervention, beside surgery, that might ameliorate the damaging inflammatory response and associated oxidative stress. The ideal intervention would simultaneously target interacting inflammatory mechanisms, abort the build up of oedema/increased ICP cycle, and protect the cells from IR injury.
We were able to demonstrate that H$_2$S may have a considerable cytoprotective role on the skeletal muscle in ACS, acting through several possible mechanisms (Figure 4.1). We also established that combining H$_2$S and CO was not superior to using either one of the substances individually. Hydrogen sulfide shows potential as an adjunctive therapy for compartment syndrome and warrants additional assessment to make it a translatable therapy for clinical applications.
Figure 4.1. **Anti-inflammatory actions of H$_2$S.** H$_2$S inhibits leukocyte adherence to the vascular endothelium, and promotes neutrophil apoptosis. Mitochondria can utilize H$_2$S as a fuel in ATP production, particularly during anoxia/hypoxia, thus reduce generation of ROS. By inhibiting phosphodiesterases (PDE), H$_2$S can elevate tissue cGMP levels, which contributes to vasodilation. The antioxidant actions of H$_2$S further reduce tissue injury. Several anti-inflammatory and antioxidant systems are activated by H$_2$S through its effects on transcription factors. H$_2$S can stimulate angiogenesis, thus promote repair of damaged tissue.

*Adapted from* (Chan & Wallace 2013), *with permission.*
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APPENDICES
APPENDIX I. Copy of the Animal Protocol Approval

Dear Dr. Lawendy:

Your animal use protocol form entitled:

Direct and Remote Organ Injury Following Hind Limb Compartment Syndrome

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

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

The protocol number for this project is 2009-083.

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

ANIMALS APPROVED FOR 4 YEARS

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REQUIREMENTS/COMMENTS

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

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

c.c. R Bihari, T Carter, K Bothwell, P Coakwell
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VITA

Name: Moustafa Mohamed Haddara

Post-secondary Education and Degrees:
Alexandria University
Alexandria, Egypt
1976-83, Bachelor of Medicine and Surgery (MBBCh)

Alexandria University
Alexandria, Egypt
1984-87, Master of Orthopaedics (MChOrth)

Royal College of Surgeons
Dublin, Ireland
1995, Fellow of Royal College of Surgeons (FRCS)

Strathclyde University
Glasgow, UK
1998-99, Diploma in Orthotics and Biomechanics

Honours and Awards:
Award of Excellence, Egyptian Ministry of Education
1976-82

Dean’s Honour List, Faculty of Medicine, Alexandria
1977-83

AO Fellowship, University of Basel, Switzerland
2001

Work Experience:
Orthopaedic Surgeon
Alexandria, Egypt
1988-2014

Publications:
