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# Pathophysiology of Compartment Syndrome

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Medical Biophysics

# PATHOPHYSIOLOGY OF COMPARTMENT SYNDROME

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**by**

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**Graduate Program in Medical Biophysics**

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## ABSTRACT

Acute limb compartment syndrome (CS), a potentially devastating complication of musculoskeletal trauma, is characterized by increased pressure within a closed osseofascial compartment, resulting in muscle-threatening and ultimately limb-threatening ischemia. Fasciotomy (to fully decompress all the muscles in the involved compartments) remains the only effective treatment and a current gold-standard surgical therapy, but it must be performed within a fairly narrow surgical window of 6-8 hours before the permanent tissue damage occurs. Despite a large body of literature dedicated to understanding the pathophysiology of CS, the mechanisms of CS-induced tissue damage are rather poorly understood. The established view is that increasing compartmental pressure compromises microcirculatory perfusion, restricting oxygen and nutrient delivery to vital tissues, resulting in cellular anoxia and severe tissue necrosis. However, unlike complete ischemia, CS causes myonecrosis in the face of patent vessels.

The purpose of this thesis was to investigate the mechanisms that contribute to the pathophysiology of CS. We developed a reproducible small-animal model of CS, utilizing saline infusion into the hind limb of the rat as the means of raising (and controlling) the compartment pressure. The microcirculatory parameters (capillary perfusion, tissue injury and leukocyte behaviour) were then assessed using intravital video microscopy (IVVM). The results of our studies described herein are the first to directly visualize the microcirculatory dysfunction and tissue damage in response to CS. A severe acute inflammatory component was detected in CS; the role of inflammation in muscle damage in compartment syndrome is unknown, but is believed to be a driving force in the generation of cell injury, and may contribute to the reduced capillary perfusion, since leukopenia was shown to be protective against cellular injury. In addition, we have demonstrated that compartment syndrome can be accompanied by a severe systemic inflammatory response. This study provides evidence of the relationship between limb compartment syndrome, systemic inflammation and remote organ dysfunction, presumably through the release of pro-inflammatory cytokines (primarily TNF-α).

The ultimate goal is to lay the groundwork for the development of rational therapeutic interventions that would, at least, extend the surgical window for fasciotomy, if not prevent the development of this condition completely.

***Keywords****:* *compartment syndrome, elevated compartment pressure, ischemia-reperfusion, fasciotomy, tissue injury, inflammation, TNF-α, cytokines, remote organ injury*

## THE CO-AUTHORSHIP

Each of the co-authors listed below made important contributions to this work. I performed the experiments, data collection and analysis. I have written the manuscripts presented in this thesis with consultation, assistance and critical review by the co-authors.

This project and its leadership have evolved since it began. Dr. Potter, followed by Dr. Badhwar, were my original basic science supervisors; however, personal and professional changes of circumstance led to Dr. Cepinskas graciously completing this work with me for the past few years. Dr. Sanders has been a mentor since we began these studies.

**Gediminas Cepinskas, DVM, PhD** and **David Sanders, MD, FRCSC**, in their role as joint supervisors, provided strong leadership on this project, offering direction, encouragement and guidance on data interpretation.

**Aurelia Bihari, MSc** taught me all of the experimental techniques used in this project, assisting with the animal protocols, model development, animal setup, data collection, analysis, much needed technical support, manuscript editing and publishing.

**Gregory McGarr, MSc** assisted with some of the leukopenia-related animal work and data collection.

**Amit Badhwar, PhD**, **Daryl Gray, MD, FRCSC** and **Neil Parry, MD, FRCSC** critically reviewed the manuscripts.

## DEDICATION

I dedicate this work to the memory of my father, who left his country of birth in the pursuit of liberty. He exchanged the convenience of a high post, for a life of labour and sacrifice. A spiritual leader, scholar, orator and poet, his effects upon my life are still felt today.

To my mother, widowed with six children, dedicated her life to raising a strong family, built business from nothing, and gave me her full support in every known meaning of the word.

To my wife, who married a student who vanished into surgical training for nearly a decade, a pillar of strength and sacrifice for me throughout these years, and a true friend.

To Safiyah and Tesneem, each equally my pride and joy.

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In the early hours of dawn, when this work was written, in solitude, I came to realize the great effort needed to contribute even a small, and perhaps insignificant amount of knowledge to the scientific endeavour. In this regard, I begin by acknowledging my own deficiencies against the One whose knowledge encompasses all. Although my name leads the work presented here, many others have had substantial contribution in shaping this project:

First, I acknowledge my supervisors, Dr. Sanders and Dr. Cepinskas, for their mentorship and patience.

Mrs. Aurelia Bihari, who since I entered the lab as a junior resident spent countless hours teaching me all the experimental techniques, animal preparation, anesthesia and microsurgery required to complete this work. Despite my surgical skills set, the requirement of tissue handling in these experiments demanded a level of refinement and training. I thank and acknowledge her assistance.

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## LIST OF ABBREVIATIONS

BB, bisbenzimide

CPC, continuously-perfused capillaries

CPS, continuously-perfused sinusoids

CS, compartment syndrome

EB, ethidium bromide

EDL, extensor digitorum longus

EICP, elevated intra-compartmental pressure

ELISA, enzyme-linked immunosorbent assay

ICP, intra-compartmental pressure

IL-1β, interleukin-1 beta

IL-8, interleukin-8

IPC, intermittently-perfused capillaries

IPS, intermittently-perfused sinusoids

IVVM, intravital video microscopy

KC, keratinocyte chemoattractant

NIR, near infra-red spectroscopy

NPC, non-perfused capillaries

NPS, non-perfused sinusoids

PI, propidium iodide

PPS, pain on passive muscle strech

RBC, red blood cells

TNF-α, tumor necrosis factor alpha

# CHAPTER 1

# INTRODUCTION AND HISTORICAL REVIEW

# CHAPTER 1: INTRODUCTION AND HISTORICAL REVIEW

## 1.1 COMPARTMENT SYNDROME

Compartment Syndrome (CS) is a condition caused by elevated pressure within a closed osseofascial compartment, leading to microvascular compromise, cellular anoxia and cell death (Mubarak et al, 1978; Rorabeck and Clarke, 1978; Matsen et al, 1980; Hartsock et al, 1998). Without urgent decompression of the compartment, significant functional impairment and ischemia may result. A myriad of traumatic injuries and medical co-morbities can lead to the development of CS. Common clinical conditions leading to CS include fractures, burns, exercise, crush injuries, and ischemia-reperfusion injury. Less common causes include bleeding disorders (Hope and McQueen, 2004), diabetes, administration of statins (Chautems et al, 1997; Jose et al, 2004), infection (Schnall et al, 1994), hypothyroidism (Hsu et al, 1995), lithotomy position (Mathews et al, 2001), snake bites (Vigasio et al, 1991), arterial rupture (Brumback, 1990) and blast injuries.

## 1.2 DIAGNOSIS OF CS

Early diagnosis of acute compartment syndrome is critical to its successful management and subsequent clinical outcome. Failure of timely diagnosis is the single most important cause of adverse outcomes (Matsen and Clawson, 1975; Rorabeck, 1984; McQueen et al, 1996; Mars and Hadley, 1998). Early diagnosis of compartment syndrome is facilitated by recognition of patient risk factors, understanding of the early clinical symptoms of compartment syndrome, and the judicious use of compartment-pressure monitoring (Matsen et al, 1980; McQueen et al, 2000). Risk factors for the development of acute compartment syndrome include male gender, young age group, tibial fracture, high-energy forearm fracture, high-energy femoral diaphyseal fracture and bleeding diathesis or anticoagulation (McQueen et al, 2000).

Missed or late diagnosis of acute compartment syndrome can result in serious complications, such as muscle infarction, muscle contracture, secondary deformity, weakness, and neurologic dysfunction (Whitesides and Heckman, 1996). Other less common sequelae include infection, gram-negative sepsis, amputation and end-organ involvement (Whitesides and Heckman, 1996). Time from onset to necrosis is variable with an accepted upper limit of 6 hours (Elliott and Johnstone, 2003). Determination of the exact time of onset of acute compartment syndrome is often difficult, as it may not parallel the onset of injury (McQueen et al, 1996). Thus, ongoing assessment of the patient at risk is important in identifying a potential delayed-onset acute compartment syndrome. Missed or late diagnosis is often a result of clinical inexperience, lack of suspicion, or a confusing clinical presentation (McQueen et al, 1996). Altered pain perception, as seen with changes in level of consciousness, regional anesthesia, patient-controlled analgesia and nerve injury are risk factors for late diagnosis (Mubarak and Wilton 1997; Harrington et al, 2000). Maintaining an appropriate index of suspicion is important in preventing the negative sequelae of late-diagnosed acute compartment syndrome, as well as malpractice litigation (Bhattacharyya and Vrahas, 2004).

### 1.2.1 Clinical Diagnosis

Disproportionate pain relative to the injury and pain on passive muscle-stretch (PPS) are recognized as the first symptoms of acute compartment syndrome (Whitesides and Heckman, 1996). Progressively increasing analgesia requirements may be a sign of disproportionate pain and an underlying compartment syndrome (Bae et al, 2001).

Pain that is produced upon plantar flexion of the foot or toes in an individual with an anterior acute compartment syndrome of the leg is an example of pain on passive stretch. Both pain out of proportion to injury and PPS are the most sensitive clinical findings (19%) and are often the only findings that precede ischemic dysfunction in the nerves and muscles of the affected compartment (Whitesides and Heckman, 1996; Ulmer, 2002). While the specificity of both pain measures is high (97%), the sensitivity is disturbingly poor (19%). Pain as a diagnostic criterion fails to identify a high percentage of individuals with acute compartment syndrome (Ulmer, 2002). The low false positive rate suggests that the absence of pain is a more useful measure in ruling out acute compartment syndrome. However, an adequate level of suspicion must be maintained as the absence of pain may indicate individual variation, altered states of pain perception, compartment syndrome of the deep posterior compartment, or missed acute compartment syndrome that has resulted in altered sensation (Whitesides and Heckman, 1996).

Sensory changes are first noted approximately 1 hour after the onset of ischemia (Whitesides and Heckman, 1996). Hypoesthesias and paresthesias in the dermatomal distribution of the nerve(s) of the involved compartment are typically the first neurological signs of acute compartment syndrome (Hargens et al, 1978; Mubarak et al, 1978; Matsen et al, 1980). As a clinical measure of acute compartment syndrome, paresthesia has a sensitivity of 13% and a specificity of 98% (Ulmer, 2002). Hypoesthesias and paresthesias of the first web space indicate involvement of the deep peroneal nerve and anterior compartment syndrome, while numbness of the dorsum of the foot may indicate lateral compartment syndrome with compression of the superficial peroneal nerve. These signs may also be caused by direct trauma to the nerve (Mubarak et al, 1978). Paresis and/or paralysis of the muscles of the involved compartment are considered to be signs of a late acute compartment syndrome that is less likely to respond to fasciotomy (Matsen and Clawson, 1975; Ulmer, 2002).

A swollen, tense compartment resulting from increased intracompartmental pressure is recognized as an early physical sign of acute compartment syndrome (Mubarak et al, 1978). These measures may not be evident with isolated involvement of a deep compartment. Dressings and casts should be removed to accurately assess swelling. The lack of a pulse is not a feature of acute compartment syndrome and the presence of a pulse does not exclude it.

Diagnosis of acute compartment syndrome requires careful evaluation of the entire clinical presentation. Ulmer (2002) found that the probability of acute compartment syndrome rose from approximately 25% when one of pain, PPS, paresthesia or paresis was present to 93% when three clinical findings were present concurrently. As noted, individual symptoms and signs are far from perfect in the diagnosis of compartment syndrome, but require careful interpretation owing to the tragic sequelae of a misdiagnosis.

## 1.3 THERAPEUTIC APPROACH TO CS

Fasciotomy of the involved compartments remains the gold standard for treatment of compartment syndrome. By contrast, non-operative measures have a limited role. There is little dispute regarding the severe consequences of delaying fasciotomy once the diagnosis of compartment syndrome has been made. Medical management at this time is restricted to an adjunctive role supplemental to fasciotomy. The therapeutic effects of mannitol have been investigated in animal studies (Better et al, 1991). Case studies have reported success at averting fasciotomy in the context of clinically diagnosed compartment syndrome (Daniels et al, 1998). Hyperbaric oxygen is thought to reduce edema within the affected compartment by oxygen-induced vasoconstriction, while maintaining oxygen perfusion at lower perfusion pressure. Although this may be an effective adjunct to fasciotomy, it has limited availability. In a recent review of the literature, Wattel et al (1998) found that hyperbaric oxygen is effective in improving wound healing, reducing amputation rate, and lowering surgical procedure rate. Tissue ultrafiltration has been used to reduce intracompartmental pressure by reducing fluid volume (Odland et al, 2005). Although clinical trials are needed, medical techniques may prove to be an effective in patients presenting with an impending compartment syndrome.

### 1.3.1 Anatomy of the Leg

The leg is divided into four compartments: anterior, lateral, posterior deep and superficial (Figure 1.1). The anterior compartment contains the extensor muscles of the foot and ankle. The compartment is bounded medially by the extensor surface of the tibia, laterally by the intermuscular septum, and posteriorly by the extensor surface of the fibula and the interosseous membrane. The anterior compartment is completely enclosed by the deep fascia of the leg. The lateral compartment contains the peroneal muscles, which evert the foot. Its medial border is the fibula, while the intermuscular septum surrounds this compartment both anteriorly and posteriorly. The posterior compartments house the flexors of the foot and ankle. Both deep and superficial groups of muscle are included, separated by a fascial layer. The posterior compartments are separated from the other compartments in the leg by a dense fibro-osseous complex. The fibula and the posterior intermuscular septum divide the posterior compartments from the lateral compartment. Anteriorly the posterior compartments are

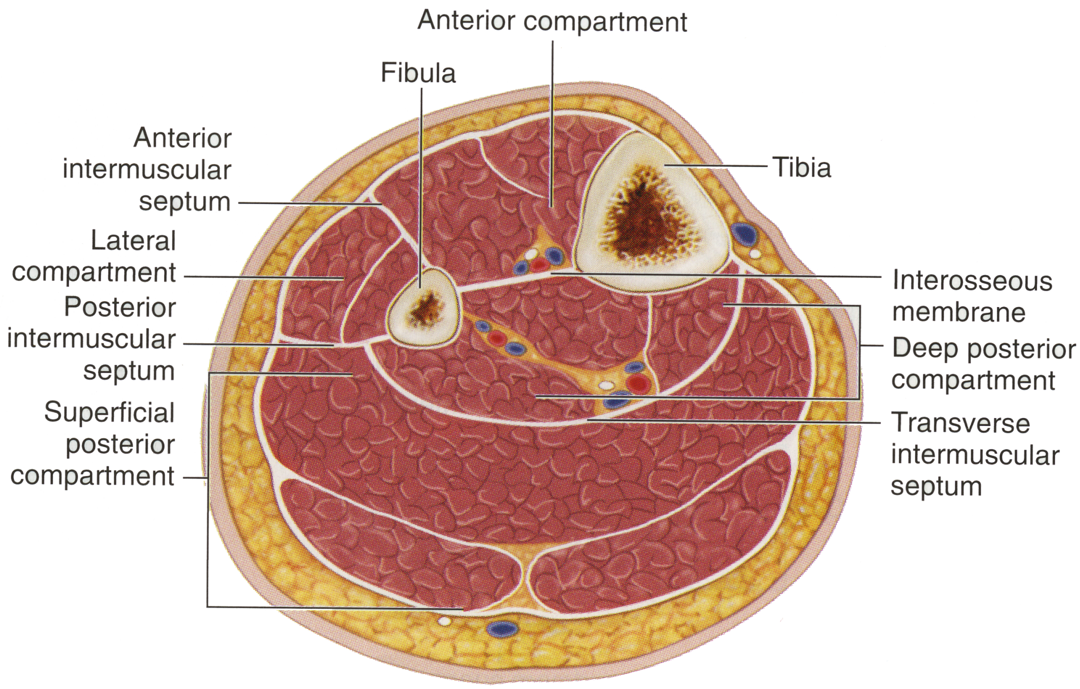


**Figure 1.1: Cross-section of the lower leg**. The lower leg consists of two bones (tibia and fibula) and 10 major muscles (tibialis anterior, extensor digitorum longus, extensor hallucis longus, peroneus longus, peroneus brevis, soleus, gascrocnemius, tibialis posterior, flexor digitorum longus, flexor hallucis longus). The muscles are separated by fascia into four compartments (anterior, lateral, superficial posterior and deep posterior).

*Reproduced with permission from Lawendy and Sanders (2010).*

separated from the extensor compartment by the interosseous membrane and the posterior surface of the tibia (Lawendy and Sanders, 2010).

### 1.3.2 Fasciotomy

The surgical techniques for complete fascial release have been well-studied in the leg. Three techniques are most commonly described: two-incision fasciotomy, single incision perifibular fasciotomy, and fibulectomy (Lawendy and Sanders, 2010). The preferred method is the double incision technique, which allows for adequate visualization of all compartments, assessment of muscle viability, and sufficient surgical control to avoid neurovascular structures (for the detailed description of the surgical procedures, see Appendix D). The single incision four-compartment fasciotomy without fibulectomy is safe and can be useful in cases where soft tissue trauma or contamination is of concern, including situations in which only a single vessel perfuses the leg, or when flap coverage may be necessary. Kelly and Whiteside (1967) described a four-compartment release with fibulectomy performed through one lateral incision. This technique takes advantage of the fascial anatomy as all the fascial membranes insert onto the fibula. However, this method is technically challenging, may place the peroneal vessels at risk, and sacrifices the fibula, which is usually unnecessary. Both the double and single incision technique are sufficiently effective at decreasing intracompartmental pressure (Mubarak and Owen, 1977; Vitale et al, 1988).

### 1.3.3 Complications of Fasciotomy

While fasciotomy is the gold standard treatment for compartment syndrome, independent of its etiology, it is a procedure that has significant risks, affecting patient morbidity and mortality. In an attempt to understand the long-term morbidity associated with fasciotomy wounds, Fitzgerald et al (2000) retrospectively assessed complications found in patients that had undergone fasciotomy over an 8-year period. Fasciotomies involved both upper and lower extremities and were all performed for the treatment of CS. They found that 77% had neurologic symptoms, such as altered sensation of wounds, and one in every ten patients had chronic pain associated with their fasciotomy wounds. Other frequent complications included dry skin, pruritus, and discolouration of wounds. Chronic swelling, tethering of tendons and scars, recurrent ulceration, and muscle herniation were also reported. The effect on patient's life was also detrimental, as 28% changed hobbies and 12% changed occupation secondary to the complications of their fasciotomy. More than 20% of patients covered their scars due to the aesthetic appearance of the wound (Fitzgerald et al. 2000).

The scars caused by fasciotomies are not inconsequential to patients or their functional outcomes. Giannoudis et al (2002) examined health related quality of life outcomes as it relates to CS, this study found that patients who find their wounds aesthetically unappealing reported significantly poorer health related quality of life as compared to patients who had no problem with the appearance of the wound. Rate of wound closure and need for skin graft were also associated with increased pain and discomfort (Giannoudis et al. 2002).

Timing of fasciotomy is critical to outcome in acute CS, as delay to treatment is associated with increased complications and negative outcomes (Finkelstein et al. 1996). Williams et al (1997) reported on the effect of delay to fasciotomy of greater than 12 hours, and found that patients treated early had 7.3% rate of infection versus 28% for delayed treatment. In one of the largest series in the literature reporting on outcomes of fasciotomies, Ritenour et al (2008) found significant complications secondary to fasciotomy revision surgery in military combat casualties. In their retrospective study of 336 patients who underwent 643 fasciotomies, they found an association between fasciotomy revision and increased rates of muscle excision, as well as a three-fold increase in mortality. Furthermore, delayed fasciotomies doubled the rate of amputation and increased the mortality rate fourfold, as compared with patients who underwent early fasciotomies (Ritenour et al. 2008). Despite being the most effective treatment for CS, fasciotomies are not trivial to patient outcomes, and techniques, timing, and alternate therapies need to be further investigated.

## 1.4 COMPARTMENT PRESSURE MONITORING

Measurement of intracompartmental pressure (ICP) is a valuable tool for providing objective criteria for the diagnosis of acute CS (Hargens and Ballard, 1995). To ensure accurate ICP measurements, proper technique is crucial. ICP measurements should be taken at the level of the fracture as well as at sites up to 5 cm proximal and distal to injury, to capture the peak ICP value (Heckman et al, 1994). Pressures should also be measured in the other compartments of the affected limb to ensure that a compartment syndrome is not missed.

### 1.4.1 Invasive Pressure Monitoring

Techniques for measuring ICP include needle manometer, wick catheter, slit catheter and electronic transducer-tipped catheters (Hargens and Ballard, 1995). The needle manometer consists of a 20 cc syringe full of air, attached to a column that contains both air and saline. The ICP is the pressure required to flatten the meniscus between the saline and the air (Whitesides et al, 1975). Matsen et al. (1980) modified this technique to measure ICP as the amount of pressure required to overcome the pressure in the circuit and infuse a small amount of saline into the compartment (Whitesides et al, 1975). While this technique is simple and low-cost, it is the least reliable, as the needle can easily be occluded (Moed and Thorderson, 1993).

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

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

Transducer-tipped catheters designed with the transducer housed in the catheter tip have improved the accuracy of compartment measurements (Hargens and Ballard, 1995). An early variant of this was the solid-state transducer intracompartmental catheter (STIC). While this system offers increased accuracy compared to the slit and wick catheters, it still relies on an infusion for pressure measurement (McDermott et al, 1984). Newer electronic transducer-tipped systems do not rely on an infusion. Electronic techniques are independent of limb position and the height of the pressure transducer and do not require calibration (Mubarak et al, 1976; Mubarak et al, 1978; McDermott et al, 1984; Moed and Thorderson, 1993; Willy et al, 1999). Disadvantages of these devices are their cost and difficulty with re-sterilization.

The indications for intracompartmental pressure (ICP) measurement, as described by McQueen in 1996, include the following:

- Unconscious patients (Gelberman et al. 1981; Hargens et al, 1989; Schwartz et al, 1989);
- Difficult-to-assess patients, such as young children (Whitesides et al, 1975);
- Patients with equivocal signs and symptoms (Gelberman et al, 1981), especially when accompanied by nerve injury (Whitesides et al, 1975; Wright et al, 1989);
- Patients with multiple injuries (Schwartz et al, 1989).

To avoid missed compartment syndromes, McQueen et al. (2000) later expanded the indications for ICP monitoring to include all tibial diaphyseal fractures (especially those in young men), high energy distal radial and forearm diaphyseal fractures in young patients, high energy fractures of the tibial metaphysis, and soft tissue injury or bleeding diathesis.

