ABSTRACT OF RESEARCH PLAN

INVESTIGATOR NAME/INSTITUTION	PROJECT TITLE
INSTITUTION: University of Western Ontario, London, Ontario, Canada	**Therapeutic application of carbon monoxide (CO), liberated from a novel CO-releasing molecule (CORM-3), in a large animal model of limb compartment syndrome.**

Abstract of research plan: Please provide a 250 word abstract with 5 underlined phrases for project summary, to fit in the box below.
Avoid summaries of past accomplishments and the use of the first person. The abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application.

Acute limb compartment syndrome (CS) is a common clinical problem occurring as a consequence of musculoskeletal trauma. CS is characterized by an increase in pressure within a closed osseofascial compartment, resulting in limb-threatening ischemia. Fasciotomy to decompress involved muscles is the primary current treatment option. Previous research has demonstrated that a leukocyte-driven inflammatory response is also involved in the development of muscle damage in addition to ischemia alone. Pharmaceutical interventions may have a role in reducing muscle damage by reducing the inflammatory component of CS. Carbon monoxide (CO), a byproduct of heme metabolism, has a protective effect against ischemia-reperfusion-based muscle injury. CO-releasing molecules (CORMs) have received attention as a safe pharmaceutical delivery system for CO. Our hypothesis is that CORM-derived CO offers protection against muscle dysfunction and remote organ injury during compartment syndrome. This study will examine the potential of CO-dependent modulations of the inflammatory response in an experimental model of CS. Saline is infused into the anterior compartment of Yorkshire-Landrace pig hindlimb, leading to a sustained elevation of compartment pressure for up to 6 hours. Fasciotomy is then performed, and the skeletal muscle studied using intravital video microscopy (IVVM). Animals will be injected with either a CO donor (CORM-3) or its inactive form. Muscle perfusion, inflammation and tissue injury will be quantified. Serum will be analyzed for cytokines, and leukocytes for activation. Skeletal muscle tissue will be examined for apoptosis and necrosis. Using this data, the efficacy of CORM-3, a potential novel pharmaceutical treatment for CS, will be evaluated.

Research Plan

A) SCIENTIFIC AIMS.

Acute limb compartment syndrome (CS) is a potentially devastating complication of musculoskeletal trauma. CS is characterized by an increase in pressure within a closed osseofascial compartment, resulting in muscle necrosis, with the potential to progress to limb-threatening ischemia[1-5]. Ischemia and inflammation both contribute to muscle necrosis. At present, surgical fasciotomy (to decompress the muscles and restore perfusion) remains the only treatment and current gold-standard surgical therapy.

CS often presents a diagnostic and therapeutic challenge in orthopedic traumatology; its consequences may be severe for both the patient and treating surgeon. The early diagnosis of CS is critical to its surgical management and avoidance of disability and litigation. The impact and magnitude of injury in CS is time-dependent, with a surgical window of 4 – 8 hours before complete and irreversible damage is sustained.

This study is designed to determine whether CORM-derived CO offers protection against ischemic and inflammatory derived tissue dysfunction. ***We hypothesize that CORM-3 administration restores microvascular perfusion, reduces leukocyte activation, and reduces muscle tissue damage in an in vivo porcine model of compartment syndrome.***

In this proposal, we will investigate two specific objectives/aims designed to define the effects and mechanisms of action of CO-dependent modulation of the inflammatory response in a large-animal (porcine) experimental model of CS:

Objective 1: To establish a large-animal model of CS; first-line *in vivo* studies;

Objective 2: To define the effects of CORM-3-derived CO on skeletal vascular endothelial cell dysfunction in CS, for development of potential CS therapeutic interventions.

We believe that the current study is the next step to advance the development of new, mechanism-based strategy to minimize CS-induced tissue damage, and thus prolong the "surgical time window" in the presence of an impending compartment syndrome. We are well positioned to define and provide pharmacological management of CS as a viable adjunct to fasciotomy. This may have profound effect in reducing the morbidity associated with CS and CS-induced disability to the patients. It will also further our understanding of specific mechanisms that lead to myonecrosis, and, potentially, open avenues for further pharmacologic interventions employing gaseous modulators of inflammation.

B) BACKGROUND & SIGNIFICANCE.

One of the most devastating complications of musculoskeletal trauma is acute limb compartment syndrome (CS). CS, characterized by an increase in pressure within a closed osseofascial compartment, results in muscle-threatening and, ultimately, limb-threatening ischemia[1-6]. Fasciotomy (to fully decompress all the muscles in the involved compartments) remains the only effective treatment and current gold-standard surgical therapy. Extremity CS occurs once swelling within a muscle compartment develops to such a degree that the tissue perfusion becomes compromised. The established view of the pathophysiological process of CS development is that increasing compartmental pressure compromises microcirculatory perfusion, thus restricting oxygen and nutrient delivery to vital tissues, ultimately resulting in cellular anoxia and severe tissue necrosis[1, 3-6]. Unlike complete ischemia, CS causes myonecrosis in the face of patent vessels. As such, the pathologic contribution of inflammation to the pathophysiology of CS is being increasingly recognized; studies from our group[7-9] and others[10, 11] have broadly implicated leukocytes as playing a primary role in both microvascular and parenchymal injury during CS.

Despite active investigation, few therapeutic options have been shown to be effective. Recently, carbon monoxide (CO), a byproduct of heme oxygenase (HO-1) activity has been shown to offer both protection to microvascular perfusion, and anti-inflammatory benefits during systemic inflammation. Although the exogenous administration of CO via inhalation (250ppm) has been shown beneficial during SIRS[12], such method of administration results in increased carboxyhemoglobin (COHb) levels, thus presenting a potential threat to the host.

Lately, transitional metal carbonyls, CO-Releasing Molecules (CORMs) have been used to deliver CO in a controlled manner without significantly altering COHb. The major advantage of using CORMs versus inhaled CO is the ability to control CO delivery without significantly increasing COHb, and choice of various routes (intravenous, intraperitoneal, subcutaneous or tissue superfusion) of CO administration to target specific organs/tissues. Consequently, CORMs have received an increased attention for the potential pharmaceutical application[13].

Our data employing rat model of CS indicates that systemic administration of a water-soluble CO donor, CORM-3, markedly attenuates inflammatory response in CS-challenged skeletal muscle (hence injury), and is able to partially restore microvascular perfusion[14, 15]. These results appear very promising; however, before CORM-3 can be tested in humans, it must be assessed in a large animal model (close to humans).

We expect that CORM-3 will be able to significantly improve function of CS-challenged muscle. This would have a profound effect in reducing the morbidity associated with CS and CS-induced disability to the patients.

C) PREVIOUS WORK DONE ON THE PROJECT.

We have developed a novel rodent model to study the microvascular effects of compartment syndrome (CS) in the hindlimb skeletal musculature[7]. Briefly, compartment syndrome is induced by an infusion of isotonic saline into the anterior compartment of the rat hind limb, producing a sustained increase in intracompartmental pressure (ICP). Following a set period of ICP, a fasciotomy is performed and the microcirculation, cellular injury and inflammatory response within the extensor digitorum longus (EDL) muscle are studied using intravital video microscopy (IVVM). Previously, we have demonstrated that the induction of compartment syndrome results in (a) a decrease in the overall level of capillary perfusion and functional capillary density, (b) an increase in acute and irreversible cell injury, and (c) an increase in inflammation[7]. We have shown that varying the duration of compartment syndrome has resulted in a variation in the severity of the microvascular dysfunction, the cellular injury and the inflammatory response.

Recently, carbon monoxide (CO), a byproduct of heme metabolism, has been shown to offer both protection to microvascular perfusion, and anti-inflammatory benefits during systemic inflammation. Transitional metal carbonyls, CO-releasing molecules (CORMs) have been used to deliver CO in a controlled manner without significantly altering COHb. The major advantage of using CORMs versus inhaled CO is the ability to control CO delivery without significantly increasing COHb, and choice of various routes (intravenous, intraperitoneal, subcutaneous or tissue superfusion) of CO administration to target specific organs/tissues. CORMs have been shown to act pharmacologically in rat aortic and cardiac tissue, where liberation of CO produced vasorelaxant effects, decreased myocardial ischemia/reperfusion damage, and reduced inflammatory response in LPS-stimulated macrophages[16-18].