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

While ICP monitoring is utilized in the diagnosis of acute CS, a specific pressure threshold at which fasciotomy is necessary remains controversial. The threshold ICP for decompression has been listed as 30 mmHg (Mubarak et al, 1978), 40 mmHg (Schwartz et al, 1989) and 45 mmHg (Matsen et al, 1980). Whitesides et al. (1975) proposed the idea that ∆P or differential pressure is indicative of tissue ischemia. He suggested that tissue ischemia began when the difference between ICP and diastolic pressure was 20 mm Hg (Whitesides and Heckman 1996). McQueen et al. (1996) recommended that the threshold ∆P be 30 mmHg, based on the retrospective observation that this value lead to no apparent missed cases of acute compartment syndrome. Many trauma surgeons prefer ∆P to the use of an absolute ICP threshold. The advantages of a differential pressure threshold include better utility in hypotensive trauma patients and a lower overall fasciotomy rate, compared to an absolute pressure threshold (Matsen et al, 1980; McQueen and Court-Brown, 1996).

### 1.4.2 Non-invasive Pressure Monitoring

Near infrared spectroscopy (NIR) has been examined as a non-invasive measurement of ischemia in compartment syndrome (Gentilello et al, 2001). NIR works by transmitting light that passes through the skin but is absorbed by hemoglobin. The amount of light absorbed by hemoglobin is dependent upon the redox state of the iron molecule in hemoglobin, such that NIR can continuously measure tissue oxygenation (Arbabi et al, 1999). Infrared imaging has also been proposed as an additional diagnostic tool. Katz et al. (2008) found that temperature differences between the thigh and the foot showed a unique pattern in individuals with acute CS. Despite the promise of both NIR and infrared imaging, further research is needed prior to routine clinical use (Arbabi et al, 1999; Katz et al, 2008).

## 1.5 A HISTORY OF OUR UNDERSTANDING OF CS

### 1.5.1 Pathophysiology: Compromised Arterial Inflow and Ischemia

The impact of elevated intra-compartment pressures and their clinical sequelae have long been documented in the surgical literature. Richard von

Volkmann (1881), a German poet and surgeon, was the first to recognize the consequences of compartment syndrome following traumatic injury. In a clinical case report, he noted deformities of the hand and wrist following supracondylar fractures of the distal humerus. Volkmann believed that these contractures were related to (and always preceded by) the application of tight bandages to the injured limb. Volkmann suggested that limb paralysis, followed by ischemic contracture, was secondary to the interruption in arterial blood supply; however, the precise cause of ischemia remained open for debate for decades to come (von Volkmann, 1881).

In 1884, Leser studied compartment syndrome experimentally, using an animal model, by tightly bandaging limbs. Commenting on Leser's contribution, Sayre (1908) states:

*"If pressure is continued for six hours or more the muscle substance rapidly degenerates, a condition of rigor mortis sets in and we may have gangrene and cutaneous blebs. If the pressure is relieved there is marked congestion of the muscles, effusion from the vessels into the muscle substance, and a myositis is set up which later on transforms the muscle wholly or in part into a fibrous cord."* Sayre (1908).

Leser believed this was primarily an insult to the muscle. He brought attention in the literature to ischemic contracture as a "condition", and described its clinical features (Leser, 1884). He supported Volkmann's idea of an ischemic insult to muscle tissue, but did not propose a pathophysiological mechanism. In 1900, Bernays published the pathologic description of ischemic muscle found in CS. He observed variability in necrosis of the involved muscle, and noted that the resultant ischemic contracture may range from a minimal involvement to complete necrosis (Bernays, 1900).

### 1.5.2 Pathophysiology: Nerve Involvement

Replicating experimental studies completed by Leser (1884), Hildebrand (1906) further defined the pathologic findings in CS. In his study, he found that experimental CS led to atrophy and fatty degeneration within muscle fibers. He believed that damage to myocytes was paramount to the injury, but brought attention to nerve involvement as part of the pathophysiology of ischemic contracture (Hildebrand, 1906). Hildebrand was the first person to coin the term 'Volkmann's contracture'. Thomas, a neurologist from Boston, reviewed the entirety of the medical literature regarding Volkmann's ischemic contracture. Cases were presented in a table format, whereby demographic data, mechanism of injury, presence of pulse, treatment, sensory findings, atrophy, contracture, trophic changes, scaring, paralysis, treatment and function were all annotated. He essentially created a complete database of clinical and pathologic findings, which allowed him to identify trends in clinical presentation of CS (Thomas, 1909). Thomas' observations developed a very systematic clinical description of CS:

*"The condition is one in which after a fracture, usually of the humerus very near the elbow-joint, or of the forearm, and after the application of fixation by one or another method, sometimes with tight bandaging, but by no means invariably so, and at times where there has been no fracture and no bandaging, there comes on usually within a short time swelling and blueness of the extremity with more or less pain, and within a varying time when the apparatus is removed, or within a short time after this, there is a swelling of the muscles more marked in the flexors, … The muscles are hard and much more dense than normal, and often there is a pressure slough or scar which may or may not be adherent to the deeper tissues."* (Thomas, 1909).

Thomas' categorical assessment of all 107 cases available in the world literature led him to believe that 61 cases could only be produced by injury to nerves of the affected extremity. Commenting on cases where flaccid paralysis occurred without contracture of the paralyzed muscle, Thomas felt that neural tissue involvement was paramount to the understanding of the clinical presentation:

*"It is evident in cases where the injury is in the forearm or to the humerus that this condition can be due only to injury of the nerves of the arm at the time of the injury or subsequently."* (Thomas 1909)

Thomas' findings were supported by the fact the deformity in Volkmann's ischemic contracture was a claw hand, which bore great resemblance to the *Main-en-grife* often caused by nerve damage to the upper extremity. However, the observed deformity in Volkmann's contracture was seen far more rapidly than what was observed with nerve compromise.

### 1.5.3 Pathophysiology: Venous Obstruction

In contrast to the idea of diminished arterial inflow and/or paralysis of involved nerves within effected compartments, Murphy (1914) drew attention to the venous circulation. By that time, it had been well established in the literature that soft tissue swelling was a clinical feature of the injured limb. Murphy believed that a downstream obstruction in the venous circulation was the cause of the observed clinic presentation and important to the pathophysiological understanding of CS. Murphy states:

*“We believe that the injury to the artery plays little or no role in the destruction of the protoplasm of the muscle cells. It is pressure which causes the cell destruction… The obstruction is in the veins and not the artery, as the great edema always indicates obstruction to return circulation and not to arterial circulation. The radial pulsations are present throughout the entire course of some of the cases.”* (Murphy, 1914).

Although current understanding of the pathophysiology of CS does not corroborate the concept of venous obstruction as the hallmark of its pathophysiology, Murphy recognized two essential features of CS: firstly, that elevated ICP leads to myonecrosis, and that the maintenance of the arterial pulse is a feature of the clinical presentation. His contribution to treatment is even more profound, as he suggested prophylactically splitting the deep fascia to relieve the venous obstruction:

*“If the cyanosis still continues with the forearm extended and elevated, the fascia on the antero-ulner side of the forearm should be split for a distance of 3 to 6 inches subcutaneously. This can be done with a tenotome without any danger of compounding the injury and should be done within twenty-four hours…”* (Murphy, 1914).

This was the first account, in the English literature, of fasciotomy as treatment for CS, several years after Bardenheuer (1911), who suggested aponeurectomy as a method of treating CS in the German literature. Bardenheuer also believed that venous obstruction resulting in venous stasis was causing the observed tissue damage, suggesting that retention of toxic metabolites in the tissue was the reason for muscle fiber degeneration (Bardenheuer, 1911).

Both Bardenheuer and Murphy supported the use of an aponeurectomy (Bardenheuer, 1911; Murphy, 1914). This was a radical procedure in the face of the available treatment options, which were aimed at treating complications of the fibrosis and contracture. Treatments included progressive splinting, shortening bones to match the length of contracted tendons, and lengthening tendons (Rowlands and Lond, 1905). At the time, prophylactic treatment was advocated by safely applying splints. Sayre (1908) felt that splints, when applied to fractures, should be split and patients seen every 4 hours to ensure no constriction to the effected extremity. Murphy (1914), however, presented a surgical solution that would prove to alter the course of the disease. He also stressed urgency in terms of preservation of function and patient outcomes. Murphy’s treatment is the mainstay of surgical practice today.

In the 1920s, Brooks (1922) and Jepson (1926) both supported the idea of venous obstruction as an important pathophysiologic cause of muscle damage in compartment syndrome. Brooks investigated both venous occlusion and arterial obstruction in a series of experiments aimed at mechanistically understanding Volkmann’s contracture (Brooks 1922). In comparing the effect of arterial occlusion to venous obstruction, Brooks believed that the clinical presentation of Volkmann’s contracture was better explained by venous obstruction as the pathologic lesion. He also believed that the re-establishment of circulation to compromised tissue could inevitably contribute to the pathology; this is in full agreement with modern understanding of ischemia reperfusion.

Jepson supported Brooks assertions. He designed a series of experiments as part of his Master’s Thesis, aimed at characterizing the definitive lesion causing “ischemic contracture”. The focus of his work was an experimental canine model that applied a series of injurious conditions to the canine limbs, attempting to reproduce Volkmann's ischemic contracture (Jepson 1926). In his first set of experiments, he utilized splints, casts and bandages to reproduce the deformity. He then utilized and Esmarch rubber bandage applied above the knee, ranging from ninety minutes to twenty-four hours, essentially producing an ischemia–reperfusion injury and reversible deformity. His final experiment was a surgical ligation of the femoral vein as a control, measured against ligation with fasciotomy, and 8 hours of Esmarch ischemia followed by fasciotomy and venous drainage tubes (Jepson 1926). Jepson noted his findings following 8 hours of ischemia:

*"At the end of this time there was considerable oedema and other signs of a sluggish circulation, and the toes were contracted. Six hours later the wound was opened and the blood and serum were evacuated… The following day the swelling had gone down markedly, and four days later the dog was walking normally. This was in marked contrast to the condition of the control animals in which drainage had not been instituted.* ***The experiment was repeated often enough to bring out the fact that the intrinsic pressure is a factor, which must be dealt with in this condition.****"*

Jepson was able to isolate a key feature regarding the pathophysiology of compartment syndrome: elevated ICP was paramount to the injury process and in fact had proven that surgical decompression was capable of restoring the function of the limb. This concept of elevated pressure driving the rapid and significant injury observed in CS remains at the core of modern understanding. Jepson, however, felt that the venous obstruction was the cause of the increased pressure and, although defining the role of the fasciotomy, he believed the drainage of the venous circulation was critical. Explaining his observations Jepson states:

*"The results of these experiments would seem to indicate that the contracture deformity is due to a combination of factors, the most important of which is the impairment of the venous flow, extravasation of blood and serum, and swelling of the tissues with consequent pressure on the blood-vessels and nerves in the involved area. If this is true, early drainage would be of value."* (Jepson 1926).

Other contemporaries supported Jepson's theory. Jones (1928), publishing on elbow injuries in children in the British Medical Journal, writes:

*"It cannot be insisted upon too often that the calamity may be due to pressure from within."* (Jones, 1928)

Jones reinforced surgical fasciotomy as necessary, to evacuate clot or "relieve pressure" (Jones, 1928). The inaugural insult, believed to be an increased ICP, caused many surgeons to treat patients at the time with a partial fasciotomy (enough to relieve the venous obstruction and evacuate clots) and yielded good results, despite the fact that the fundamental understanding at the time was incomplete. In 1937, Wertheimer and Dechaume injected charcoal and gelatin into the venous system, which resulted in venous obstruction and capillary occlusion. With this insult to the venous system and, more importantly, the microvascular system these authors demonstrated similar histopathological features as those seen in Volkmann's contracture (Wertheimer and Dechaume, 1937). Other authors, however, discarded the idea of pressure-induced ischemia as the driving cause of this pathology, and turned to the arterial side as the primary cause of ischemia and injury.

### 1.5.4 Pathophysiology: Arterial Injury?

Griffiths delivered a Hunterian lecture to the Royal College of Surgeons in England in 1940, attempting to restore Richard Van Volkmann's notion that ischemic contracture and paralysis was primarily an arterial vascular insult. His belief was that Volkmann's contracture was "due to arterial injury with reflex spasm of the collateral circulation" (Griffiths, 1940). He believed that the observed venous obstruction or rise in pressure was secondary to the arterial spasm, and should not be viewed as the primary pathology; here, Griffiths was attempting to depart from the mainstream paradigm of understanding.

Griffiths advocated that the treatment should be aimed at exploring the artery in an impending Volkmann's contracture, which unknowingly led to the secondary application of fasciotomy as a necessity of the surgical dissection. Once again, fasciotomy improved patient outcomes and Griffiths, as well as others (Kinmonth, 1952), took this evidence as support for their theory. Interestingly, Griffiths cited the injection studies done by Wertheimer and Dechaume (1937) that experimentally resulted in increased venous pressure causing microvascular occlusion to support his findings of "traumatic arterial spasm". Griffiths continued to publish on his theory for 2 decades in the surgical literature. Although it would later be disproven and fall out of favour, his contribution in attempting to understand the pathophysiology led to significant understanding to the clinical presentation of CS. Griffiths categorically described presenting signs and symptoms defining the early diagnostic criteria of compartment syndrome: painful onset, pain with passive extension, pallor, and puffiness, which remain an essential part of clinical diagnostic criteria.

### 1.5.4.1 World Wars: Lessons Learned

In 1941, the concentrated bombing raids over London, known as the London Blitz, lead to the discovery of a crush syndrome causing traumatic rhabdomyolysis and renal failure secondary to reperfusion syndrome. Bywaters and Beall (1941) reported in the British Medical Journal on 4 cases of air raid casualties that had similar clinical presentations. Patients had crushed extremities with compartment syndrome, after being buried for several hours. Patients were stable on admission to hospital; shortly after they had decreased urine output, systemic deterioration, multi-organ failure and eventually died, even when limbs had been amputated (Bywaters and Beall, 1941). It became known as the “crush syndrome”, defining the clinical findings associated with severe reperfusion syndrome. This understanding lead to improved medical management in cases of severe CS, but further shifted surgical thinking away from pressure and venous stasis as driving the CS injury back in the direction of ischemia and arterial injury.

Following World War II, Sir Reginald Watson-Jones (1952) supported the notion of arterial injury and spasm as the cause of ischemic contracture. In order to treat the spasm and arterial injury, another line of research emerged, attempting to prevent this phenomenon by means of sympathetic blockade (Foisie, 1942). In his publication in the New England Journal of Medicine, Foisie argued that when Volkmann's ischemic contracture is explored, no arterial lesion is found and hence, a spasm upstream must exist. He discredited the theory of venous occlusion and elevated pressure, stating that incisions in the deep fascia aimed at relieving the pressure were only treating the downstream effect of the proximal arterial injury (Foisie, 1942). Hence, Foisie supported, with many of his contemporaries, the idea that arterial spasm was a reflex mediated through the autonomic nervous system and could be appropriately interrupted by sympathetic blockade, which would, with time, prove to be very ineffectual at treating CS. This diversion from the true underlying pathophysiology caused debate in the literature. Seddon (1966) provided evidence that challenged this theory. Based on his observations, he noted that all cases of ischemic contracture exhibited early massive swelling, with nearly half of patients presenting with palpable peripheral pulses (Seddon, 1966). In describing the early clinical signs of impending compartment syndrome, Seddon wrote:

*"Neither pain, pallor, cyanosis, pulselessness, paralysis, nor contracture was noted in over half the cases that were carefully documented. The most reliable sign of all is painful limitation of extension of the fingers."* (Seddon 1966).

Seddon further emphasized the importance of prophylactic fasciotomy as a treatment for CS:

*"The first and most essential step is to recognise the early signs of ischaemic damage. Incision of the deep fascia may then save the threatened underlying muscle, though it may also be necessary to seek for and evacuate a haematoma beneath the muscle."* (Seddon, 1966)

#### 1.5.4.2 Lower Extremity – Arterial Spasm or Elevated Pressure?

The early surgical literature had almost exclusively focused on upper extremity injuries in defining the understanding of Volkmann's contracture. Few case reports scattered through the literature regarding lower extremity involvement had been documented. Hughes (1948), and later Mavor (1956) presented clinical cases of atraumatic onset of ischemic necrosis in the lower extremity secondary to strenuous exercise. Hughes (1948), in his discussion, noted:

*"Prolonged activity of a muscle may increase its weight by 20 per cent, the bulk being increased by the retention of excessive fluid within the tissue spaces. If for this, or any other reason, tension within the fascial compartment rises, the circulation within the intramuscular vascular networks must be embarrassed."* (Hughes, 1948).

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

Blandy and Fuller (1957) reviewed the available literature regarding what they termed the "march gangrene". They presented 3 of their own cases and reviewed 16 others that were available, of young injured service men. All cases were male who were subjected to prolonged activity, few had a traumatic limb injury but all developed compartment syndrome. They detailed symptoms, histology, outcomes, and discussed the importance of fasciotomy. They noted that with a rise in fluid pressure in the fascial compartment

*"even to a level well below the systemic arterial pressure, it is capable of producing muscular ischaemia"* (Blandy and Fuller, 1957).

Pressure-induced ischemia, although not widely accepted, was beginning to be recognized as a key etiological factor in causing compartment syndrome. Blandy and Fuller provided a detailed account of the benefit of fascial release. Emphasis was now placed on the role of fascial decompression in relieving symptoms. Benjamin (1957), in a case series aimed at supporting the theory of arterial spasm, noted that *"patients suffering from limb injuries with vascular complications suggest that the presence of oedema alone in a neighbouring fascial compartment may be an important etiological factor."* (Benjamin 1957).

The theory of arterial spasm causing Volkmann's ischemia still dominated the understanding in the surgical literature. Despite this bias, Benjamin's observations, as a senior registrar, pointed to a very significant finding that pressure in, or around, the compartment is part of the pathophysiology of CS. Although Benjamin thought that a rise in pressure within the compartment was what had caused the arterial spasm, he felt that increased pressure was critical to understanding this injury. Griffiths, who for more than 20 years tried to solidify the idea of arterial spasm, rejected this evidence, calling it an attractive theory,

*"which may often be accepted because they cannot be rejected. Surgery has been led astray too often by just this fallacy".* (Griffiths, 1957).

Ironically, Griffiths' theory would soon fall out of favour – an accepted theory that could not be rejected. A growing body of evidence was now pointing to increased ICP as an important part of the pathophysiology of compartment syndrome. Reneman (1968), while working in the Military Hospital at Utrecht, published on the compartment syndrome in the lower extremity. He was able to distinguish both the acute and chronic form in its presentation. Reneman reinforced the idea that vascular disturbances led to increased intra-compartmental pressure requiring acute fascial decompression, to avert muscular necrosis. Not only did he emphasize the importance of fasciotomy, but also discussed the surgical exposure of the muscles in the anterior and lateral compartments, as well as incision in the fascia overlying other compartments.

#### 1.5.4.3 Modern Understanding of Compartment Syndrome

The modern understanding of CS was based on several very important developments. The first, CS was not isolated to the upper extremity, as had been suggested by the early literature. The second, increased pressure was the critical feature in the pathophysiology. The third, fasciotomy was an effective treatment to this very significant disease. Matsen put together many of these in his paper on the unified concept of compartment syndrome (Matsen, 1975). He reiterated that increased tissue pressure, caused by either increased compartment volume or a decrease in the myofascial compartment size, was the hallmark of this disease. The cause of increased pressure (tight cast, splint, edema, vascular reperfusion), although important clinically, was not as relevant as recognizing the fact that the resultant increased pressure was the cause of the ischemia. Hence relieving the pressure was paramount to treatment.

This notion of relieving the pressure is common knowledge in any medical school today. However, Matsen (1975) significantly altered the discourse surrounding pathophysiology and treatment of CS. The main priority now was the treatment; furthermore, the research was now focused towards the measurement of pressure, in defining thresholds for treatment. The role of fasciotomy became a crucial focus of treatment and research in years to come. The contribution of Whitesides et al (1975) was also significant, as they helped to define a methodology for measuring intracompartmental tissue pressure, and suggested thresholds for fasciotomy. One year later, Mubarak et al (1976) published a means of continuous monitoring using a wick catheter. Matsen et al (1977) described an infusion technique for monitoring compartments. Rorabeck (1978) measured pre-fasciotomy compartment pressures in the lower extremity, and found pressures up to 50mmHg in CS patients. While pressure thresholds and techniques of measurement and diagnostics were being defined, understanding the insult at the level of microcirculation was simultaneously developing.

### 1.6 MICROCIRCULATION AND CS

Three theories that attempted to explain the pathophysiology of microvascular dysfunction and ischemia in CS as they relate to tissue pressure emerged: microvascular occlusion theory, critical closing pressure theory and arteriovenous gradient theory.

### 1.6.1 Microvascular Occlusion Theory

According to the microvascular occlusion theory, CS results from the occlusion of capillaries in response to the increase in the absolute compartment pressure (Hargens et al, 1978). It hypothesizes that, in response to an increase in the tissue pressure above normal resting capillary pressure, there is a concomitant reduction in capillary blood flow leading to an ischemic state in the muscle, and a subsequent tissue necrosis (Hargens, 1978). Normal resting capillary pressure measured with the capillary at heart level ranges from 10.5 to 22.5 mmHg (Shore, 2000). Hence, this theory postulates that a modest rise in pressure can result in a critical insult to the patency of the capillaries causing microvascular compromise. Hartsock et al (1998) designed a series of experiments to test this: using rodent cremasteric muscle subjected to sequential elevation in pressure with direct observation of blood flow they observed diminished capillary flow with elevation of pressure. However, they also noted:

*"In our study, we did not observe collapse of any vessels, even when the compartment pressure was raised to a level that caused complete arrest of capillary blood flow… As external pressure was increased on the vessels, the intraluminal pressure may have increased to prevent collapse of the vessel."* (Hartsock et al, 1998)

This study essentially discredited, in part, the microvascular occlusion theory.

### 1.6.2 Critical Closing Pressure Theory

The critical closing pressure theory assumes that there is a critical pressure at which active closure of arterioles will occur, secondary to a drop in the transmural pressure (i.e. the difference between intravascular pressure and tissue pressure) (Burton, 1951). Since the arterioles are small, the walls experience high tension and hence require high arteriolar tissue pressure gradient to maintain patency (Burton, 1951). The theory predicts that compartment syndrome is caused by extreme elevation of pressure or a physiologically significant reduction in the arteriolar tissue pressure gradient leading to arteriolar collapse (Ashton, 1975). This would essentially render the tissue ischemic. However, a study aimed at assessing the response of arterioles, capillaries and post-capillary venules of various diameters to graded pressure elevation, using a skinfold chamber in Syrian golden hamsters, demonstrated no signs of arteriolar spasm or collapse (Vollmar et al, 1999).

### 1.6.3 Arterio-venous Gradient Theory

The arterio-venous (AV) gradient theory predicts that an increase in tissue pressure, as seen in CS, will reduce the AV pressure gradient, resulting in a net decrease in blood flow (Matsen et al, 1980). This suggests that an increase in pressure results in a rise in the intraluminal venous pressure. Flow from high-pressure arteries to low-pressure veins depends on the maintenance of this pressure gradient. Hence, elevation in ICP diminishes the gradient between vessels, and thus the AV gradient falls. With the decreased gradient, skeletal muscle blood flow is reduced and muscle damage occurs (Matsen et al, 1980). Furthermore, the decrease in AV gradient not only impairs the delivery of oxygenated blood, but also decreases the rate at which venous blood is cleared, leading to a cascade of fluid extrusion into the interstitium. The result is increased edema within a closed space and hence elevation in the compartment pressure (Matsen and Krugmire 1978).