CO is delivered to the organ of interest in the form of a novel, water-soluble CO-releasing molecule (CORM-3), with its inactivated version (iCORM-3) as a control. We have demonstrated that the systemic application of CORM-3 (10mg/kg IP, given upon fasciotomy) in the rat was able to attenuate the CS-associated skeletal muscle microvascular dysfunction, tissue injury and inflammation[14].

To further explore the causes and mechanisms responsible for microvascular dysfunction during CS, we examined the role of pro-inflammatory cytokines, both systemically and locally (within the skeletal muscle) during CS. We have demonstrated that leukocyte activation appears to play a role in the tissue injury. While the mechanism(s) driving the CS-induced injury are not clear, it appears that it may be, at least in part, mediated by a systemic release of inflammatory cytokines/chemokines, especially TNF-α (Figure 1).

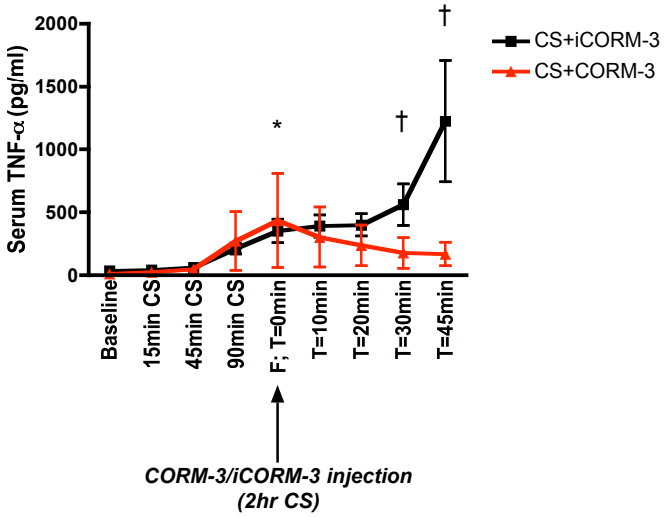

Figure 1. **The effect of CORM-3 on systemic TNF-a levels in CS**. Rats were subjected to 2hr CS and injected with CORM-3 (or its inactive form, iCORM-3) immediately upon fasciotomy. Serum TNF-a levels were quantified at each time point indicated. *Post-fasciotomy TNF-a elevation was reversed by CORM-3 application* (*p<0.01 from baseline; †p<0.001 from CS+iCORM-3). *F*, fasciotomy.

D) METHOD.

In order to test the effects of CORM-3 on humans, it must be tested in a large animal model of CS – something more akin to humans than rodents. Therefore, in this proposal, we will focus on two specific aims designed to address the effects and potential mechanisms of CO-dependent modulation of the inflammatory response in a large-animal (porcine) experimental model of CS: 1. Establish a large animal model of CS (first-line *in vivo* studies); 2. Define the effects of CORM-3 derived CO on skeletal vascular endothelial cell dysfunction in CS, for the development of potential CS therapeutic interventions. We hypothesize that *CORM-3 administration will restore microvascular perfusion, reduce leukocyte activation, and reduce muscle tissue damage in an in vivo porcine model of compartment syndrome.*

Objective 1: To establish a large-animal model of CS: first-line *in vivo* studies.

All animals will be taken care of in accordance with the Canadian Council on Animal Care standards. Protocol approval from the University of Western Ontario has been obtained (see the attached approval letter).

Porcine model of Compartment Syndrome (CS): We will utilize an infusion of isotonic saline into the anterior compartment of the swine hind limb, resulting in a sustainable increase in intracompartmental pressure (ICP) up to 30mmHg. Following a defined time interval of elevated ICP (6 hours, to maintain clinical relevance), fasciotomy will be performed and the limb will be allowed to reperfuse for 3 hours; the microcirculation within the peroneus longus muscle will be visualized using hand-held intravital video microscopy (IVVM), utilizing orthogonal polarization spectral (OPS) imaging.

Blood sampling: Blood will be sampled at various time intervals during CS (1hr, 2hr, 3hr, 4hr, 5hr, 6hr) and post-fasciotomy (10min, 20min, 30min, 40min, 50min, 1hr, 2hr, 3hr), to measure levels of LDH, serum myoglobin and inflammatory cytokines (X-MAP multiplex analysis, sampling 24 different cytokines/chemokines).