Vollmar et al (1999) lends modest support to this theory. In an experimental model examining the response of the microcirculation to graded external pressure changes in hamster striated muscle, Vollmar found that minimal increase in external pressure was able to halt the flow though capillaries and venules while arteriolar blood flow was maintained. Venules responded to pressure with decreased diameter and flow; relief of the external pressure and thus restoration of the pressure gradient was able to restore blood flow. The study demonstrated that a pressure gradient is needed for flow from capillaries to venules, which show the vulnerability of the microvasculature to pressure fluctuation. It also pointed to the idea of a yield stress in the microvascular response, since an increase in perfusion pressure gradient was required to restart blood flow when it had been interrupted (Vollmar et al, 1999).

The AV gradient theory does provide some guidance to the possible mechanisms of how elevated tissue pressure disrupts blood flow, but it relies on certain assumptions, particularly that the microvasculature passively responds to pressure. It does not account for the local adaptive mechanism that serve to protect the microvasculature under ischemic stress, such as changes in vasodilation, shunting of blood, changes in endothelium structure and function, as well as the role inflammation plays in this process (Gourgiotis et al, 2007).

## 1.7 THE AIM OF THIS THESIS

The high metabolic demand of skeletal muscle makes it the most vulnerable tissue in a limb affected by acute CS. Both the magnitude and duration of increased compartment pressure have major effects on muscle viability (Hargens et al, 1978; Heckman et al, 1993; Matava et al, 1994). The reduced local blood flow to skeletal muscle causes ischemia and eventually leads to cell death. Despite the volume of work dedicated to understanding the mechanisms by which CS can become a limb- or life-threatening disease, there are many questions that still remain unanswered. Starling, a British physiologist, already demonstrated in 1896 that, under normal conditions, the fluid filtering outward through the arterial capillaries is in a state of equilibrium with the fluid returning to the circulation through absorption of the post capillary venules (Starling 1896). The role of the microcirculation in understanding the pathophysiology of CS has only been studied in the last 3 decades in the orthopaedic literature, despite the basic science understanding of fluid transport.

The aim of this thesis is to develop a clinically relevant small animal model of CS, in order to study the effect of elevated ICP on capillary perfusion, inflammation and cellular injury. A further focus of the study was to define the role of inflammation in local and systemic CS-induced pathology. It is important to note that for the purpose of this thesis elevated ICP is utilized interchangeably with CS however CS is defined clinically only in humans.

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## CHAPTER 2

### Compartment Syndrome-Induced Microvascular Dysfunction: An Experimental Rodent Model.

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# CHAPTER 2: COMPARTMENT SYNDROME-INDUCED MICROVASCULAR DYSFUNCTION: AN EXPERIMENTAL RODENT MODEL

## 2.1 INTRODUCTION

Acute limb compartment syndrome (CS) is characterized by raised pressure within a closed fascial compartment (Tornetta and Templeman, 1996; Matsen et al, 1980; Mubarak et al, 1978; Whitesides et al, 1975; Matsen, 1975; Rorabeck and Clarke, 1978; Mabee and Bostwick, 1993). Untreated it may lead to tissue necrosis and permanent functional impairment (Whitesides et al, 1975; Heckman et al, 1994; Heckman et al, 1993; McQueen et al, 1996). The clinical sequelae of compartment syndrome, first described by Richard von Volkmann in 1875, relates irreversible contractures of the hand to an ischemic process in the forearm. Volkmann put forward the idea that the pathophysiology of the contracture is caused by arterial insufficiency combined with venous stasis (Jepson, 1926). Despite the breadth of research dedicated to understanding the pathophysiology of CS, the mechanisms causing the tissue and microvascular injury associated with acute compartment syndrome are complex and remain only partly understood. Factors hindering our understanding of CS pathophysiology include limitations in clinical trials due to the severe acuity of CS, absence of a clinically relevant standardized animal model and the difficulty of applying invasive tools to help delineate the pathways that propagate the CS injury at a cellular level.

Intravital video microscopy (IVVM) is a modern technique allowing for the visualization and study of microvascular perfusion (Potter et al, 1993). This technique has previously been used in the study of ischemia-reperfusion, ischemic preconditioning, sepsis and other disease states that may compromise blood flow (Potter et al, 1993; Piper et al, 1996; Badhwar et al, 2004; Badhwar et al, 2003; Forbes et al, 1995). Microvascular perfusion may readily be assessed in vivo using various techniques including positron emission tomography scan, laser Doppler and Intra-vital video microscopy. Many advanced techniques have been published in the literature for assessment of flow under experimental conditions. Here IVVM was employed due to familiarity of the technique in the rodent model.

The purpose of this study was to develop a clinically relevant small animal model of elevated intracompartmental pressure and to employ IVVM in order to study the microvascular and inflammatory response to compartment syndrome.

## 2.2 METHODS

### *2.2.1 Animal Description and Care*

Male Wistar rats utilized for these experiments had access to food and water *ad libitum*. All protocols and experiments were conducted in agreement with the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council, and approved by the institutional Council on Animal Care.

### *2.2.2 Experimental Protocol*

Ten rats (175-250 g) were anesthetized with inhalational isoflurane. Following induction at 5% isoflurane in a 1:1 $O_2$:$N_2$ mixture, anaesthesia was maintained at 2% isoflurane and titrated to maintain general anesthesia. The carotid artery was cannulated for continuous blood pressure monitoring and fluid replacement to maintain a normal mean arterial pressure at 100mmHg**.** Once anesthetized, compartment pressure was elevated by slowly infusing isotonic normal saline via a 24-gauge angiocatheter into the anterior compartment of the left hindlimb for the experimental group. Compartment pressure was raised to 30mmHg and maintained between 30-40mmHg for the duration of the protocol. An electronic compartmental pressure monitoring system (Synthes USA, Paoli PA) was inserted into the anterior and then posterior compartment through a 14-gauge angiocatheter. As the pressure rose within the hindlimb, both the anterior and posterior compartments became isobaric (both anterior and posterior compartment pressures were raised to 30-40mmHg). In order to test the effect of time on capillary perfusion and cellular injury, elevated intracompartmental pressure (EICP) was maintained for 45min (n=5) prior to the release of the EICP via fasciotomy. Control animals (n=5) had all the same preparation, however no saline was infused into the compartment via the catheter and the intracompartmental pressure was held at control levels for the duration of the experiment prior to fasciotomy.

### *2.2.3 Surgical Technique*

The Extensor Digitorum Longus (EDL) muscle was prepared for intravital microscopy, as previously described (Potter et al, 1993; Forbes et al, 1995; Tyml and Budreau, 1991). In brief, the exposure of the EDL muscle began by incising the skin over the posterior aspect of the hindlimb. The underlying biceps femoris muscle was retracted to expose the tibialis anterior and the lateral gastrocnemius muscles. These muscles were divided to expose the EDL. The overlying fascia was incised. A suture ligature was applied around the distal tendon of the EDL. The tendon was then cut from its bony insertion to allow the EDL to be reflected onto the microscope stage with its proximal arterial and venous pedicle intact. Once prepared, animals were placed onto the stage of an inverted microscope (Nikon Diaphot 300) and the EDL was reflected onto a slide moistened with saline. A cover slip was placed on top of the EDL, and all exposed tissues were covered with a plastic film, to isolate the preparation from the atmosphere and to prevent drying. A heat lamp maintained the EDL muscle temperature (32°C) as well as the core temperature (37°C) of the rat. Care was taken to ensure that the time from fasciotomy to the first microscopy recording was no more than 5 minutes.

### *2.2.4 Intravital Microscopy and Video Analysis*

The muscle preparations remained on the microscope with intact circulation post fasciotomy. Five fields of view within the EDL were randomly chosen containing a complete microvascular unit (arteriole, capillary bed, and post capillary venule). These fields were recorded onto video using a 20X objective, for a final magnification of 700X at the monitor. The microscope was connected to a charged-coupled device camera (Dage-MTI VE1000), a time-date generator (WJ-810, Panasonic), and a computer. Appropriate white light illumination was obtained using fiber-optic guides. One-minute video recording of each field of view was obtained post-fasciotomy and stored on the computer for later analysis. An additional 15 seconds was recorded for the nuclear dye staining. This period is limited to reduce exposure to excitation wavelength in order to preserve the fluorochrome contained within the dyes.

### *2.2.5 Perfusion analysis*

An index of compartment syndrome-induced microvascular dysfunction was determined by counting the number of perfused capillaries crossing three equidistant parallel lines drawn on the computer monitor, perpendicular to the capillary axis and expressed as the number of perfused capillaries by red blood cells per millimeter line length (Npc/mm) following our previously validated methodology (Potter et al, 1993; Badhwar et al, 2004; Badhwar et al, 2003; Forbes et al, 1995; Forbes et al, 1996).

### *2.2.6 Injury analysis*

Following fasciotomy, fluorescent vital dyes ethidium bromide (EB, 5μg/mL) and bisbenzimide (BB, 5μg/mL) were added to the saline bath as previously described (Forbes et al, 1996; Potter et al, 1995). The topical use of bisbenzimide and ethidium bromide does not alter microvascular perfusion and is a reliable technique for cellular labelling in the live animal (Potter et al, 1995).

Bisbenzimide, a membrane-permeant dye, stains the nucleus of all cells. Ethidium bromide, a larger molecule, is membrane impermeant, and hence it acts to stain the nuclei of cells with injured (permeable) membranes (Forbes et al, 1995; Potter et al, 1995). Since ethidium bromide labels cells with a range of injury from minor (increased permeability) to cellular death, this technique cannot distinguish injury from lethality. Fluorescent illumination with the appropriate filters for EB (Ex = 482 nm; Em = 610 nm) and BB (Ex = 343 nm and Em = 483 nm) were applied. Tissue injury was examined in 5 fields of view for each group (control and CS) of EICP. Cellular injury was expressed as the ratio of ethidium bromide-labelled nuclei to bisbenzimide-labelled nuclei (EB/BB) (Forbes et al, 1995; Potter et al, 1995).

### *2.2.7 Analysis of Leukocytes*

Leukocyte rolling and adherence were observed in post-capillary venules using the 40x objective (final magnification, 1400X) post fasciotomy. The total number of rolling and adherent leukocytes were measured over 30 seconds and expressed as the number per 1000μm$^2$. An adherent leukocyte was defined as a cell that remained stationary for a minimum of 30 seconds. Measurements of rolling and adhered leukocytes from each of the 5 fields of view were observed in both the control and experimental group.

### *2.2.8 Statistical Analysis*

Statistical analysis consisted of a repeated measures two-way analysis of variance testing (ANOVA) to compare the degree of perfusion, muscle injury, leukocyte rolling and leukocyte adherence with the presence of compartment syndrome. Statistical significance was defined as $p<0.05$.

## 2.3 RESULTS

### *2.3.1 Microvascular Dysfunction*

The effects of increased duration of elevated intracompartmental pressure on capillary flow are shown (Figure 2.1). The capillary profile observed in control animals demonstrates predominately continuous perfusion, representing normal healthy perfusion.

The number of continuously perfused capillaries (mean ± SEM) decreased from 78.4 ± 3.2/mm in the control group to 41.4 ± 6.9/mm at 45-minute compartment syndrome ($p<0.05$). Perfusion shifted from a predominantly continuous profile in the control animals, to an intermittent and non-perfused profile in the compartment syndrome group. There was an increase in the number of intermittently perfused capillaries from 10.4 ± 2.7/mm to 31.4 ± 6.0/mm in the experimental group ($p<0.05$). The number of non-perfused capillaries increased from 12.7 ± 1.4/mm in the control group, to 30.0 ± 6.7/mm following 45 min of EICP (CS group) ($p<0.05$).

### *2.3.2 Inflammation*

Leukocyte number and flow characteristics increased in response to compartment syndrome. The mean number of activated leukocytes increased from 3.6 ± 0.7/30s in the control group to 8.6 ± 1.8/30s in the 45-minute compartment syndrome. Rolling leukocytes observed increased from 2.5 ± 0.7/30s in the control animals to 4.1 ± 0.4/30s in the experimental group. Adherent leukocytes significantly increased from 1.6 ± 0.4/30s in control group to 5.4 ± 0.8/30s in experimental animals ($p < 0.005$) (Figure 2.2).

### *2.3.3 Tissue Injury*

Muscle injury was quantified as the ratio of EB/BB stained nuclei and represents the percent injured cells per field (Figure 2.3). After application of the fluorescent dyes, the control group demonstrated a baseline level of tissue injury (5.0 ± 2.1%), presumed to be secondary to tissue handling during surgical preparation. There was a sudden and significant ($p < 0.05$) increase in the percentage of injured cells (16.3 ± 6.8%) in the CS group.

### *2.3.4 Model Characteristics*

Carotid artery cannulation demonstrated a normotensive model throughout the duration of CS. Mean arterial pressure was maintained within physiologic limits (Figure 2.4).

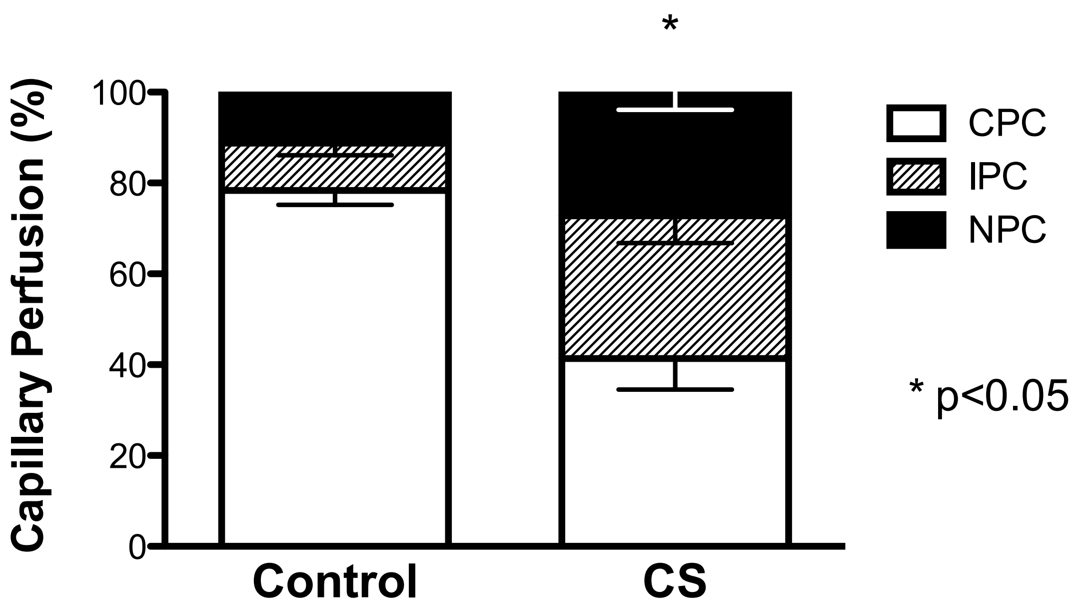


**Figure 2.1**. **The effect of elevated intra-compartmental pressure on microvascular perfusion measured using intravital videomicroscopy**. The graph represents the overall surface microvascular perfusion within the EDL muscle when subject to elevated pressure. Continuous and intermittently perfused capillaries at 45 min are significantly different than controls ($p<0.05$). The number of non-perfused capillaries increased ($p<0.05$) at 45 min as compared to controls. N=5 in each group.

## 2.4 DISCUSSION

We studied the effect of elevated intracompartmental pressure on microvascular perfusion, tissue injury and inflammation in a small animal model of compartment syndrome using intravital video microscopy and nuclear fluorescent dyes. A rodent model was chosen in order to use IVVM and have a reproducible, feasible means of study. Direct imaging of capillaries demonstrated a significant decrease in continuously perfused capillaries ($p<0.05$) with a significant increase in intermittent and non-perfused capillaries ($p<0.05$)(Figure 2.1). This observation characterizes the early microvascular5 response to the compartment syndrome insult. Continuous perfusion is normal physiologic perfusion observed in uninjured microvasculature. The immediate response to CS is a shift to intermittent and non-perfused capillaries. This state of diminished microvascular flow produces a non-nutritive perfusion with compromised gas exchange. Intermittent perfusion demonstrates a marked decrease in red cell flow whereas in non-perfused capillaries red cells have no movement. Post-fasciotomy intermittently perfused capillaries may recover flow; however, non-perfused capillaries do not (Brock et al, 1999; Lawlor et al, 1999).

This microvascular dysfunction is accompanied by a substantial inflammatory response (Figure 2.2). Activated leukocytes are categorized as rolling or adherent, and were measured in the post-capillary venule. Leukocyte adherence was significantly increased ($p< 0.05$) in CS animals as compared to controls. There was no observed difference in leukocyte rolling between groups. At 45 minutes the observed leukocyte adherence reflects a relatively early time

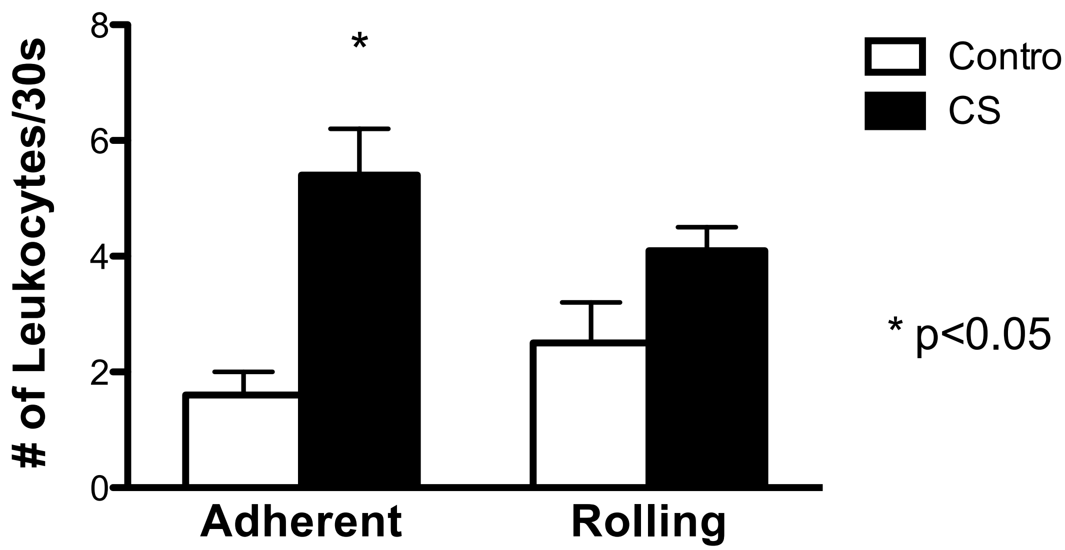


**Figure 2.2**. **Leukocyte rolling and adherence in post-capillary venules observed in control and at 45 min of elevated intra-compartmental pressure.** An early and significant ($p<0.05$) difference in leukocyte adherence is noted. In inflamed tissue, leukocyte rolling leads to a stationary state in which the leukocyte remains firmly attached to the endothelial cell surface without motion. This high-affinity adhesive interaction (leukocyte sticking or adherence) denotes the absence of movement of the leukocyte along the length of the venule.

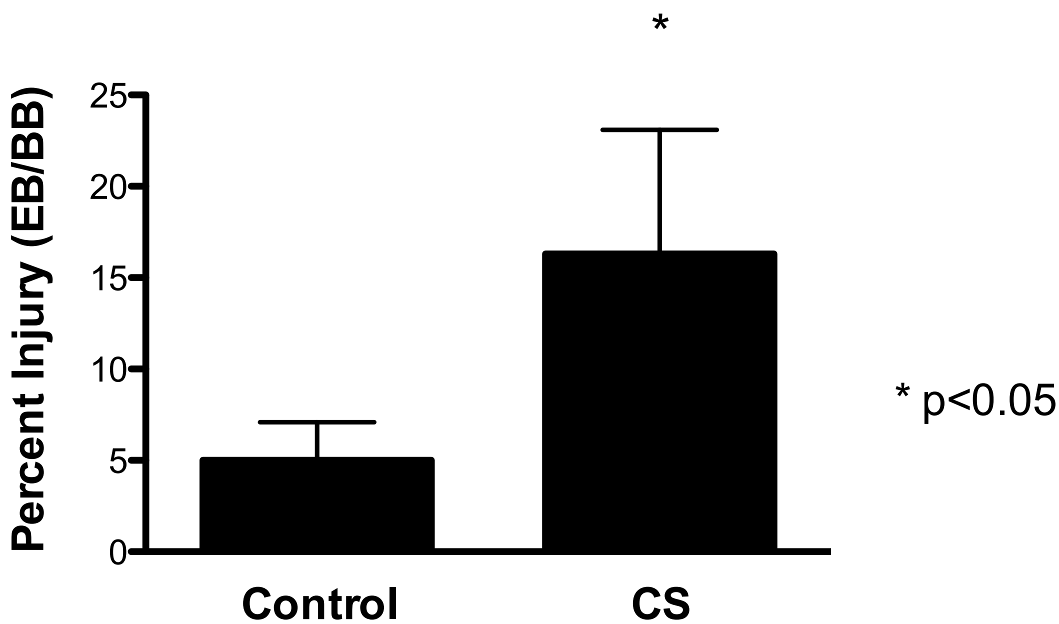


**Figure 2.3**. **The effect of elevated intra-compartmental pressure on parenchymal tissue injury within the EDL muscle.** Sham muscles (0 min) have a low baseline level of parenchymal injury (indexed by the number of ethidium bromide (EB)-labelled nuclei relative to the bisbenzimide (BB)-labelled nuclei). At 45 minutes of CS a significant increase ($p<0.05$) in muscle cellular injury is noted.

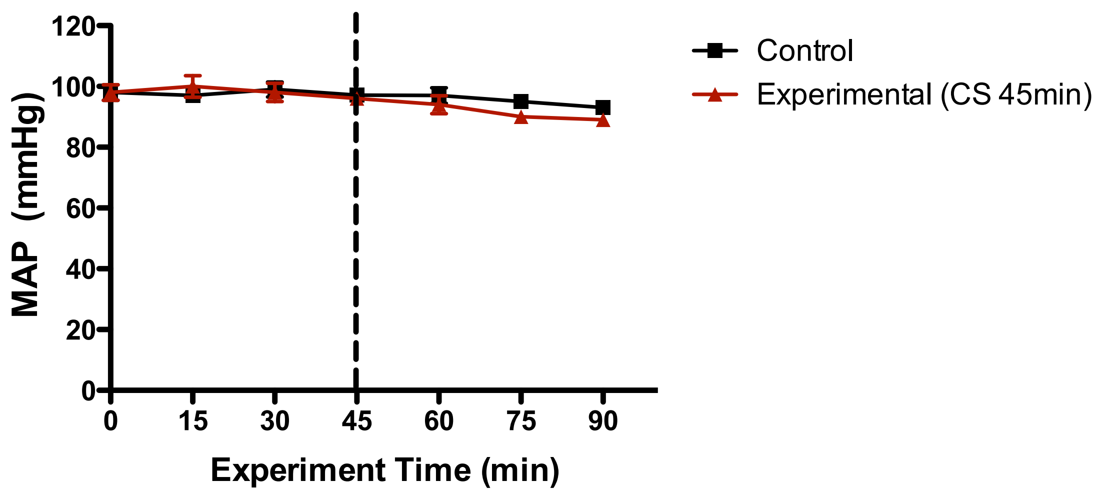


**Figure 2.4**. **Mean arterial pressure of rats**. Mean arterial pressure measurements of control and compartment syndrome animals. The values were not significantly different and remained within physiologic limits.

course for leukocyte accumulation (Forbes et al, 1996; Harris and Skalak, 1996). Leukocyte arrest during rolling is triggered by chemoattractants and is mediated by the interaction of integrins to immunoglobulins expressed by endothelial cells (Campbell et al, 1996; Campbell et al, 1998). The arrest of leukocytes under conditions of flow and the leukocyte recruitment and emigration observed suggests that compartment syndrome induces a pro-inflammatory environment. The inflammatory activity seen in this model of compartment syndrome exceeds the degree of inflammation noted in complete ischemia and early reperfusion models (Forbes et al, 1996). The exact role of inflammation in muscle damage in compartment syndrome is unknown, but may contribute to the non-reflow of capillaries as well as cellular injury.

Parenchymal injury was evidenced by the sudden significant increase in number of EB-labelled nuclei in the CS group as compared to control animals ($p<0.05$) (Figure 2.3). Ethidium bromide is a fluorescent dye, which does not penetrate the cell membrane of uninjured cells (Potter et al, 1995). Injured cells develop increased membrane permeability and allow EB to enter the cell and stain the nucleus, thereby reflecting the amount of injury within the capillary networks observed. Whether these cells are able to recover or become functionally viable remains unknown. This technique for detecting injury has been used in vivo for many years in studying microcirculation and ischemia reperfusion (Potter et al, 1993; Badhwar et al, 2004; Forbes et al, 1995; Potter et al, 1995).

### *2.4.1 CS as Low-Flow Ischemia*

After 45 minutes of compartment syndrome nearly all of the capillaries observed in the EDL muscle displayed altered perfusion. Despite microvascular dysfunction in acute CS, some degree of perfusion remains at all times, creating a partial ischemic environment, of reduced or "low-flow" ischemia within the limb. This allows neutrophils to be activated immediately, which may contribute to the degree of cellular injury noted (Harkin et al, 2001; Kurose et al, 1994).

Following complete ischemia, revascularization leading to the reintroduction of oxygen into ischemic tissue results in an increase in reactive oxygen metabolites, initiating an acute state of inflammation (Gute et al, 1998; Lum and Roebuk, 2001; Schlag et al, 2001. These reactive metabolites serve as a trigger to increase the overall rate of cellular apoptosis and necrosis (Schlag et al, 2001). During EICP (30mmHg) in a normotensive model with partially sustained perfusion, a concurrent amplification of the inflammatory system from reactive metabolites may occur since oxygenated blood continues to perfuse the compartment, in contrast to complete ischemia. In a murine model comparing complete hindlimb ischemia to partial ischemia, Conrad et al (2005) reported that partial ischemia causes a significant early increase in the pro-inflammatory cytokine KC which is analogous to human IL-8 expressing neutrophil chemotactic activity. This finding corroborates the early inflammatory response we observed in compartment syndrome, which we believe is physiologically similar to a partial ischemic state. In a canine model comparing complete ischemia to compartment syndrome, Heppenstall *et al* (1986) observed that the compartment syndrome stimulus causes severe acidosis and metabolic stress. He also concluded that compartment syndrome renders a more severe degree of muscle ultrastructural deterioration than ischemia alone. CS was found to be more injurious to muscle than complete ischemia, possibly due to the cytotoxic inflammation induced by this low flow ischemic state. Our physiologic model of CS includes a "low-flow" ischemic state with associated inflammatory activation and muscle tissue injury (Figure 2.5).

### *2.4.2 Compartment Syndrome Modelling*

The severity and acuity of compartment syndrome restricts the study of its pathophysiology in humans. Animal models have been applied in the study of compartment syndrome since 1926 when Jepson published an inaugural study in canines. He experimentally induced compartment syndrome and detailed the functional benefit of decreasing "venous obstruction" via fasciotomy. Animal models of acute lower-extremity compartment syndrome have been developed using various techniques in both large and small animals. Skin fold chambers, arterial occlusion via Fogerty balloon, arterial ligation, inflation of latex balloons within compartments, external compression and tourniquet application are some of the techniques published (Sheridan and Matsen, 1975; Sheridan et al, 1977; Strauss et al, 1983; Mortensen et al, 1985; Hargens et al, 1978; Mubarak et al, 1976; Matsen et al, 1977; Perler et al, 1990; Vollmar et al, 1999). Large animal canine models deemed clinically relevant have induced compartment syndrome

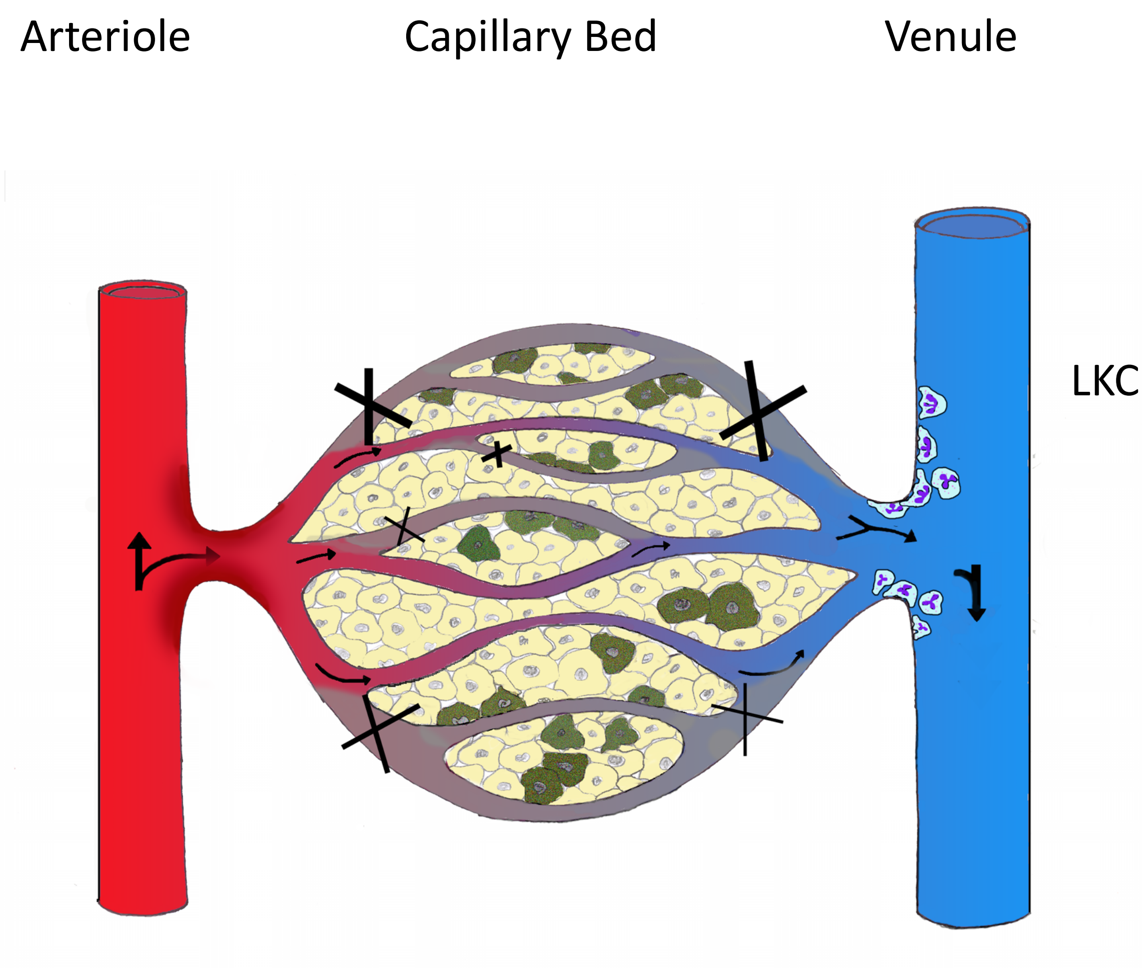


**Figure 2.5. Proposed conceptual model of compartment syndrome-induced microvascular dysfunction**. Oxygenated blood flows from the arteriole through the capillary, unloading oxygen to cells. With elevated compartmental pressure, non–perfused and intermittently-perfused capillaries become visible within capillary beds and are ineffective at gas exchange (X) contributing to cellular injury (green). Furthermore, maintenance of capillary perfusion during CS allows for oxygenated blood into the compromised compartment, which may lead to reactive oxygen metabolites contributing to the chemotactic stimuli for the expression and activation of leukocytes (LKC). In the post-capillary venule, activated leukocytes are observed which may contribute further to tissue injury.

using pressure-controlled autologous blood or plasma infusion into compartments.

In the present study, pressure-controlled isotonic normal saline infusion was utilized to elevate intra-compartmental pressure as it is a standardized concentration and would thus be more reliable then blood or plasma from various donor sources. An absolute pressure threshold was utilized for ease of experimentation in a normotensive model. We studied the EDL muscle, as it is composed of a mixture of muscle fiber types, with up to 54% of the muscle being fast twitch (Tyml and Budreau, 1991), similar to human anterior compartment musculature. The EDL preparation has been established in the study of microcirculation (Badhwar et al, 2004; Badhwar et al, 2003; Forbes et al, 1995; Tyml and Budreau, 1991; Forbes et al, 1996; Potter et al, 1995), its advantages being that it is a deep muscle and sustains minimal mechanical manipulation in its preparation and therefore minimal reactive hyperemia and injury. The majority of the muscle remains *in situ* when its microcirculation is studied, its surgical preparation does not demonstrate deterioration of perfusion with time and hence experimental controls can be easily applied.

The time chosen for elevation of compartment pressure (45 min) was based on previous work demonstrating that 1 hour of ischemia in a rodent approximates 4 hours of ischemia in a human (Sheridan et al, 1977). The experimental time of 45 minutes was applied in order to observe the early microvascular response to EICP and its subsequent effects on the surrounding tissue. Small animal models are not identical to metabolic and cellular derangements in humans and hence experimental effects need to be compared to the existing body of literature. This model is reliable and simple to use for the study of microcirculation, inflammation and injury in acute compartment syndrome and allows for detailed study of the mechanism underlying compartment syndrome.

To our knowledge, this study provides the first evidence of the *in vivo* microvascular perfusion changes that occur with early compartment syndrome. The use of intravital microscopy in conjunction with fluorescent stains in a small animal model has demonstrated the specific perfusion changes, inflammation and tissue injury that occur in early CS. This data suggests that the injury process in CS begins early and causes a severe inflammatory response. Further study is required to fully delineate the mechanism causing the severe injury observed clinically in CS.

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# CHAPTER 3

## Inflammatory Contribution to Cellular Injury in Compartment Syndrome In an Experimental Rodent Model.

# CHAPTER 3: INFLAMMATORY CONTRIBUTION TO CELLULAR INJURY IN COMPARTMENT SYNDROME IN AN EXPERIMENTAL RODENT MODEL

## 3.1 INTRODUCTION

Compartment syndrome (CS) is a devastating complication of musculoskeletal trauma, caused by increased pressure within a closed osseofascial compartment (Matsen 1975, Whitesides et al. 1975, Matsen 1980, Rorabeck 1984, Tornetta and Templeman 1997) (Matsen 1975, Whitesides et al. 1975, Mubarak et al. 1978, Rorabeck and Clarke 1978, Matsen et al. 1980, Hartsock et al. 1998). A large body of literature has determined that the inaugural pathophysiological event in the development of CS is a result of increased intra-compartmental pressure, leading to microcirculatory dysfunction. This, in turn, limits oxygen and nutrient delivery, giving rise to cellular anoxia and tissue necrosis (Sheridan and Matsen 1975, Whitesides et al. 1975, Rorabeck and Clarke 1978, Matsen et al. 1980). The final common pathway is severe myonecrosis, which often results in permanent functional impairment or even loss of the limb. Unlike complete ischemia, however, CS causes tissue necrosis in the face of patent vessels; paradoxically, ischemia ensues with a distal pulse present (Seddon 1966), indicating the pathophysiology is more complex than previously understood.

Direct live *in vivo* imaging of the capillaries in CS has demonstrated significant microvascular impairment coupled with a substantial increase in activated leukocytes in skeletal muscle postcapillary venule (Lawendy et al. 2011). The observed low-flow ischemic state maintains a diminished level of microvascular blood flow associated with a rapid activation of leukocytes, suggesting that early cellular injury in CS may result from a combination of ischemia and acute inflammatory damage. Intravital video microscopy (IVVM) studies in animal models of complete hindlimb ischemia and reperfusion (I/R) have demonstrated that activated leukocytes adhering to postcapillary venules directly impair capillary perfusion (Forbes et al. 1996, Harris and Skalak 1996), while increasing vascular protein leakage and edema (Kurose et al. 1994). Leukocytes also cause direct parenchymal injury following reperfusion (Forbes et al. 1995, Forbes et al. 1996).

The pathologic contribution of inflammation to the pathophysiology of CS is being increasingly recognized; studies from our group (Manjoo al. 2010, Lawendy et al. 2011) and others (Heppenstall et al. 1986, Perler et al. 1990, Sadasivan et al. 1997, Kearns et al. 2004) have broadly implicated leukocytes as playing a primary role in both microvascular and parenchymal injury during CS. Inflammation, being subject to modulation, may therefore provide an opportunity to attenuate injury in the muscle subjected to elevated intra-compartmental pressure (ICP).

In this study, normal rodents exposed to elevated ICP were compared with leukopenic rodents, to determine the direct contribution of inflammation to the cellular injury in CS using both IVVM and histochemical staining techniques. It was hypothesized that leukopenia would provide significant microvascular and parenchymal protection compared to rodents with intact immunity. These results may thus provide evidence toward a potential therapeutic benefit for anti-inflammatory treatment of elevated ICP.

## 3.2 METHODS

### *3.2.1 Animal Handling and Care*

Male Wistar rats (175- 250 g) utilized for these experiments had access to food and water *ad libitum*. Animal housing, care and associated protocols were conducted in agreement with the Canadian Council on Animal Care. The animal protocol was this study was approved by the Animal Use Subcommittee at the University of Western Ontario.

### *3.2.2 Experimental Protocol*

Fifty rats were randomly assigned into two groups: control (n=25) and leukopenia (n=25). Rats were rendered leukopenic by a single injection of high-dose cyclophosphamide (250mg/kg IP, Procytox™, Deerfield IL) three days prior to induction of CS. Complete blood count (CBC) was ordered for each animal to ensure leukopenia at 72 hours post-injection; samples were processed at the clinical biochemistry laboratory at the London Health Sciences Centre (London, Ontario, Canada). Leukopenia was defined as number of leukocytes$<0.5x10^9$/L at the time of experimentation.

The animals were anaesthetized with isoflurane (5% induction, 2% maintenance) in a 1:1 O2:N2 mixture for the whole duration of the experiment. The left carotid artery was cannulated to monitor mean arterial pressure.

### *3.2.3 Compartment Syndrome*

Compartment pressure was elevated by an infusion of isotonic normal saline via a 24-gauge angiocatheter into the anterior compartment of the left hind limb, as described previously (Lawendy, Sanders et al. 2011). The ICP was measured by an electronic compartmental pressure monitoring system (Synthes USA, Paoli, PA), inserted through 14-gauge angiocatheter. Sham animals (n=10) underwent all procedures as CS groups, but the ICP was kept at the baseline of 0 mm Hg. In CS animals, the ICP was maintained between 30-40 mmHg for 45- (n=10), 90- (n=10), 120- (n=10) and 180-minute (n=10) time intervals. These were then followed by fasciotomy and intravital video microscopy (IVVM), in order to assess the degree of microvascular dysfunction, leukocyte activation and irreversible injury to muscle cells.

### *3.2.4 Intravital Video Microscopy (IVVM)*

Following fasciotomy, the extensor digitorum longus (EDL) muscle was prepared for IVVM, as previously described (Potter et al. 1993, Forbes et al. 1995, Manjoo et al. 2010, Lawendy et al. 2011). Briefly, the EDL was dissected to the level of its distal tendon, which was then tied with a suture and cut from its bony insertion. The animal was transferred onto the stage of an inverted microscope (Nikon); the EDL was reflected into a saline bath containing 5mg/ml each of the fluorescent vital dyes bisbenzimide (BB; exc. 343nm, em. 483nm) and ethidium bromide (EB; exc. 482nm, em. 616nm). BB stains the nuclei of all cells while EB stains the nuclei of only those cells with damaged cell membrane; thus, EB/BB ratio provided an index of tissue injury.

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

### *3.2.5 Offline Video Analysis*

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

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

### *3.2.6 Statistical Analysis*

Statistical analysis consisted of a repeated measures two-way analysis of variance (ANOVA) to compare the degree of perfusion, muscle injury, leukocyte rolling and leukocyte adherence in the presence of compartment syndrome, in both the control and leukopenic animals at 45, 90, 120 and 180 minutes of elevated ICP. Statistical significance was defined as $p<0.05$.

## 3.3 RESULTS

### *3.3.1 Microvascular Perfusion*

The effect of elevated ICP on microvascular perfusion is shown in Figure 3.1. Both control and leukopenic groups demonstrated an observed reduction in capillary perfusion at all experimental time points. The capillary profile observed in sham animals demonstrates predominately continuous perfusion, representing the expected normal healthy perfusion. In the control CS group, the number of CPC (mean ± SEM) decreased from 76.5 ± 5.1% in sham to 38.8 ± 7.1%, 36.4 ± 5.7%, 32.0 ± 1.7%, and 30.5 ± 5.35 at 45, 90, 120 and 180 min CS animals, respectively ($p < 0.05$). In the leukopenic group, the perfusion profiles demonstrated a similar trend in microvascular dysfunction: CPC decreased from 71.5 ± 2.1% in sham to 39.2 ± 8.6%, 43.5 ± 8.5%, 36.6 ± 1.4% and 50.8 ± 4.8% at 45, 90, 120 and 180 min CS, respectively ($p < 0.05$). Thus, the perfusion

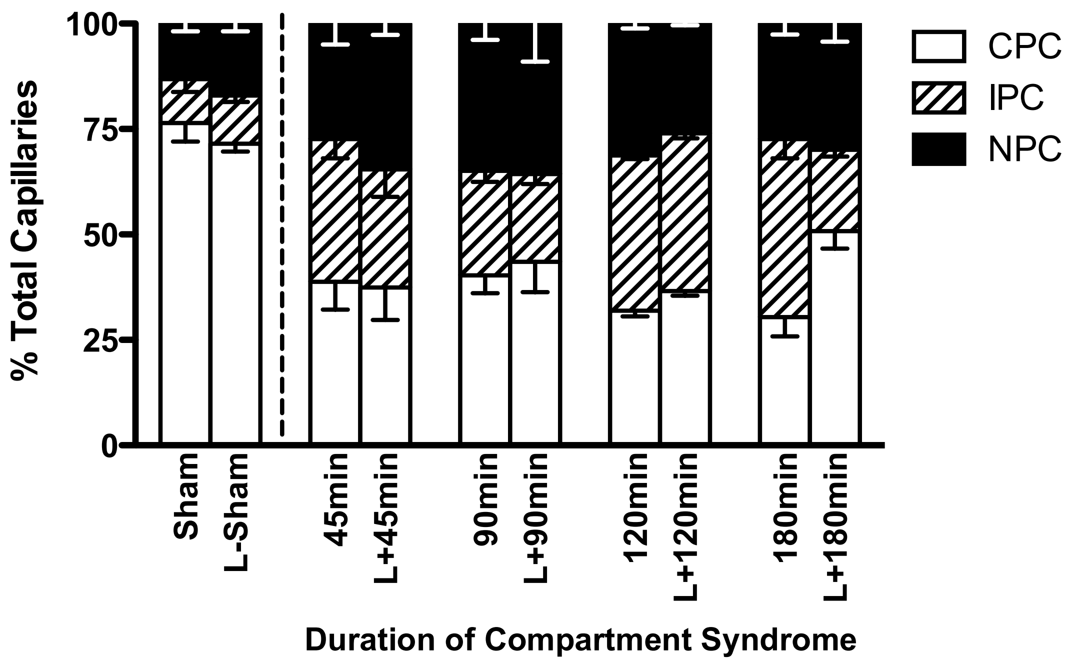


**Figure 3.1: The effect of leukopenia on microvascular perfusion following CS, measured using intravital video microscopy**. There were no significant differences in capillary perfusion between control and leukopenic (*L*) animals. *CPC*, continuously-perfused capillaries; *IPC*, intermittently-perfused capillaries; *NPC*, non-perfused capillaries.

shifted from a predominantly continuous profile in the sham to an intermittent and non-perfused profile in the CS animals, in both the control and leukopenic groups. No statistical significance was demonstrated between the experimental (i.e. leukopenic) and control groups.

### *3.3.2 Tissue Injury*

Muscle injury was quantified as the ratio of EB/BB stained nuclei, and is represented as the percent of injured cells per field of view (Figure 3.2). Muscle injury was significantly increased in the control group (i.e. normal leukocyte count) from 5.0 ± 3.0% in sham animals to 18.0 ± 4.0% at 45 minutes, 23.0 ± 4.0% at 90 minutes, 32.0 ± 7.0% at 120 minutes, and 20.0 ± 5.0% after 180 minutes of elevated ICP. Leukopenia itself had no effect on muscle injury, as seen in the leukopenic sham animals. When leukopenic animals were subjected to elevated ICP, there was a significant decrease in tissue injury observed at all time intervals: 7.0 ± 2.0% at 45 minutes, 7.0 ± 1.0% at 90 minutes, 9.0 ± 1.0% at 120 minutes, and 5.0 ± 2.0% at 180 minutes of elevated ICP; this level of injury was significantly lower in the leukopenic group, as compared to control animals (Figure 3.2).