Fluorescence labelling: Just before the conclusion of the experiment, animal will be injected, through the femoral artery, with fluorescent (vital) dyes (ethidium bromide/bisbenzimide staining, EB/BB) to obtain levels of cellular injury, and an *in vivo* fluorescent marker of apoptosis (FLIVO).

Histology: At the conclusion of the experiment, just before euthanasia, the peroneus longus muscle will be biopsied and fixed in formalin. Samples will be paraffin-embedded and sectioned. Slides will be analyzed for inflammation/leukocyte infiltration (H&E staining) and fluorescence (apoptosis-FLIVO, cell death-EB/BB).

We estimate 2-3 pigs will be required to obtain the baseline data. One to two IVVM experiments per month can be undertaken (subject to large animal OR availability); thus, all data from this part of the proposal should be accumulated within 3 months.

Objective 2: To define the effects of CORM-3-derived CO on skeletal vascular endothelial cell dysfunction in Compartment Syndrome (CS): *in vivo* studies

CORM-3 application in porcine model of CS: Pigs will undergo 6 hours of CS, as described in Objective 1. Animals will be treated with CO-donor, CORM-3 (5mg/kg, IV), or its inactive form, iCORM-3 (CORM-3 deprived of CO) as a control immediately upon fasciotomy, and allowed to reperfuse for 3 hours. CORM-3 will be synthesized by us, as described previously[15, 17]. Microcirculation will be imaged with hand-held IVVM (OPS imaging). Blood will be sampled at various time intervals (during CS and post-fasciotomy), to measure levels of LDH, serum COHb, myoglobin and pro-inflammatory cytokines.

Inflammatory cytokines: Using our rat model of CS, we have shown that TNF-α appears to play a role in the initiation of the CS injury. Thus, we will determine the time-course of TNF-α levels in the

serum of pigs treated with CORM-3 or iCORM-3 during various time points in the development of CS (1hr, 2hr, 3hr, 4hr, 5hr, 6hr) and after fasciotomy (10min, 20min, 30min, 40min, 50min, 1hr, 2hr, 3hr). In addition, 23 other different cytokines/chemokines will be tested through the use of X-MAP Luminex technology (Multiplex assay).

Leukocytes, apoptosis and cellular injury: The markers of organ/tissue dysfunction such as leukocyte infiltration, cellular injury (BB/EB staining) and apoptosis (FLIVO) will be assessed, in tissue sections, employing standard histological techniques.

Polymorphonuclear cells (PMN) activation: PMNs will be isolated from blood at each time point; their activation (i.e. superoxide production) will be tested immediately, by oxidation of superoxide-specific substrate, L-012.

Statistical analysis: Results for cytokine expression, leukocyte activation, leukocyte accumulation within the tissue, microvascular perfusion, apoptosis and cellular injury for each of the groups (CORM-3, inactive CORM-3 and control) will be compared using ANOVA; $p<0.05$ will be considered significant.

We estimate 5-6 pigs per group will be required to show a significant difference at an 85% confidence level; therefore, an additional total of 12 pigs (6 CORM-3, 6 iCORM-3) will be required for this part of the proposal. Following the use of 6 pigs, analysis will be performed to determine if significance was reached. One to two IVVM experiments per month can be undertaken; thus, all data from this part of the proposal should be accumulated within 12 months.