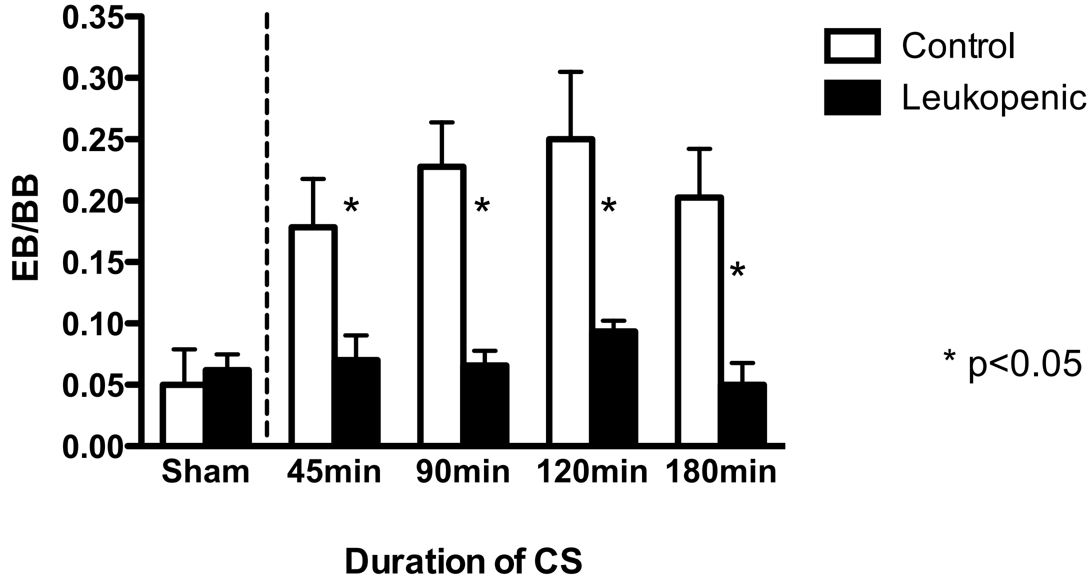


**Figure 3.2: The effect of leukopenia on parenchymal tissue injury within the EDL muscle following CS**. Leukopenia significantly decreased (p<0.05) the EB/BB ratio at all time points of elevated ICP, while it had no effect on sham levels. This graph signifies that injury was diminished in leukocyte deplete animals. This may indicate both decrease in initiation and propagation injury in response to elevated Intra-compartmental pressure.

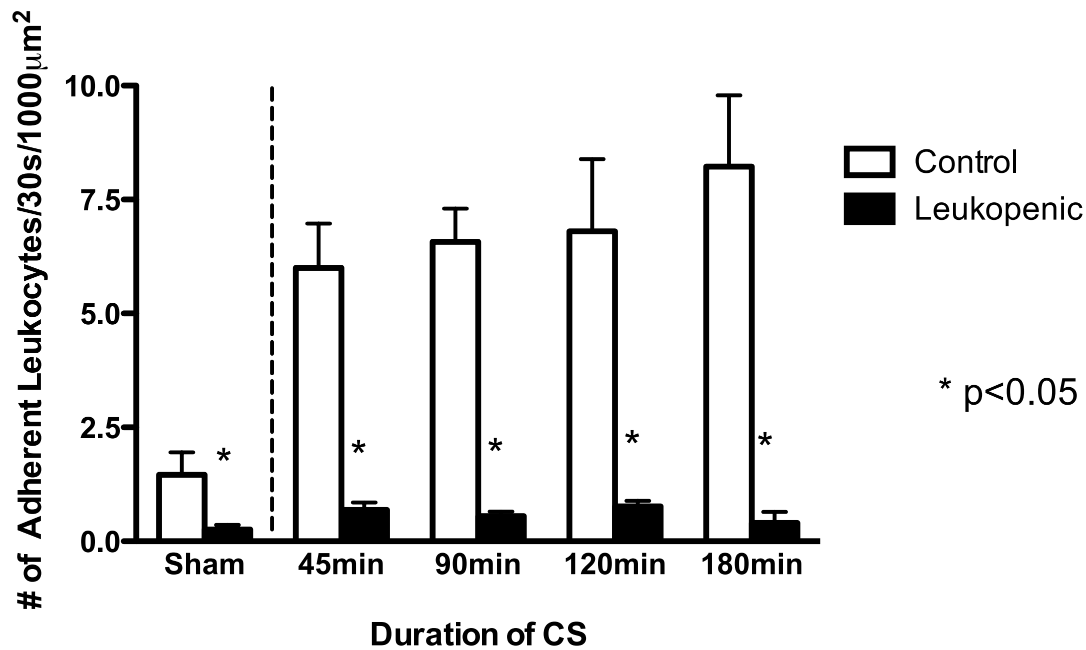


**Figure 3.3. The effect of leukopenia on leukocyte activation (adherent leukocytes) following CS.** Leukopenic animals showed a significant decrease (*p<0.05) in leukocyte activation, as demonstrated by the lack of adherence, across all time points, including sham animals.

### *3.3.3 Inflammation*

Leukocyte activation and flow characteristics were significantly up-regulated by the CS insult (Figure 3.3). Leukocyte adhesion to the vascular endothelium increased from 1.5 ± 0.55 leukocytes/30s/1000mm$^2$ in sham animals to 6.0± 1.06, 6.6 ± 0.77, 6.8 ± 1.84 and 8.2±1.81 leukocytes/30s/1000mm$^2$ at 45, 90, 120 and 180 min CS, respectively ($p < 0.05$). Leukopenia significantly blocked leukocyte activation at all experimental time points: adhesion was diminished in sham rodents to 0.3 ± 0.11 leukocytes/30s/1000mm$^2$, and continued to remain blunted to 0.7 ± 0.18, 0.6 ± 0.11, 0.8 ± 0.15, and 0.4 ± 0.27 leukocytes/30s/1000mm$^2$ at 45, 90, 120 and 180 min CS, respectively ($p < 0.05$) (Figure 3.3).

A similar trend was demonstrated in rolling leukocytes, with a significant increase at all experimental time points as compared to sham in normal rodents. Rolling behaviour increased from 1.8 ± 0.59 leukocytes/30s/1000mm$^2$ to 3.9 ± 1.6, 4.8 ± 1.65, 7.3 ± 2.90 and 9.8 ± 2.73 leukocytes/30s/1000mm$^2$ at 45, 90, 120 and 180 min CS, respectively (Figure 3.4). Leukopenic animals did not mount a significant inflammatory response; leukocyte rolling did not increase between sham and 45 min of elevated ICP, and remained at 0.5 ± 0.19 leukocytes/30s/1000mm$^2$. Rolling also remained low at 0.3 ± 0.10 leukocytes/30s/1000mm$^2$ at 90 min CS, with just a slight, non-significant increase to 2.7 ± 1.34 leukocytes/30s/1000mm$^2$ at 120 min CS. Finally, at 180 min CS, the rolling returned back to sham levels, at 0.6 ± 0.07 leukocytes/30s/1000mm$^2$ (Figure 3.4).

### 3.4 DISCUSSION

The pathophysiological mechanisms that underlie the severe and acute myonecrosis observed in CS are complex and not fully understood. This study was designed to examine the relative contribution of inflammation to tissue injury in a small animal model of CS. By rendering the animals leukocyte deplete, a very rigid control was applied in order to accurately quantify the relative contribution of inflammation to parenchymal injury in animals subjected to elevated ICP over time. We studied the effect of elevated ICP in a leukocyte deplete rodent model, assessing microvascular perfusion, inflammation and tissue injury, utilizing IVVM and fluorescent dye staining.

#### 3.4.1 Tissue Perfusion

Perfusion under normal, non-traumatic conditions exhibits continuous physiologic flow, with a constant stream of red blood cells travelling though capillaries. The CS insult demonstrated a significant shift from continuous perfusion in sham animals to increased intermittent and non-perfused capillaries across all time points. Interruption of flow rate and volume leads to intermittent perfusion which, in turn, compromises gas exchange. Non-perfused capillaries exist when complete arrest of red cell are observed in the capillary bed, leading to no nutrient or gas exchange: essentially, a state of ischemia (Lawendy et al. 2011). This shift in flow demonstrates a pathologic microvascular perfusion in response to the CS insult, shown *in vivo,* under live conditions. The microvascular dysfunction occured early, and appeared to persist over time. With

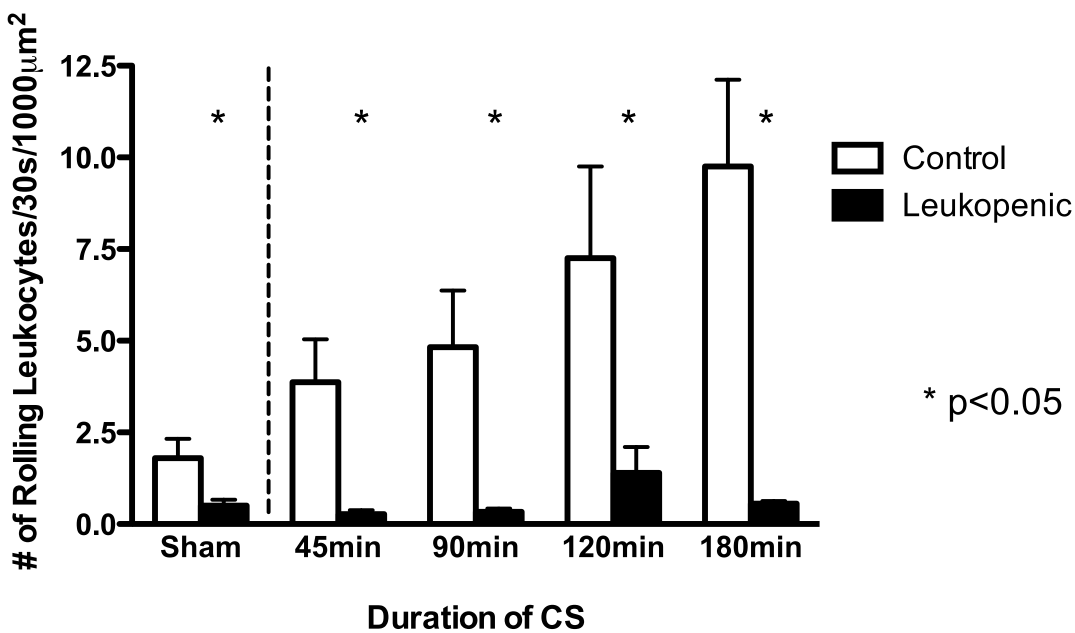


**Figure 3.4. The effect of leukopenia on leukocyte activation (rolling leukocytes) following CS.** Leukopenic animals showed a significant decrease (*$p<0.05$) in leukocyte activation, as demonstrated by the lack of rolling behaviour, across all time points, including sham animals.

increased proportion of non-perfused capillaries in the presence of continuous perfusion within the same capillary bed, a low flow ischemic state is established in CS. The effect of no-flow ischemia on skeletal muscle has been well studied in the literature (Harman 1948, Strock and Majno 1969, Swartz et al. 1978, Labbe et al. 1987, Belkin et al. 1988, Lindsay et al. 1990, Hickey et al. 1992, Sabido et al. 1994)As the duration of ischemia increases, predictable changes in the microcirculation such as increased vascular permeability to plasma proteins and progressive interstitial edema ensue (Sexton et al. 1990, Kurose et al. 1994, Lu et al. 1997). In CS, leukocyte deplete animals demonstrated no significant difference ($p<0.05$) in the blood flow rate or flow characteristics at 45, 90, 120 an 180 minutes of CS, as compared to controls (Figure 3.1).

Microvascular perfusion was essentially unchanged in leukopenic animals, as compared to controls; hence leukopenia was not protective in restoring or maintaining perfusion in the face of elevated compartment pressure. This data suggests that the effects of leukocytes on the microvascular perfusion in CS are, perhaps, pathophysiologically different from a pure ischemia-reperfusion insult with respect to skeletal muscle microcirculation. In a leukocyte deplete ischemia reperfusion model, microvascular dysfunction (i.e. no reflow phenomenon) was prevented, and parenchymal injury diminished in the presence of leukopenia (Forbes et al. 1996). In our studies, however, while perfusion was altered in CS, leukopenia did not have a direct effect on the magnitude of microvascular dysfunction, suggesting that although ischemia-reperfusion pathophysiology may share features with CS, there may be a distinct pathophysiology causing microvascular dysfunction.

### 3.4.2 Inflammation

The results of our study demonstrate that the CS insult is accompanied by a substantial inflammatory response. At 45 minutes of CS, we observed the arrest of leukocytes under conditions of flow, recruitment of activated leukocytes and extravasation, which strongly suggests that CS induces a pro-inflammatory environment. Leukopenia significantly diminished leukocyte activation, both in terms of rolling and firm adhesion in the post-capillary venules at 45, 90, 120 and 180 minutes of CS as compared to controls ($p<0.05$) (Figure 3.2). Leukocyte-endothelial interactions in the conditions of trauma, injury, infection and ischemia are known to create a pro-inflammatory environment secondary to the upregulation of cytokines and chemokines, which stimulate leukocyte activation and recruitment of polymorphonuclear leukocytes (PMNs) into the area of injury (ref). Activated leukocytes produce reactive oxygen species and proteolytic enzymes, causing cellular damage, increasing permeability and edema, resulting in increased interstitial pressure; this may lead to non-perfused segments in the microvascular beds (Forbes et al. 1996, Kurose et al. 1997, Gute et al. 1998).

### 3.4.3 Tissue Injury

Parenchymal injury was evidenced by the significant increase in the number of EB-labelled nuclei in the CS group, as compared to control animals ($p<0.05$). All experimental groups demonstrated a more than 50% significant reduction in tissue injury as compared to controls (Figures 3.3 and 3.4). This data suggests that inflammation is a significant pathophysiologic mechanism driving injury in experimental CS. Leukocyte adhesion and interaction with the endothelium appears to be important to the development of tissue injury without significant effect on capillary perfusion. This would suggest that in early CS, inflammation may be more important and perhaps with prolonged exposure to CS late ischemia may be more pathophysiologically relevant.

This study demonstrates that inflammation should be considered central to the understanding of the pathogenesis of cellular injury in CS. Perhaps, modulation of inflammation may diminish myonecrosis in CS. The specific inflammatory pathways or signaling systems still need to be clearly delineated, as well as whether the leukocyte activation and adhesion remain temporally uncoupled from the observed microvascular dysfunction.

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# CHAPTER 4

# Compartment Syndrome Causes Systemic Inflammatory Response in the Rat.

## CHAPTER 4: COMPARTMENT SYNDROME CAUSES SYSTEMIC INFLAMMATORY RESPONSE IN THE RAT.

### 4.1 INTRODUCTION

Acute compartment syndrome (CS) is a devastating complication of musculoskeletal trauma. Elevated pressure within a closed osseofascial compartment can lead to microvascular compromise and tissue necrosis within the affected compartment (Ashton 1975, Hargens et al. 1981, Botte and Gelberman 1995, McQueen et al. 1996, Botte and Gelberman 1998, Hope and McQueen 2004, Gourgiotis et al. 2007). Untreated, critical tissue ischemia may develop, leading to loss of life or limb. The complications of compartment syndrome, although often isolated to the affected extremity, can also have systemic consequences. An example is “crush injury”: a clinical entity caused by severe CS leading to hypovolemia, traumatic rhabdomyolysis, electrolyte and acid-base abnormalities, renal failure and sometimes death (Bywaters and Beall 1941, Bywaters and McMichael 1953, Kikta et al. 1987, Better et al. 1990, Better and Stein 1990). Myonecrosis, with the release of cellular contents into the circulation, causes the observed metabolic derangements and acute renal injury (Montagnani and Simeone 1953, Kikta et al. 1987, Odeh 1991).

Isolated trauma is known to have significant host effects when local inflammation becomes dysregulated, leading to a systemic inflammatory response (SIR), characterized by the activation of complement and the coagulation cascade, the secretion of acute-phase proteins, as well as activation of neutrophils, macrophages, and lymphocytes (Ogura et al. 1999, Wakai et al. 2001, Blaisdell 2002, Lenz et al. 2007). The SIR response occurs in concert with the local inflammatory stimulus. The classic example is prolonged ischemia followed by tissue reperfusion, leading to an intense inflammatory response causing sequellae of the diffuse cellular injury and inflammation, which may ultimately lead to remote organ failure. (Friedl et al. 1991, Gottlieb et al. 1994, Rubin et al. 1996, Gute et al. 1998, Grisotto et al. 2000, Harkin et al. 2001, Krishnadasan et al. 2003) In this paradigm of understanding, remote organ injury is caused primarily by the immune response. Experimental studies have demonstrated that CS alone can produce a significant pro-inflammatory environment within the affected compartment. Unlike a period of discrete ischemia followed by a period of reperfusion, CS injury occurs with patency of the arterial supply to the extremity and hence the microvascular perfusion deficits observed produce a low-flow ischemic state; hence ischemia and reperfusion are essentially occurring simultaneously, leading to marked inflammatory response that has immediate access to the systemic circulation.

The goal of this study was to further our understanding of the pathophysiology of CS. The study was carried out to determine whether acute CS, a partial ischemia-reperfusion phenomenon, is able to cause systemic inflammatory process, lending support to the inflammatory basis of its injury mechanism. To test this hypothesis, we induced acute CS in rodents and assessed the animals for hepatic microvascular perfusion, inflammation and hepatocellular damage.

## 4.2 METHODS

### *4.2.1 Animal Description and Care*

Male Wistar rats utilized for these experiments had access to food and water *ad libitum*. All protocols and experiments were conducted in agreement with the Canadian Council on Animal Care, and approved by the University of Western Ontario Animal Use Subcommittee.

### *4.2.2 Experimental Protocol*

Fifteen rats (175-250 g) were anesthetized with inhalational isoflurane. Following induction at 5% isoflurane in a 1:1 $O_2$:$N_2$ mixture, anaesthesia was maintained at 2% isoflurane and titrated to maintain an adequate level of general anesthesia. The carotid artery was cannulated for continuous blood pressure monitoring and fluid replacement to maintain a normal mean arterial pressure at 100mmHg**.** The animals were randomized into two groups: control (n=5) and CS (n=10).

### *4.2.3 Compartment Syndrome*

Once anesthetized, limb compartment pressure was elevated by slowly infusing isotonic normal saline via a 24-gauge angiocatheter into the anterior compartment of the left hindlimb in the experimental group. Compartment pressure was raised to 30mmHg and maintained between 30–40mmHg for the duration of the protocol; this technique has been previously described (Manjoo et al. 2010, Lawendy et al. 2011). An electronic compartmental pressure monitoring system (Synthes USA, Paoli PA) was inserted into the anterior and then posterior compartment through a 14-gauge angiocatheter. As the pressure rose within the hindlimb, both the anterior and posterior compartments became isobaric. In order to test the effect of time on capillary perfusion and cellular injury, elevated intracompartmental pressure (ICP) was maintained for 2 hours prior to the pressure release via fasciotomy.  Control animals underwent all the same preparation, however no saline was infused into the compartment via the catheter and the ICP was held at control levels for the duration of the experiment prior to fasciotomy.

#### *4.2.4 Intravital Video Microscopy*

Following fasciotomy, animals were allowed to reperfuse for 45 minutes, followed by liver IVVM. Liver preparation had been previously described in detail (Lawlor et al. 1999, Hundt et al. 2011). Briefly, the liver was exteriorized through midline laparotomy; animals were placed onto the stage of an inverted microscope (Nikon Diaphot 300) and the liver was reflected onto the slide moistened with saline. All exposed tissues were covered with a plastic film, to isolate the preparation from the atmosphere and to prevent drying. A heat lamp maintained the core temperature (37°C) of the rat.

Eight to 12 fields of view of liver microcirculation were randomly chosen, and recorded using a 20X objective, for a final magnification of 700X at the monitor. Additional 8 fields of view of sinusoidal microcirculation, and up to 12 post-sinusoidal venules were recorded using 40X (final magnification 1400X), to assess the volumetric flow and leukocyte behaviour, respectively.

#### *4.2.5 Liver Microcirculation Analysis*

Sinusoidal diameters (D) were measured using ImageJ (NIH, Bethesda, MD) software, by averaging 3 different points along each sinusoid, and expressed in mm. Centreline velocity of red blood cells (RBC) (V) was assessed within each sinusoid by using frame-by-frame analysis and expressed as μm/s. Volumetric flow (VQ) in pL/s, and shear ($\gamma$) ($s^{-1}$) were calculated using the formulas $VQ = \pi r^2 \times V$ and $\gamma = 8V/D$, respectively.

Sinusoidal perfusion was evaluated using well-described established stereological techniques (Brock et al, 1999). Hepatic microcirculation was classified as continuous, intermittent or non-perfused, based on flow characteristics observed during IVVM in 1-minute intervals. Continuous RBC perfusion during direct observation was classified as a continuously perfused sinusoid (CPS). A sinusoid whereby RBC perfusion was arrested and then regained flow was classified as an intermittently perfused sinusoid (IPS). Sinusoids that had no observable red cell movement for the duration of the observation period were classified as non-perfused sinusoids (NPS). The number of sinusoids was expressed as a percentage of the total number of sinusoids evaluated.

### *4.2.6 Hepatocellular Death*

Fluorescent vital dye, propidium iodide (PI) (5μg/mL) (Sigma Aldrich, Mississauga, ON), was added to the saline bath. PI is highly membrane-impermeant, and hence it stains only the nuclei of lethally injured cells. Fluorescent illumination with the appropriate filter for PI (Ex = 535 nm; Em = 617 nm) was applied. Hepatocyte death was assessed, and expressed as the number of PI-labelled cells per $0.1mm^3$.

### *4.2.7 Inflammation*

Leukocytes were observed in sinusoids and post-sinusoidal venules (PSV) from each of the recorded fields of view. The total number of rolling and adherent leukocytes in PSV were measured over 30 seconds and expressed as the number per $10{,}000μm^2$. Venular area was measured using ImageJ software (NIH, Bethesda, MD). An adherent leukocyte was defined as a cell that remained stationary for a minimum of 30 seconds.

### *4.2.8 Systemic TNF-α measurements*

TNF-α levels were measured from arterial blood samples drawn at 8 time points: (1) baseline, (2) 1 hour into CS, (3) 2 hours into CS – just prior to fasciotomy, (4) 10 minutes post-fasciotomy, (5) 20 minutes post fasciotomy, (6) 30 minutes post fasciotomy, (7) 40 minutes post fasciotomy, (8) 45 minutes post fasciotomy, just before IVVM. TNF-α was assessed using enzyme-linked immunosorbent assay (ELISA, Pierce Biotechnology, c/o Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The TNF-α ELISA was sensitive to less than 5 pg/mL.

#### *4.2.9 Statistical Analysis*

Statistical analysis consisted of a series of t-tests to compare the degree of liver perfusion, hepatocyte injury, volumetric flow, leukocyte rolling and adherence in sinusoids and PSV with the presence of compartment syndrome. Systemic levels of TNF-α were analyzed by one-way analysis of variance (ANOVA). Statistical significance was defined as $p<0.05$.

## 4.3 RESULTS

### 4.3.1 Leukocyte Activation

Leukocyte number and flow characteristics increased in response to CS. The number of adherent leukocytes within the post-sinusoidal venules was significantly higher for the CS group (3.2 ± 1.7 leukocytes/30s/10,000$\mu m^2$) compared to that in sham (0.2 ± 0.2 leukocytes/30s/10,000$\mu m^2$) ($p<0.05$). The number of non-adherent leukocytes after CS injury was similar between sham and CS injured rats, while the number of rolling leukocyte was also significantly upregulated from 1.2 ± 0.7 leukocytes/30s/10,000$\mu m^2$ in sham to 8.4 ± 5.2 leukocytes/30s/10,000$\mu m^2$ ($p<0.05$) (Fig. 4.1). Thus, CS appears to have resulted in leukocyte recruitment to the liver and hepatic inflammation.

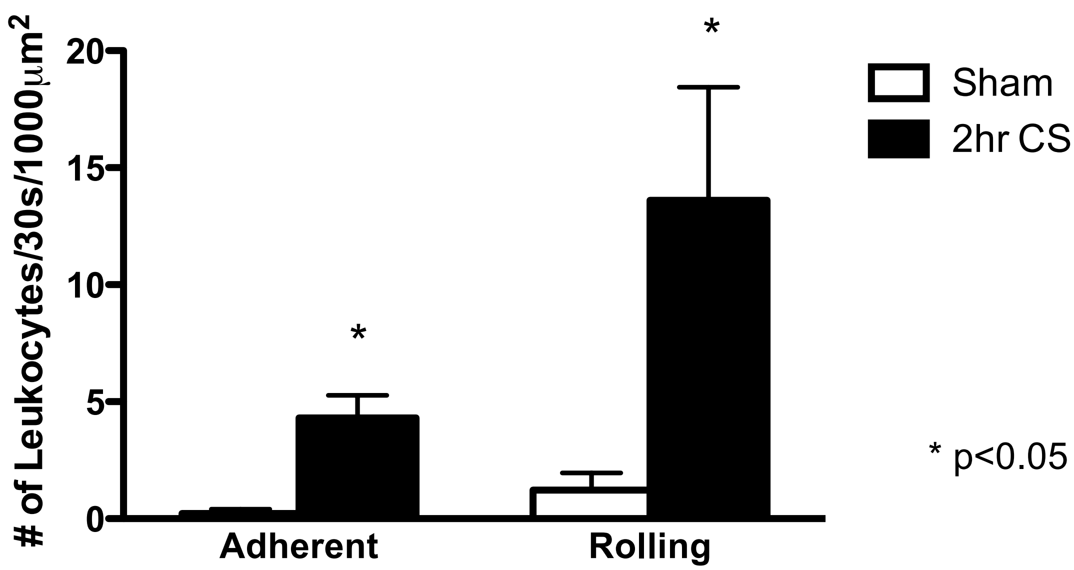


**Figure 4.1: Hepatic leukocyte activation following CS**. Two hours of CS resulted in significant leukocyte recruitment to the liver, as shown by an increase in adherent and rolling leukocytes ($p<0.05$).

### 4.3.2 Hepatic Microcirculation

Microvascular perfusion of the sinusoids was evaluated by quantification of volumetric flow within the sinusoids (comprising of sinusoidal diameters and RBC velocity), and the degree of sinusoidal perfusion (continuously-perfused, intermittently-perfused and non-perfused sinusoids). No changes in the mean diameter of sinusoids were observed between experimental and sham groups (Figure 4.2); volumetric blood flow also remained unchanged, although a slight increase in perfusion heterogeneity was observed (Figure 4.3). There was a mild perfusion deficit following CS, as evidenced by a decreased percentage of continuously perfused sinusoids and an increased percentage of intermittently and non-perfused perfused sinusoids (Figure 4.4).

### 4.3.3 Hepatocellular Injury

Hepatocellular death was significantly higher in the 2hr CS group (192 ± 51 PI-labelled cells/$10^{-1}$ $mm^3$) as compared to controls (30 ± 12 PI-labelled cells/$10^{-1}$ $mm^3$) ($p<0.05$) (Figure 4.5). PI is impermeable to normal cells; thus only cells with irreversible membrane injury (which allows influx of PI into the cells) are labelled by this method.

### 4.3.4 Serum TNF- α measurements

Elevation of ICP led to a progressive serum TNF-α release, reaching its maximum level at 2 hours (just prior to fasciotomy; $p<0.05$) (Figure 4.6). TNF-α levels continued to rise in the post-fasciotomy and reperfusion period. This result

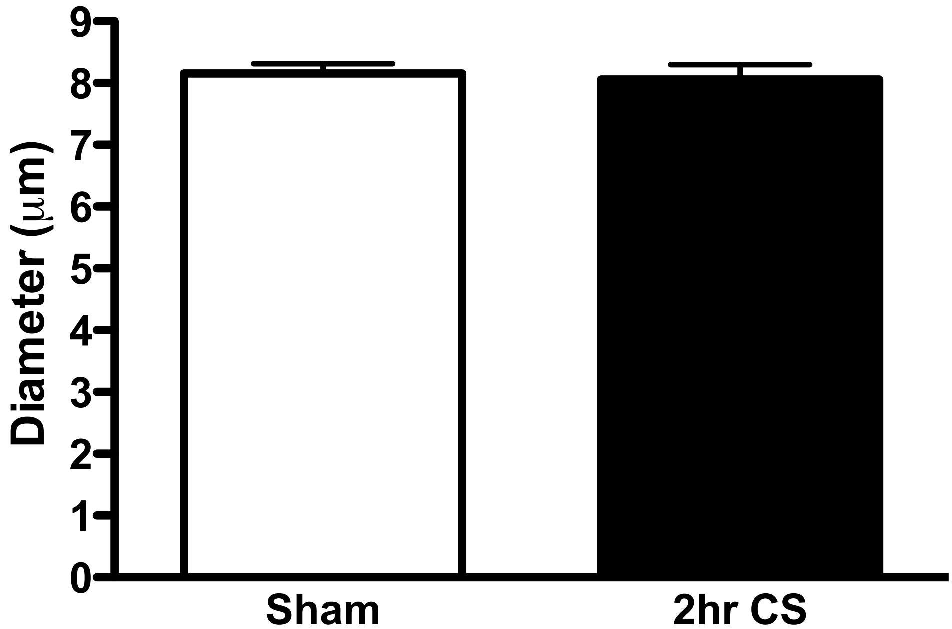


**Figure 4.2: The effect of CS on hepatic sinusoidal diameters**. There was no significant change between sham and 2hr CS groups.

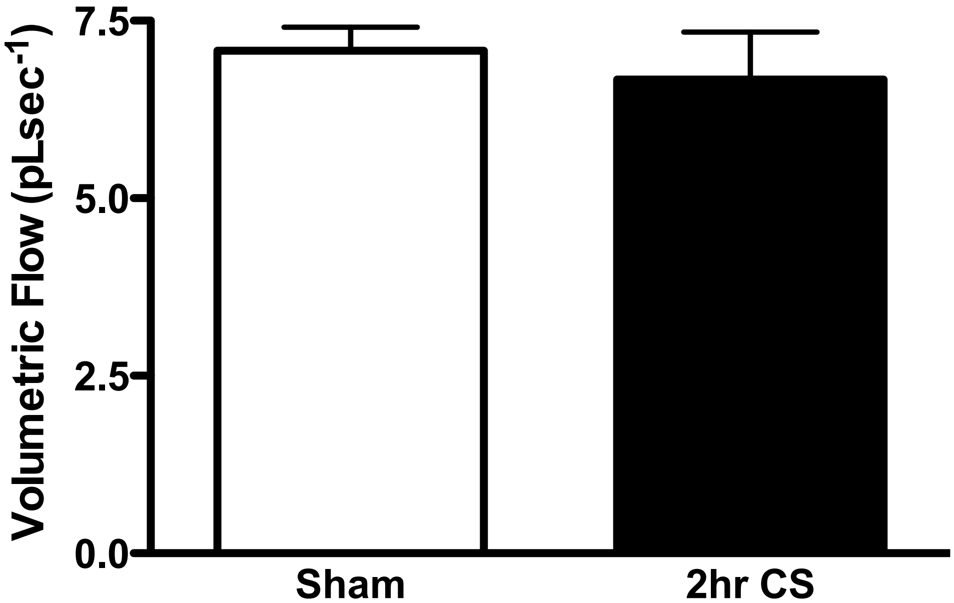


**Figure 4.3: The effect of CS on hepatic sinusoidal volumetric flow**. Two hours of CS had no significant effect on either one of these parameters (i.e. RBC velocity or sinusoidal diameters), although the heterogeneity of the flow appears to have slightly increased.

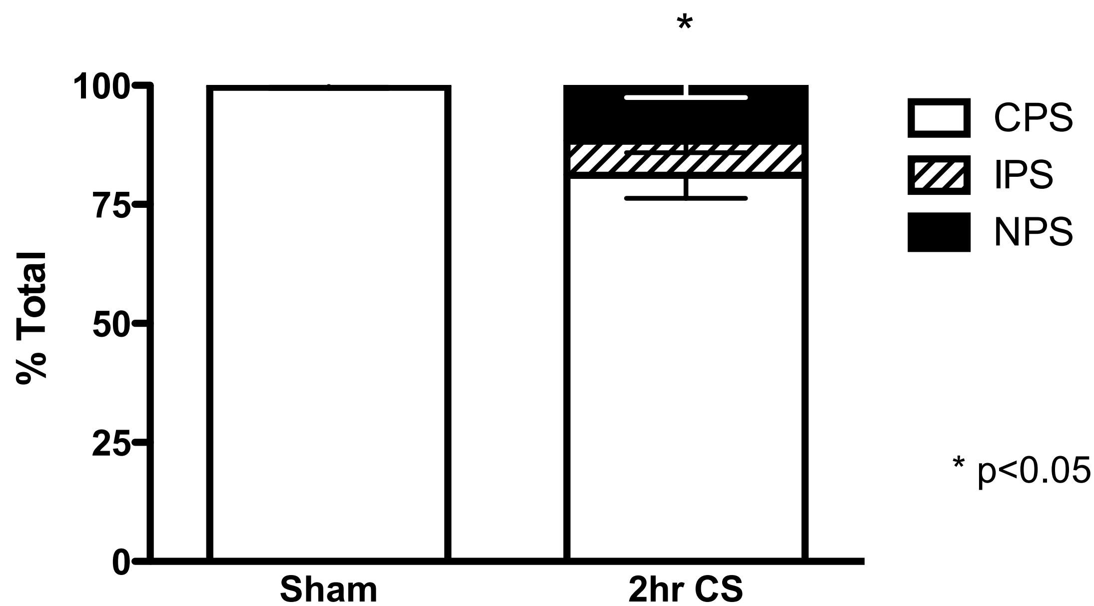


**Figure 4.4: Sinusoidal perfusion following CS**. There was a significant increase ($p<0.05$) in the heterogeneity of the flow, as evidence by a decrease in CPC and an increase in NPC in animals undergoing 2 hours of CS. *CPS,* continuously-perfused sinusoids; *IPS*, intermittently perfused sinusoids; *NPS*, non-perfused sinusoids.

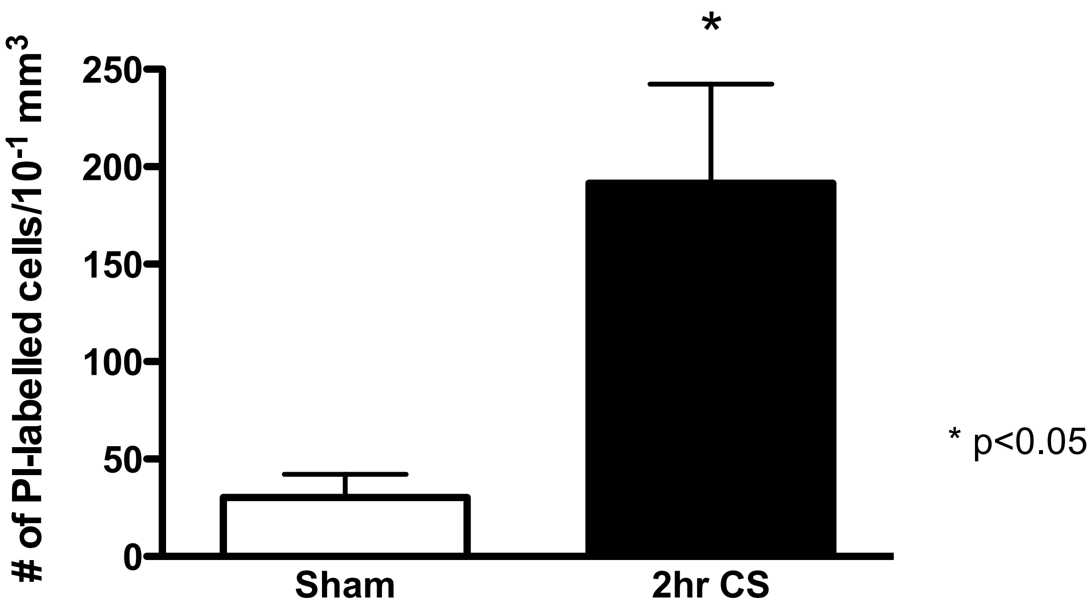


**Figure 4.5: The effect of CS on the degree of hepatocellular death**. There was a significant increase in hepatocellular injury following 2hr CS as compared to sham animals ($p<0.05$).

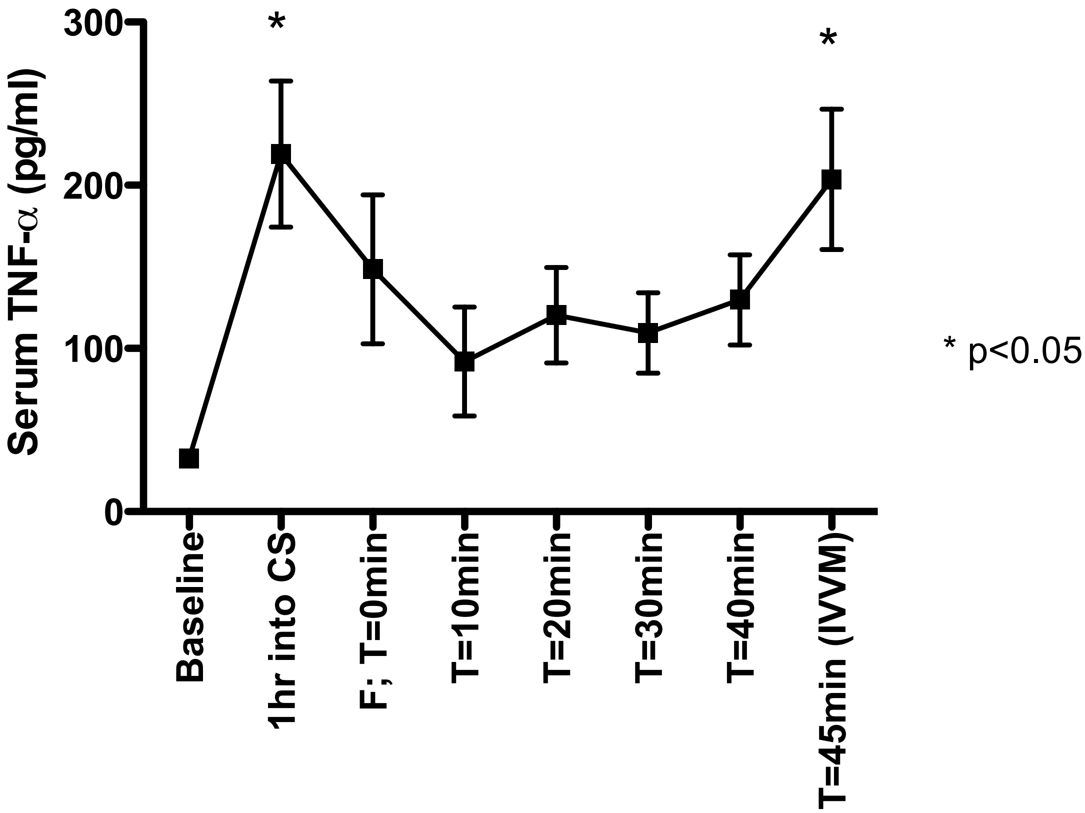


**Figure 4.6: Time course of systemic TNF-α levels following CS**. There was a progressive increase in TNF-α during the period of elevated ICP, with maximal statistically significant level just prior to fasciotomy ($p<0.05$). TNF-α continued to further increase after fasciotomy.

demonstrates a two-hit inflammatory model caused by CS and fasciotomy.

## 4.4 DISCUSSION

The purpose of this study was to determine if acute CS, a low-flow ischemia-reperfusion phenomenon, could produce a pro-inflammatory environment sufficient enough to cause remote organ injury, in order to further understand the mechanisms underlying the pathophysiology of CS. Our study demonstrates that partial ischemia due to unilateral hindlimb CS resulted in a 7-fold increase in hepatocellular injury, and a 25-fold increase in the number of activated leukocytes, as compared to sham ($p<0.05$). CS can be accompanied by a systemic inflammatory response and end organ damage, as evidenced by increased hepatic venular and sinusoidal leukocyte count, and hepatocyte death.

### 4.4.1 Model Characteristics

The model utilized to study the systemic effects of CS was based on a previously-published, clinically relevant experimental rodent model of CS (Manjoo et al. 2010, Lawendy et al. 2011), combined with a published technique for the study of SIR and remote organ injury, in response to trauma in the absence of infection (Brock et al. 1999a, Brock et al. 1999b, Lawlor et al. 1999, Brock et al. 2001, Wunder et al. 2002, Wunder et al. 2004). We exposed Wistar rats to 2 hours of unilateral hind limb CS, followed by fasciotomy. Blood samples were collected at regular intervals to assess TNF-α levels. Following fasciotomy, and laparotomy, liver sinusoidal and venular blood flow was measured via liver exposure and IVVM. PI staining was used to measure hepatocellular death; leukocyte counts, taken in both hepatic venules and sinusoids, were used as a visual marker of the underlying inflammatory process. A normotensive model was used in order to eliminate the possibility of a systemic inflammatory response that may be caused by hypoxic stress secondary to hypo-perfusion and shock.

### 4.4.2 Hepatic Microcirculation

The CS challenge to the rodent hindlimb had no effect on volumetric flow, RBC velocity or mean diameter of hepatic sinusoids when compared to sham group, demonstrating that hepatic microcirculatory homeostasis was not disturbed. However, perfusion deficits were present following CS, as evidenced by a decreased percentage of CPS and an increased percentage of IPS and NPS. Perfusion heterogeneity in the liver exists under normal and pathologic states; however, it is thought to occur more readily under conditions of physiologic stress (MacPhee et al. 1995, Vollmar et al. 1996). The perfusion changes observed in the experimental group may or may not affect cellular viability, as the vast majority of the perfusion was preserved (this was demonstrated by the maintenance of a substantial amount of continuously perfused sinusoids).

Although an intact microcirculation is required for adequate organ integrity and function, the microcirculation observed was grossly unchanged. Furthermore, temporary red cell stasis does not have significant effect on the liver parenchyma (MacPhee et al. 1992). In previous studies of remote organ injury, hepatocyte death occurred without concomitant sinusoidal perfusion failure, despite demonstrating perfusion heterogeneity (Brock et al. 1999a, Brock et al. 1999b).

### 4.4.3 Inflammation and Injury

Examination of post-sinusoidal venules demonstrated a significant increase in activation and recruitment of leukocytes in the experimental group (Figure 4.1). Inflammation of the liver was demonstrated by an increase in rolling and adherent leukocytes. This finding is understood within the framework of a very simple but powerful paradigm of leukocyte recruitment, which takes place in a step-wise fashion by engaging the endothelium through P-selectin (platelets), E-selectin (endothelium) and L-selectin (leukocyte) ligands (Asako et al. 1994, Lozano et al. 1999, Kyriakides et al. 2000, Russell et al. 2003, Ley et al. 2007). The interaction causes leukocyte rolling, which affords the leukocytes the ability to sample the local environment. Here, chemoattractants such as chemokine's act in guiding cell migration, resulting in firm adhesion. The adhesion process is complex and requires the activation of integrins binding to ICAM-1 and other ligands; this creates the necessary environment for leukocyte emigration into surrounding tissue (Ley and Reutershan 2006, Ley et al. 2007).

The hepatic microcirculation diverges from this classic understanding, due to its unique vascular biology. The liver microcirculation receives venous portal blood flow returning from the gut, draining into the sinusoids before terminating in the central venules. The sinusoids have a unique architecture, which not only facilitates nutrient and metabolite exchange, but also allows for neutrophils to accumulate in liver sinusoids (the equivalent of the capillaries), as well as the post-sinusoidal venules (Fox-Robichaud and Kubes 2000). In states of acute inflammation, such as endotoxemia, neutrophils have demonstrated significant accumulation in sinusoids, leading to parenchymal injury (Wong et al. 1997). Direct visualization of leukocyte behaviour secondary to CS demonstrated a rapid neutrophil-endothelial interaction occurring primarily in the post-sinusoidal venules (where a 25-fold increase in the number of activated leukocytes was observed, as compared to controls).

The observed inflammation was accompanied by a rise in TNF-α, an acute-phase chemoattractant acting on neutrophils, promoting the expression of adhesion molecules and resulting in selective adhesion/transmigration of leukocytes (Ascer, Gennaro et al. 1992, Yi and Ulich 1992, Seekamp, Warren et al. 1993, Zhang, Hu et al. 2005). Our results demonstrated a two-hit inflammatory model due to the CS insult and the subsequent fasciotomy. It appears that CS is a sufficient insult to cause a significant initial rise in TNF-α (Figure 4.6), followed by fasciotomy and a second peak in the systemic TNF-α levels. This is probably due to the cellular debris, pro-inflammatory mediators and cytokines gaining access to the systemic circulation when the previously-ischemic tissue is reperfused, leading to a systemic inflammatory response (Forbes et al. 1995, Harkin et al. 2001, Wakai et al. 2001, Katada et al. 2009). In CS, the washout of debris is suspected to be simultaneous to the pressure insult, and is further compounded by the fasciotomy. This indicates that CS is more pro-inflammatory than ischemia-reperfusion injury. Brock et al (1999) found hepatocyte death and systemic inflammation in rats that had undergone 4 hours of bilateral hindlimb tourniquet-induced ischemia; both Kupffer cells and TNF-α were involved in the initiation of hepatic injury. In an attempt to further characterize the specific role of TNF-α, Lawlor et al. (1999) showed that scavenging TNF-α with a polyclonal antibody decreased the lethal hepatocyte injury, but did not eliminate it. This demonstrated that TNF-α is partially responsible for early hepatocellular injury with distant ischemia-reperfusion injury. It is clear that while Kupffer cells and their release of TNF-α played an important role in remote hepatic injury, they were only partially responsible.

Our findings suggest that unilateral hindlimb CS is a significantly injurious process, producing both local and systemic injury through an inflammatory mechanism. This lends support to the crucial role of inflammation in understanding the underlying pathophysiology of CS.