E) REFERENCES

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F) FIGURES

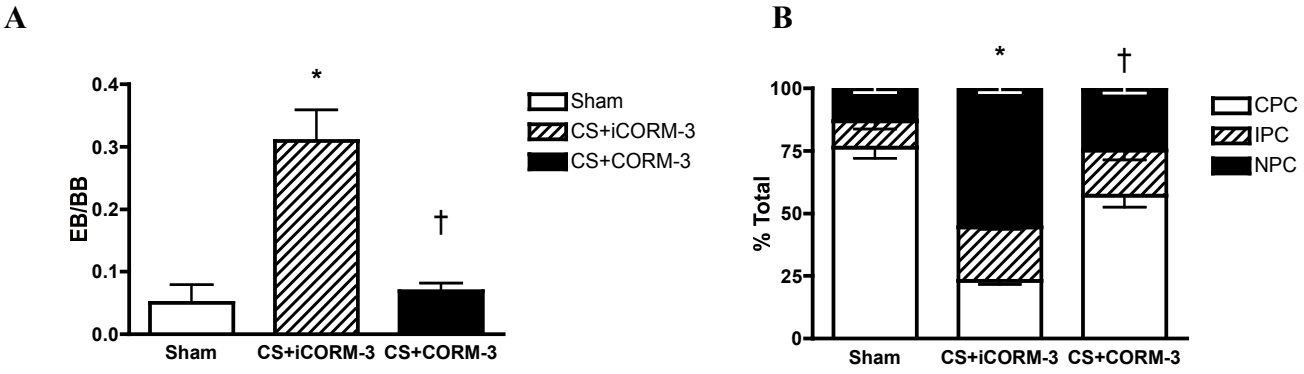

Figure 2. **The effect of CORM-3 on A) levels of cellular injury (EB/BB staining) and B) perfusion in the skeletal muscle following CS**. Rats were subjected to 2hr CS, injected with CORM-3 (or its inactive form, iCORM-3) immediately upon fasciotomy, and assessed for tissue injury/perfusion 45min later by IVVM (CPC, continuously-perfused; IPC, intermittently-perfused; NPC, non-perfused capillaries). N=4 in each group; *p<0.001 from sham; † p<0.001 from CS+iCORM-3.

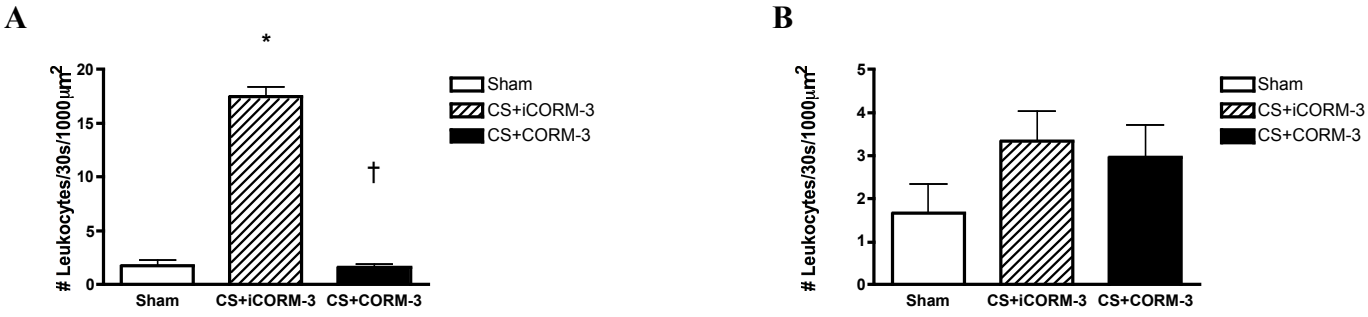

Figure 3. **The effect of CORM-3 on modulation of leukocyte behaviour in the skeletal muscle following CS.** Rats were subjected to 2hr CS, injected with CORM-3 (or its inactive form, iCORM-3) immediately upon fasciotomy, and assessed for **A) leukocyte adhesion** and **B) leukocyte rolling** 45min later by IVVM (# of leukocytes/1000mm^2/30s). N=4 in each group; * p<0.001 from sham; † p<0.001 from CS+iCORM-3.

SALARIES AND WAGES (List all personnel for whom money is requested)	% Of Time on this project	Requested from OTA Funds (Omit Cents)
Aurelia (Relka) Bihari, Research Technician	80%	$10,200
(data collection and analysis)	%	
	%	
	%	
Fringe Benefits _____% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	

PERMANENT EQUIPMENT (Justification to be appended)	
n/a	
Subtotal	

CONSUMABLE SUPPLIES (Exclude animals and animal care)	
FLIVO in vivo apoptosis kits, 5 kits (@$490/kit)	$2,450
L-012	$610
Porcine TNF-a kit, 8x96-well plates (@$870/kit)	$6,960
Histology supplies	$530
Subtotal	

ANIMALS AND ANIMAL CARE	
15 Yorkshire-Landrace pigs (@$1,950/pig)	$29,250
(includes all OR-associated supplies)	
Subtotal	

ALL OTHER EXPENSES	
Subtotal	

TOTAL DIRECT COSTS **$50,000**