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# CHAPTER 5

## General Discussion and Conclusions.

# CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

## 5.1 OVERVIEW OF RESULTS

### 5.1.1 Pathophysiology of Compartment Syndrome

Since Richard von Volkmann's description of ischemic contracture in the upper extremity of children, CS has been recognized as a complication of trauma, leading to severe acute tissue necrosis (von Volkmann 1881). Volkmann hypothesized the pathophysiology as an ischemic insult secondary to the interruption in arterial blood supply, and for more than a century the pathophysiology of CS has been debated. In Chapter 1, an attempt was made to review and highlight the major conceptual contributions that have shaped our understanding since Volkmann put forward his theory. Central to the modern pathophysiological understanding of CS is that it is pressure-induced ischemic event leading to microvascular dysfunction, limiting oxygen and nutrient delivery, leading to cellular anoxia and tissue necrosis (Sheridan and Matsen 1975, Whitesides et al. 1975, Rorabeck and Clarke 1978, Matsen et al. 1980). However, unlike complete ischemia, CS causes tissue necrosis in the face of patent vessels; paradoxically, ischemia ensues in the presence of a pulse (Seddon 1966), indicating that the pathophysiology is more complex than previously understood. The severity and acuity of CS poses a great challenge to its study in humans, and hence animal models have been utilized to further our understanding for more than eight decades (Jepson 1926). Models of injury have included skin fold chambers, blood or plasma infusion, arterial occlusion, arterial ligation, inflation of latex balloons, external compression and tourniquet application (Sheridan and Matsen, 1975; Sheridan et al, 1977; Strauss et al, 1983; Mortensen et al, 1985; Hargens et al, 1978; Mubarak et al, 1976; Matsen et al, 1977; Perler et al, 1990; Vollmar et al, 1999). The first aim of this thesis was to develop a clinically relevant small animal model of CS, in order to understand the effect of elevated ICP on capillary perfusion, inflammation and cellular injury. A further focus of the study was to define the role of inflammation in local and systemic CS-induced pathology. The framework on which these studies were conducted and analyzed was largely influenced by the last four decades of ischemia-reperfusion literature. This paradigm was applied as the techniques for the study of the microvscular and immune system had been validated and supported by an extensive experimental literature in the field of ischemia-reperfusion. Here a clinically relevant, feasible, reproducible model was developed based on the previous studies of CS, utilizing the techniques available for the study of the microvascular system and its response to trauma.

### 5.1.2 Model Development

The effect of elevated ICP on microvascular perfusion, tissue injury and inflammation in a normotensive model of CS using intravital video microscopy and nuclear fluorescent dyes is described in Chapter 2. Direct imaging of capillaries demonstrated early microvascular response to the CS insult. The time chosen for elevation of compartment pressure (45 min) was based on previous work demonstrating that 1 hour of ischemia in a rodent approximates 4 hours of ischemia in a human (Sheridan et al. 1977, Hulbert et al. 2007). The model was an attempt to characterize the early microvascular changes secondary to a compartment syndrome challenge.

Normal, continuous physiologic perfusion demonstrated an immediate response to CS, with a shift to intermittent and non-perfused capillaries producing non-nutritive perfusion with compromised gas exchange. This microvascular dysfunction was accompanied by a substantial inflammatory response measured in post-capillary venules (Figure 2.2). Leukocyte adherence was significantly increased, as demonstrated by the arrest of leukocytes under conditions of flow. The observed leukocyte recruitment suggested that CS induces a pro-inflammatory environment. A significant early increase in parenchymal injury was also detected.

#### *5.1.2.1 CS as Low-Flow Ischemia*

Despite the microvascular dysfunction secondary to the CS insult, a degree of continuous perfusion remained present, causing a partial ischemic environment, or “low-flow” ischemia within the limb. This allows neutrophils to be activated immediately, which may contribute to the degree of cellular injury noted (Harkin et al, 2001; Kurose et al, 1994). In contrast to complete ischemia, where revascularization leads to the reintroduction of oxygen into ischemic tissue in distinct phases of injury (ischemia, then reperfusion), the wash out of debris following reperfusion results in an increase in reactive oxygen metabolites, initiating an acute state of inflammation, with reactive metabolites triggering an increase in apoptosis and necrosis (Gute et al, 1998; Lum and Roebuk, 2001;

Schlag et al, 2001). The idea that CS is more injurious than pure ischemia has been corroborated by Heppenstall *et al* (1986) in comparing complete ischemia to CS in a canine model. Heppenstall *et al* (1986) observed that the CS stimulus caused severe acidosis and metabolic stress, rendering a more severe degree of muscle ultra-structural deterioration than ischemia alone. CS produced greater injury to muscle than complete ischemia, which may be due to the cytotoxic inflammation induced by this low-flow ischemic state. The experimental model designed and described in this thesis is reliable and simple to use for the study of microcirculation, inflammation and injury in acute CS, allowing for detailed study of the mechanisms underlying compartment syndrome.

### 5.1.3 Leukocyte Depletion

#### *5.1.3.1 Leukocyte Depletion and Perfusion*

This study was designed to define the contribution of inflammation to tissue injury in CS. By rendering the animals leukocyte deplete, a rigid experimental control was applied, affording the ability to define the relative contribution of inflammation to parenchymal injury in animals subjected to elevated ICP over time. In the original model discussed in Chapter 2, an early CS insult was utilized; with greater familiarity of the model use, the CS challenge could be carried out to 180 minutes. In this series of experiments we studied the effect of elevated ICP in a leukocyte deplete rodent model, assessing perfusion, inflammation and tissue injury, utilizing IVVM and fluorescent dye staining. Here, normal animals with intact immunity undergoing a CS challenge were compared with leukocyte-deplete experimental groups. Leukocyte depletion provided no advantage in the red blood cell flow rate or flow characteristics at 45, 90, 120 and 180 minutes of CS, as compared to controls. Leukopenia was not protective in restoring or maintaining perfusion in the face of elevated compartment pressure. This is divergent from the findings in similar experiments conducted, utilizing an ischemia-reperfusion model, which demonstrated benefit in microvascular perfusion in the absence of leukocytes (Forbes et al. 1996). Our data suggests that the effects of leukocytes on perfusion in CS are pathophysiologically distinct from a pure ischemia-reperfusion insult with respect to skeletal muscle microcirculation.

#### *5.1.3.2 Leukocyte Depletion: Inflammation and Injury*

Leukopenia significantly diminished leukocyte activation in the post-capillary venules at 45, 90, 120 and 180 minutes of CS as compared to controls ($p<0.05$) (Figure 3.2). This leukocyte reduction was accompanied by a greater than 50% reduction in tissue injury. This data suggests that inflammation contributes to cellular injury in experimental CS. Leukocyte-endothelial interactions in the conditions of trauma, injury, and ischemia are known to create a pro-inflammatory environment secondary to the upregulation of cytokines and chemokines, stimulating leukocyte activation and recruitment of polymorphonuclear leukocytes (PMNs) into the area of injury (Harlan  et al. 1991, Sabido et al. 1994, Gute et al. 1998, Ley and Reutershan 2006, Ley et al. 2007). Activated leukocytes produce reactive oxygen species and proteolytic enzymes, causing cellular damage, increasing permeability and edema, and resulting in increased interstitial pressure (Forbes al. 1996, Kurose et al. 1997, Gute et al. 1998), further compounding the pressure-induced injury seen in CS. Thus, our study demonstrates that inflammation should be considered central to the pathogenesis of cellular injury in CS.

### 5.1.4 CS and Systemic Inflammation

The purpose of this study was to determine if CS could produce a pro-inflammatory environment sufficient enough to cause remote organ injury, in order to further understand the mechanisms underlying the pathophysiology of CS. A hybrid model was applied for these experiments. The rodent normotensive CS model was combined with published models and technique designed for the study of SIR and remote organ injury, in response to hindlimb ischemia (Brock et al. 1999a, Brock et al. 1999b, Lawlor et al. 1999, Brock et al. 2001, Wunder et al. 2002, Wunder et al. 2004). We exposed Wistar rats to 2 hours of unilateral hindlimb CS, followed by fasciotomy. Blood samples were collected at regular intervals to assess TNF-α levels. Following fasciotomy and laparotomy, liver sinusoidal and venular blood flow was measured via liver exposure and IVVM. PI staining was used to measure hepatocellular death; leukocyte activation was used as a visual marker of the underlying inflammatory process.

This study found that the CS challenge resulted in a 7-fold increase in hepatocellular injury, and a 25-fold increase in the number of activated leukocytes, as compared to sham ($p<0.05$). The observed inflammation was accompanied by a rise in TNF-α, an acute-phase chemoattractant acting on neutrophils, promoting the expression of adhesion molecules and resulting in selective adhesion/transmigration of leukocytes (Ascer et al. 1992, Yi and Ulich 1992, Seekamp et al. 1993, Zhang et al. 2005). Our results demonstrated a two-hit inflammatory model due to the CS insult and the subsequent fasciotomy. It appears that CS is a sufficient hit to cause a significant initial rise in TNF-α (Figure 4.6), followed by fasciotomy causing a second peak in the systemic TNF-α levels.

In ischemia-reperfusion, cellular debris, pro-inflammatory mediators and cytokines gain full access to the systemic circulation leading to a systemic inflammatory response (Forbes et al. 1995, Harkin et al. 2001, Wakai et al. 2001, Katada et al. 2009). In CS, the washout of debris is suspected to be simultaneous to the pressure insult, and hence a rise in TNF-α levels is observed, which is further compounded by the fasciotomy allowing unrestricted access to the circulation. This indicates that CS may be even more pro-inflammatory than ischemia-reperfusion injury (Figure 5.1).

Thus, CS can be accompanied by a systemic inflammatory response and end organ damage, as evidenced by increased hepatic leukocyte count, and hepatocyte death.

Our findings suggest that unilateral hindlimb CS is a significantly injurious process, producing both local and systemic injury through an inflammatory mechanism. This lends support to the crucial role of inflammation in understanding the underlying pathophysiology of CS.

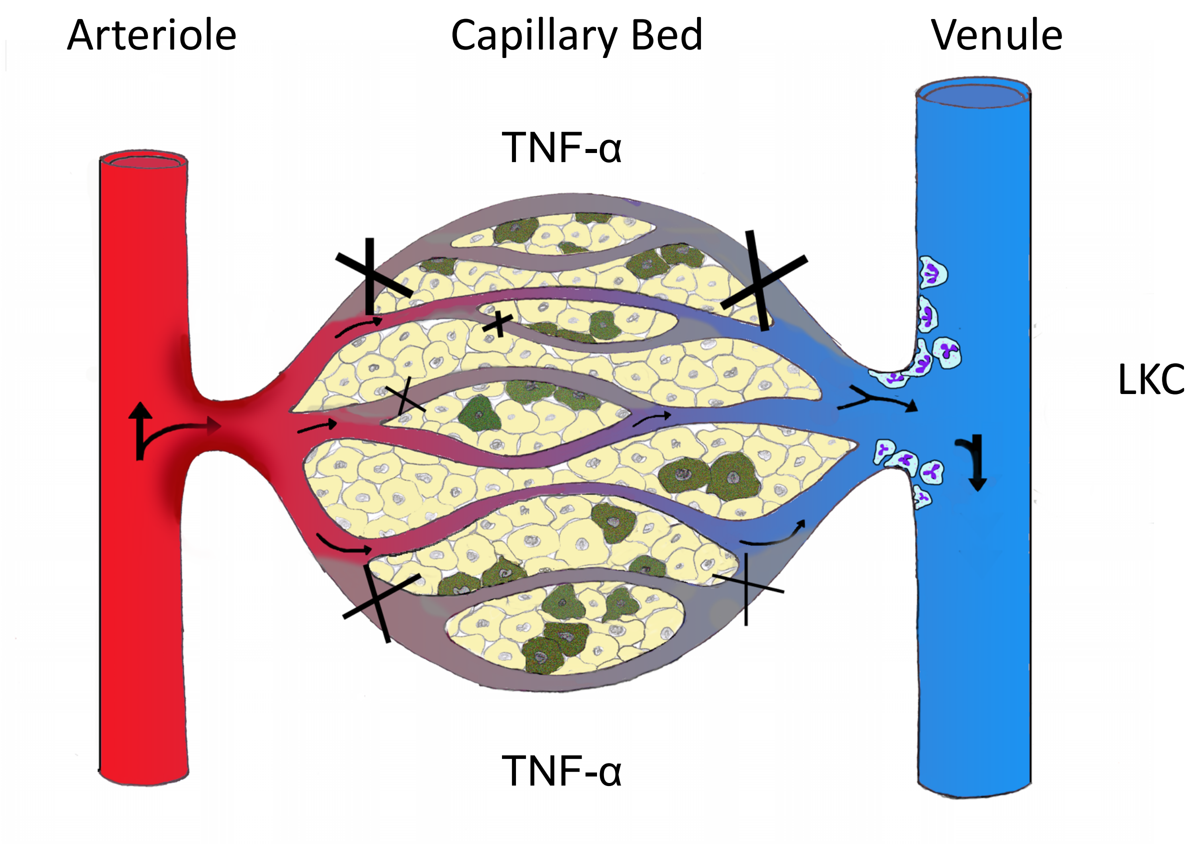


**Figure 5.1: New proposed conceptual model of CS.** Oxygenated blood flows from the arteriole through the capillary, unloading oxygen to cells. With elevated CS, NPC and IPC become visible within capillary beds and are ineffective at gas exchange (X), contributing to cellular injury (green). Furthermore, maintenance of capillary perfusion during CS allows for oxygenated blood into the compromised compartment, which may lead to reactive oxygen metabolites contributing to the chemotactic stimuli for the expression and activation of leukocytes (LKC). These in turn, stimulate production of TNF-α, further perpetuating tissue injury, affecting remote organs and producing end organ damage.

## 5.2 LIMITATIONS AND FUTURE DIRECTIONS

The significance of this thesis, lies in part, with the use of a clinically relevant model aimed at defining a revised conceptual framework for understanding the pathophysiology of CS. Distinguishing the pathophysiology of CS from ischemia-reperfusion has been emphasized in order to properly define the pathophysiology of CS. Inflammation has been demonstrated to be important to the injury process but how that may translate to human models is yet to be determined. The rodent is an extremely resilient animal and CS may have completely different functional outcomes, as compared to humans, secondary to CS. Understanding the functional deficits in rodents, is required to further validate the model. Recognizing the role of inflammation to the injury process raises the possibility of an entirely new role for pharmacologic adjuncts that may help salvage muscle when CS is diagnosed. Modulation of inflammation may or may not diminish myonecrosis in CS; however, further study is required to fully elucidate the mechanism causing such severe acute injury in CS.

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## APPENDIX A

## Animal Protocol Approval Letter

## APPENDIX A: ANIMAL PROTOCOL APPROVAL LETTER

Western

**12.02.09**
***This is the original approval for this protocol***
*A full protocol submission will be required in 2013*

Dear Dr. Lawendy:

Your animal use protocol form entitled:

Direct and Remote Organ Injury Following Hind Limb Compartment Syndrome

Funding agency Canadian Institute of Health Research – Remote Organ Injury Following Hind Limb Ischemia-Reperfusion – Grant #MOP 68848 has been approved by the University Council on Animal Care.

This approval is valid from **12.02.09 to 12.31.13** with yearly renewal required.

The protocol number for this project is **2009-083**.

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

**ANIMALS APPROVED FOR 4 YEARS**

| Species | Strain | Other Detail | Pain Level | Animal # Total for 4 years |
|---|---|---|---|---|
| Rat | Wistar | 150-350 g | C | 460 |

**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.**

***The* University *of* Western Ontario**
Animal Use Subcommitte/University Council on Animal Care
Health Sciences Centre London • Ontario • CANADA – N6A 5C1
PH: 519-661-2111, ext. 86770 • FL: 519-661-2028 • www.uwo.ca/animal

# APPENDIX B

# GENERAL METHODOLOGY

# APPENDIX B: GENERAL METHODOLOGY

## B1 EXPERIMENTAL ANIMALS

Seventy-five adult male Wistar rats (Charles River, Quebec, Canada) were used for the studies. The rats were individually housed in clear plastic cages with water and Purina rat chow available *ad libitum* in an illumination-controlled room (12:12 h light/dark cycle), at a temperature of 22±1ºC. The animals were randomly divided into 3 main groups: experimental rodent model studies, neutropenia experiments, and remote organ injury studies.

## B2 SURGICAL PREPARATION

All rats, weighing 175-250 g at the time of surgery, were anesthetized with isoflurane (5% induction, 2% maintenance) (Baxter, co) in a 1:1 $O_2$:$N_2$ mixture. Carotid artery was cannulated for the continuous monitoring of mean arterial pressure (MAP), blood sampling and fluid replacement.

## B3 COMPARTMENT SYNDROME INITIATION

An electronic compartmental pressure monitoring system (Synthes USA, Paoli, PA) was inserted into the anterior and/or posterior compartment through a 14-gauge angiocatheter (BD, Mississauga, ON). In animals undergoing CS, compartment pressure was elevated by slowly infusing isotonic normal saline via a 24-gauge angiocatheter into the anterior compartment of the left hind limb (Figure B1). Compartment pressure was raised to 30mmHg and maintained

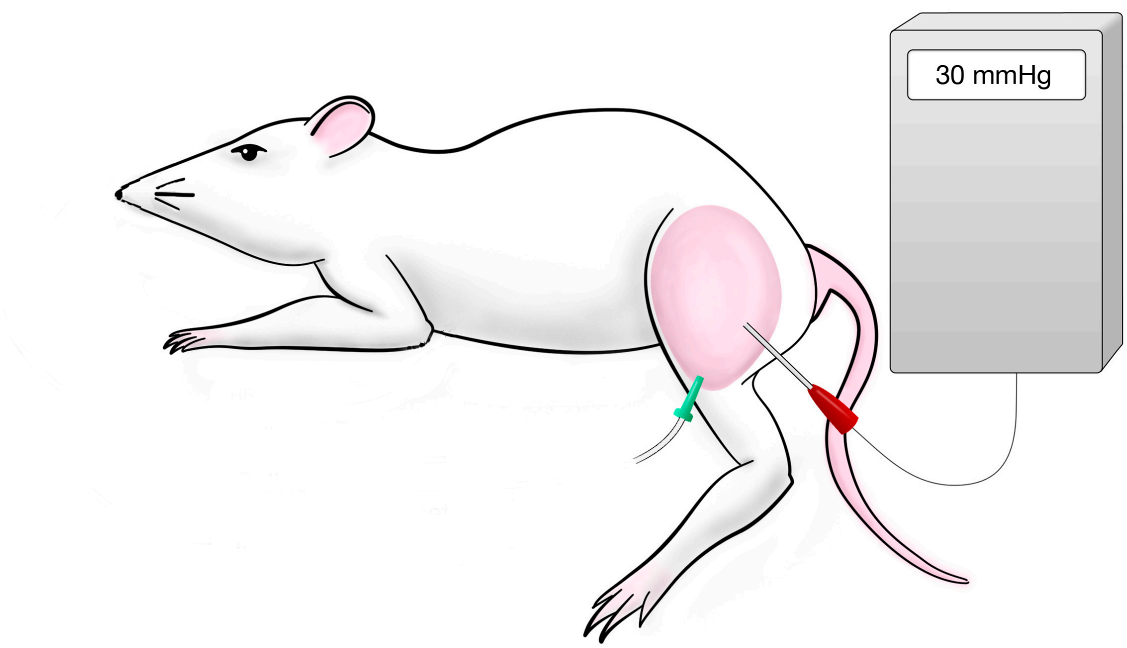


**Figure B1: Experimental Setup for Rat Compartment Syndrome**. Fluid is infused into the left hindlimb, raising the compartment pressure to, or above, 30 mmHg.

between 30-40mmHg for the duration of the protocol. In the control group, 24-gauge angiocatheter was inserted into the anterior compartment without the saline infusion.

Elevated compartment pressure was maintained for a specific period of time (described in detail in each subsequent study). The pressure was then relieved by the means of fasciotomy, and the animals were prepared for intravital video microscopy.

**B4 Leukopenia**

Neutropenia was induced in XX rats by a single intra-peritoneal injection of cyclophosphamide (CP) (250 mg/kg, Cytoxan®, Baxter Corporation), 72 hours prior to experiments.

**B5 INTRAVITAL VIDEO MICROSCOPY (IVVM)**

***B5.1 IVVM OF SKELETAL MUSCLE***

**B5.1.1 EDL Exposure**

The Extensor Digitorum Longus (EDL) muscle was used for all IVVM studies. The EDL dissection began by incising the skin over the posterior aspect of the hindlimb. The underlying *biceps femoris* muscle was retracted to expose the *tibialis anterior* and the *lateral gastrocnemius* muscles. The latter two muscles were divided to expose the EDL. The overlying fascia was incised. A suture ligature was applied around the distal tendon of the EDL. The tendon was then cut from its bony insertion to allow the EDL to be reflected onto the microscope stage with its proximal arterial and venous pedicle intact. Once prepared, animals were placed onto the stage of an inverted microscope (Nikon Diaphot 300) and the EDL was reflected onto a slide with saline bath. A cover slip was placed on top of the EDL, and all exposed tissues were covered with a plastic film, to isolate the preparation from the atmosphere and to prevent drying. A heat lamp maintained the EDL muscle temperature (32°C) as well as the core temperature (37°C) of the rat. Care was taken to ensure that the time from fasciotomy to the first microscopy recording was no more than 5 minutes.

### B5.1.2 EDL Microcirculation

Five fields of view containing a complete microvascular unit (arteriole, capillary bed, and post capillary venule) were randomly chosen, and recorded onto video using a 20X objective, for a final magnification of 700X at the monitor. The microscope was connected to a charged-coupled device camera (Dage-MTI VE1000), a time-date generator (WJ-810, Panasonic), and a computer equipped with a video capture card (Pinnacle DV500). Appropriate white light illumination was obtained using fiber-optic guides. One-minute video recording of each field of view was obtained post-fasciotomy and stored on the computer for analysis.

An index of compartment syndrome-induced microvascular dysfunction was determined by counting the number of continuously-perfused, intermittently-perfused and non-perfused capillaries crossing three equidistant parallel lines drawn on the computer monitor, perpendicular to the capillary axis, and expressed as the percent of total number of capillaries (Figure B2). A non-perfused vessel had no red blood cell movement at all, while an intermittently-perfused capillary exhibited some stoppage of the flow during the recording.

### B5.1.3 Tissue Injury

Following fasciotomy, fluorescent vital dyes ethidium bromide (EB) (5μg/mL) (Sigma Aldrich, Mississauga, ON), and bisbenzimide (BB) (5μg/mL) (Sigma Aldrich, Mississauga, ON) were added to the saline bath. BB, a membrane-permeant dye, stains the nucleus of all cells. EB, a larger molecule, is membrane impermeant, and hence it stains only the nuclei of cells with injured (permeable) membranes. Fluorescent illumination with the appropriate filters for EB (Ex = 482 nm; Em = 610 nm) and BB (Ex = 343 nm and Em = 483 nm) were applied. Tissue injury was examined in 5 fields of view. Cellular injury was expressed as the ratio of ethidum bromide-labelled nuclei to bisbenzimide-labelled nuclei (EB/BB).

### B5.1.4 Leukocyte Behaviour

Leukocyte rolling and adherence were observed in post-capillary venules using the 40x objective (final magnification, 1400X) post fasciotomy from each of the 5 fields of view. The total number of rolling and adherent leukocytes were measured over 30 seconds and expressed as the number per 1000$\mu m^2$ (Figure B3). Venular area was measured using ImageJ software (NIH, Bethesda, MD). An adherent leukocyte was defined as a cell that remained stationary for a minimum of 30 seconds.

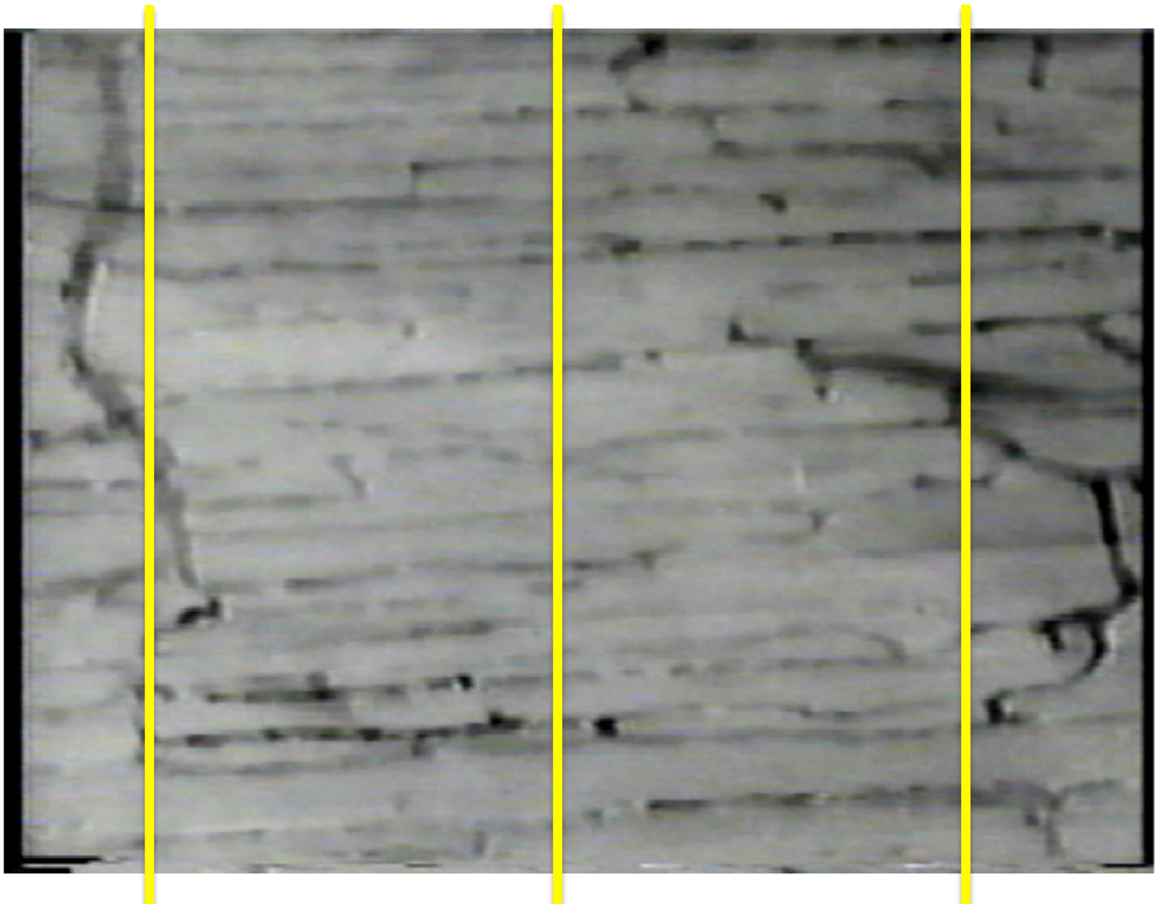

**Figure B2: Example of Skeletal Muscle Microvascular Perfusion**. An index of CS-induced microvascular dysfunction was determined by counting the number of continuously-perfused, intermittently-perfused and non-perfused capillaries crossing three equidistant parallel lines (*yellow lines*) drawn on the computer monitor, perpendicular to the capillary axis, and expressed as the percent of total number of capillaries.

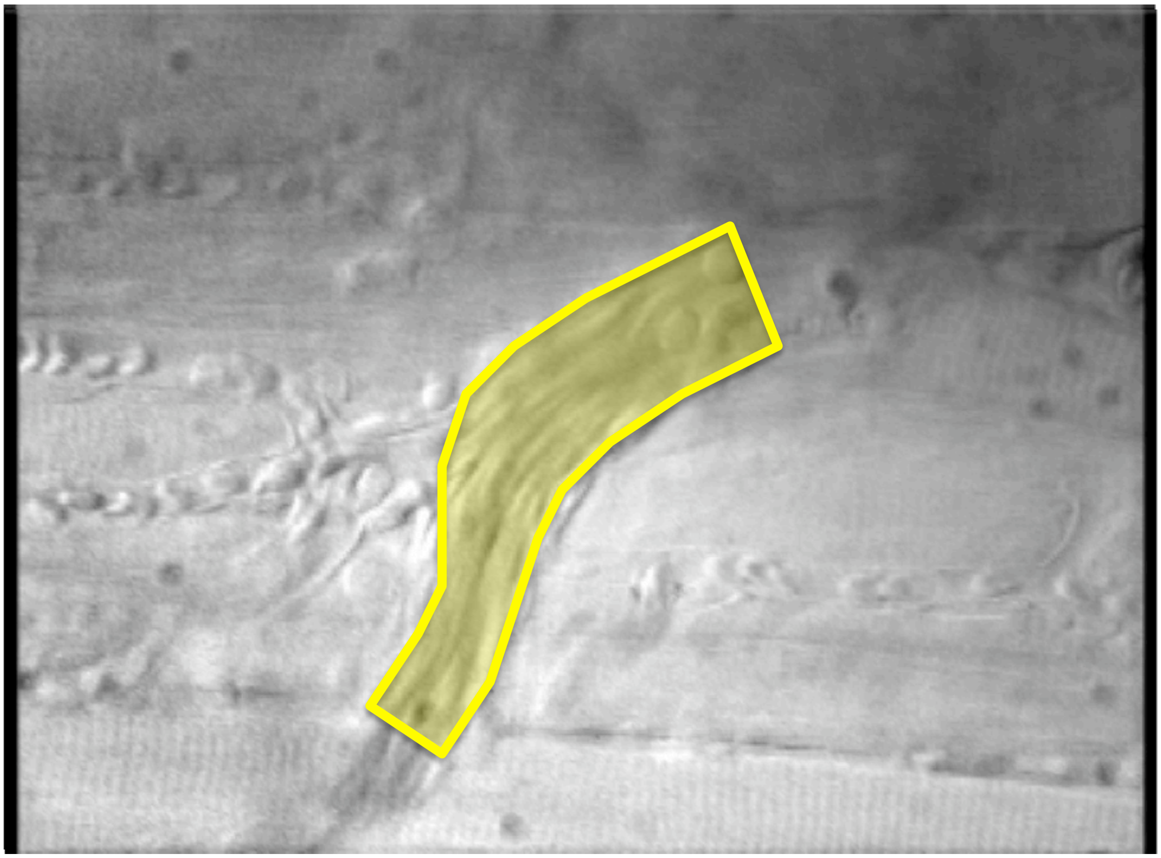

**Figure B3: Leukocyte Behaviour Analysis within the Post-Capillary Venule**. The total number of rolling and adherent leukocytes (*asterisk*) were measured over 30 seconds and expressed as the number per 1000μm$^2$. Venular area was measured using ImageJ software (*yellow mask*).

*B5.2 LIVER IVVM*

### B5.2.1 Liver Preparation

Following midline laparotomy, animals were placed onto the stage of an inverted microscope (Nikon Diaphot 300) and the liver was reflected onto the slide moistened with saline. All exposed tissues were covered with a plastic film, to isolate the preparation from the atmosphere and to prevent drying. A heat lamp and a warm saline bath maintained the liver temperature (32°C) as well as the core temperature (37°C) of the rat.

### B5.2.2 Liver Microcirculation

Eight to 12 fields of view of liver microcirculation were randomly chosen, and recorded using a 20X objective, for a final magnification of 700X at the monitor. Additional 8 fields of view of sinusoidal microcirculation, and up to 12 post-sinusoidal venules were recorded using 40X (final magnification 1400X), to assess the volumetric flow and leukocyte behaviour, respectively.

### B5.2.3 Sinusoidal Diameters, Perfusion and Volumetric Flow

Sinusoidal diameters were measured using ImageJ software, by averaging 3 different points along each sinusoid, and expressed in μm. Velocity of red blood cells was assessed within each sinusoid by using frame-by-frame analysis and expressed as μm/s. Volumetric flow (VQ) in pL/s, and shear ($\gamma$) ($s^{-1}$) were calculated using the formulas $VQ = \pi r^2 \times V$ and $\gamma = 8V/D$, respectively.

Sinusoidal perfusion was evaluated using well-described established stereological techniques (Brock et al, 1999) Hepatic microcirculation was classified as continuously-, intermittently-, or non-perfused based on flow characteristics observed during IVVM in 1-minute intervals. Continuous RBC perfusion during direct observation was classified as a continuously perfused sinusoid. A sinusoid whereby RBC perfusion was arrested and regained flow was classified as an intermittently-perfused sinusoid. Sinusoids that had no observable RBC movement for the duration of the observation period were classified as non-perfused sinusoids. The number of sinusoids was expressed as a percentage of the total number of sinusoids evaluated.

### B5.2.4 Hepatocyte Death

Fluorescent vital dye, propidium iodide (PI) (5μg/mL) (Sigma Aldrich, Mississauga, ON), was added to the saline bath. PI is highly membrane-impermeant, and hence it stains only the nuclei of lethally injured cells. Fluorescent illumination with the appropriate filter for PI (Ex = 535 nm; Em = 617 nm) was applied. Hepatocyte death was assessed, and expressed as the number of PI-labelled cells per $0.1mm^3$.

### B5.2.5 Leukocytes in Sinusoids and Post-Sinusoidal Venules

Leukocytes were observed in sinusoids and post-sinusoidal venules (PSV) using the 40X objective (final magnification, 1400X) from each of the recorded fields of view. The total number of rolling and adherent leukocytes in PSV were measured over 30 seconds and expressed as the number per 10,000μm$^2$. Venular area was measured using ImageJ software (NIH, Bethesda, MD). An adherent leukocyte was defined as a cell that remained stationary for a minimum of 30 seconds.

**B6 CYTOKINE ANALYSIS**

Blood, skeletal muscle and liver tissues were collected from all animals. The tissues were flash-frozen in liquid nitrogen and stored at -80°C until needed. Serum was obtained by allowing the collected blood to clot at room temperature for 30 minutes, followed by centrifugation at 1,500xg for 20 minutes at 4°C. The supernatants (serum) were collected and stored at -80°C.

Liver and skeletal muscle tissues were homogenized in 0.1M phosphate buffer saline with PMSF at 1:10 ratio, centrifuged at 6,000xg, 4°C to get rid of cellular debris. The collected supernatants were used to run the ELISAs. Total protein concentration was assessed by serial dilutions of 20 μl of each sample and comparing it to the known concentrations of bovine serum albumin (BSA), using detergent-compatible assay (DC Assay, Bio-Rad, Mississauga, ON).

**B6.1 TNF-α ELISA**

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 run the standard curve. Serum samples were diluted at 1:1 ratio with sample diluent buffer. All plate incubations were carried out at room temperature. 50μl of each sample or standard were added to the appropriate designated wells on a 96-well plate, and incubated for 1 hour. After washing the plate three times, 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 of 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.

**B7 STATISTICAL ANALYSIS**

Statistical analysis of all data was performed using Prism software, version 4.0c for Mac (GraphPad Software Inc., San Diego, CA). All parameters were analyzed using a repeated measures two-way analysis of variance testing (ANOVA). Statistical significance was defined as $p<0.05$.

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Lawendy AR, Sanders DW, Bihari A, Parry N, Gray D, Badhwar A. Compartment syndrome-induced microvascular dysfunction: an experimental rodent model. Canadian Journal of Surgery 2011, Jun 54 (3): 194-200.

Dr. Lawendy is the primary author of the study; he would like to include a version of the article as a chapter in his PhD thesis. The proof of permission is required to be included as an appendix in the thesis.

The contact info is as follows:

Abdel-Rahman Lawendy, MD, FRCSC

If you have any questions or concerns, do not hesitate to contact me, or Dr. Lawendy directly.
Thank you in advance.

Best regards,

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# APPENDIX D

# SURGICAL APPROACH TO LEG COMPARTMENT SYNDROME

# APPENDIX D: SURGICAL APPROACH TO LEG COMPARTMENT SYNDROME

The surgical techniques for complete fascial release have been well studied in the leg. Three techniques are most commonly described: two-incision fasciotomy, single incision perifibular fasciotomy, and fibulectomy. Our preferred method is the double incision technique, which allows for adequate visualization of all compartments, assessment of muscle viability, and sufficient surgical control to avoid neurovascular structures. The single incision four-compartment fasciotomy without fibulectomy is safe and can be useful in cases where soft tissue trauma or contamination is of concern, including situations in which only a single vessel perfuses the leg, or when flap coverage may be necessary. Kelly and Whiteside (1967) described a four-compartment release with fibulectomy performed through one lateral incision. This technique takes advantage of the fascial anatomy as all the fascial membranes insert onto the fibula. However, this method is technically challenging, may place the peroneal vessels at risk, and sacrifices the fibula which is usually unnecessary. Both the double and single incision technique are sufficiently effective at decreasing intracompartmental pressure (Mubarak and Owen, 1977; Vitale et al, 1988).

Subcutaneous fasciotomy is a technique in which the fascia is incised blindly with dissecting scissors through a small skin incision (Hutchinson and Ireland, 1994). Advantages to this technique include technical ease and cosmesis. However, access is limited to the deep posterior compartment and the neurovascular bundle. As well, it is now recognized that intact skin may not allow for complete release of intracompartmental pressure.

Small incision fasciotomy, as well as endoscopically assisted fasciotomies, may have a role in chronic exertional compartment syndrome (Apaydin et al, 2008; Hutchinson et al, 2003; Leversedge et al, 2002). However these techniques should not be used in acute compartment syndrome as recurrence of limb threatening ischemia may occur despite fascial release when the skin is left intact (Illig et al, 1998). In acute compartment syndrome, the skin is an important boundary of all compartments that must be released to achieve the greatest decrease in intracompartmental pressure.

**D1. Surgical Technique: Single-Incision Fasciotomy (Davey et al, 1984)**

The patient is positioned supine with a bump under the hip. Tourniquet is applied and not insufflated. The limb is prepped and draped free. Begin with a single longitudinal, lateral incision in line with the fibula (Figure D1). The incision extends from the fibular head to 3 cm proximal to the lateral malleolus. The superficial peroneal nerve is at risk toward the distal aspect of the incision. Skin flaps are developed anterior and a longitudinal fasciotomy of the anterior and lateral compartments is performed with dissecting scissors. Next, develop a posterior flap and perform a fasciotomy of the superficial posterior compartment. Identify the interval between and superficial and lateral compartments distally and develop this interval proximally by detaching the soleus from the fibula. Subperiosteally dissect the flexor hallucis longus from the fibula. At this point, all

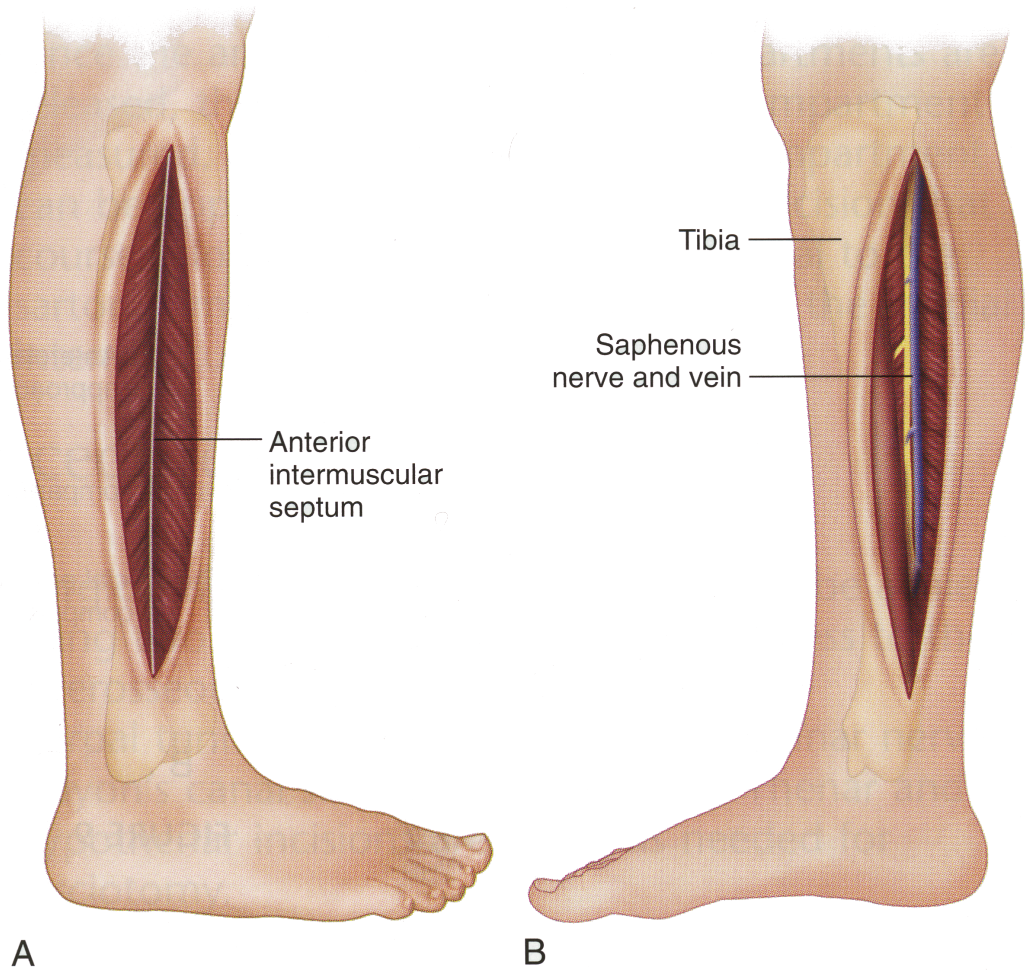


**Figure D1: Single-incision fasciotomy**. (**A**) Lateral aspect and (**B**) medial aspect of the leg. The incision extends from the fibular head to 3 cm proximal to the lateral malleolus. Skin flaps are developed anterior, and a longitudinal fasciotomy of the anterior and lateral compartments is performed with dissecting scissors. Posterior flap is developed and a fasciotomy of the superficial posterior compartment is performed. Wounds are packed open or the skin may be loosely closed over suction drains.

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four compartments have been decompressed. However, on occasion tibialis posterior exists within a self-contained fascial envelope and therefore, it is beneficial to continue the deep dissection until tibialis posterior is decompressed. Retract the muscle and the peroneal vessels posteriorly. Identify the fascial attachment of the tibialis posterior muscle to the fibula and incise this fascia longitudinally. Wounds are packed open or the skin may be loosely closed over suction drains.

**D2. Surgical Technique: Two-Incision Fasciotomy (Mubarak and Hargens, 1981)**

Patient is positioned supine, tourniquet applied and not insufflated. The limb is prepped and draped free. Begin with a 25 cm incision in the anterior compartment, centered halfway between the fibular shaft and the crest of the tibia (Figure D2). Subcutaneous dissection is utilized for wide exposure of the fascial compartment. Expose the lateral intermuscular septum and identify the superficial peroneal nerve lying posterior to the septum. Using dissecting scissors, release the anterior compartment proximally and distally in line with the tibialis anterior. Access the lateral compartment and perform a fasciotomy of the lateral compartment proximally and distally in line with the fibular shaft. A second longitudinal incision 2 cm posterior to the posterior margin of the tibia is made. Skin flaps are elevated and the saphenous vein and nerve are identified and protected. The septum between the deep and superficial posterior compartments is identified and the fascia over the gastrocsoleus complex is released over its

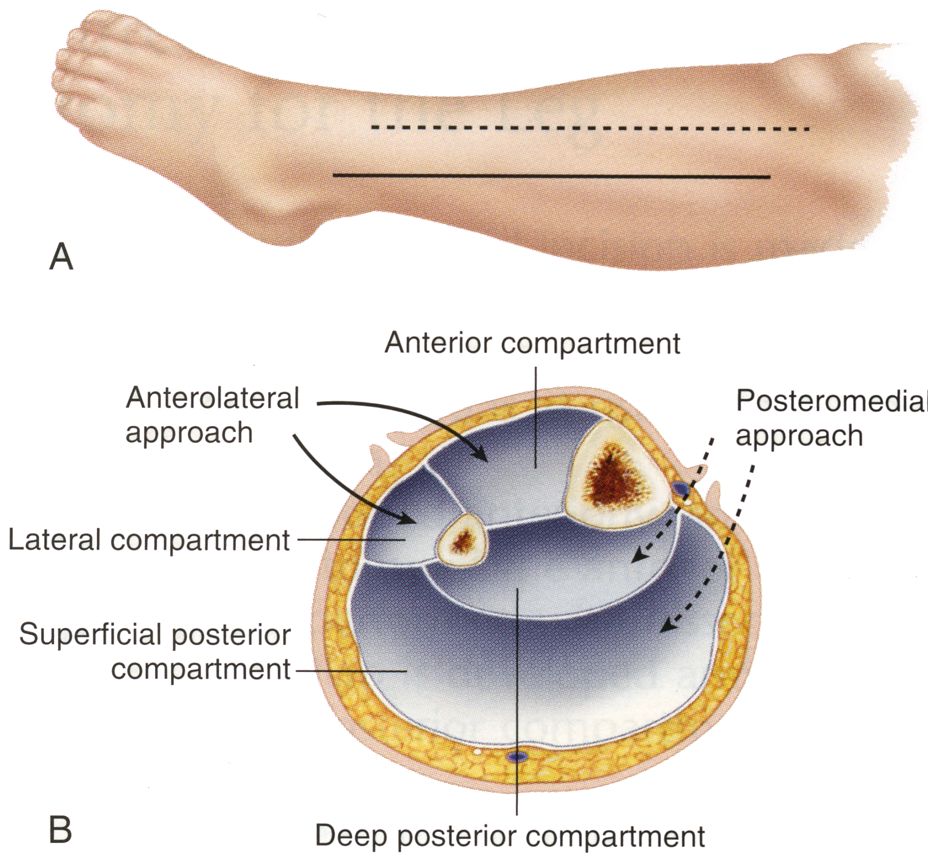


**Figure D2: Two-incision fasciotomy**. An incision in the anterior compartment is centered halfway between the fibular shaft and the crest of the tibia. Lateral intermuscular septum is exposed and the superficial peroneal nerve is identified. The anterior compartment is released proximally and distally in line with the tibialis anterior. Fasciotomy of the lateral compartment proximally and distally in line with the fibular shaft is performed. A second longitudinal incision 2 cm posterior to the posterior margin of the tibia is made; fascia over the gastrocsoleus complexis released over its entire length. Then, the deep posterior compartment is released via fascial incision over the flexor digitorum longus.

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entire length. Make another fascial incision over the flexor digitorum longus muscle and release the entire deep posterior compartment. As the dissection is carried proximally, if the soleus bridge extends more than halfway down the tibia, release this extended origin. After release of the posterior compartment, identify the tibialis posterior muscle compartment. If increased tension is evident in this compartment, release it over the extent of the muscle belly. Pack the wound open and apply a posterior plaster splint with the foot plantigrade.

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