Evaluation of Hydrogen Sulfide for Peripheral Nerve Regeneration and Neuromuscular Recovery Following Traumatic Neurovascular Injury in Rats

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EVALUATION OF HYDROGEN SULFIDE FOR PERIPHERAL NERVE REGENERATION AND NEUROMUSCULAR RECOVERY FOLLOWING TRAUMATIC NEUROVASCULAR INJURY IN RATS (RATTUS NORVEGICUS)

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14. ABSTRACT-

Traumatic injury to peripheral nerves is a common clinical problem affecting the warfighter due to combat-related injuries. Blast, penetrative and blunt force injuries are also accompanied by severe neurovascular, musculoskeletal, and pulmonary injuries as a result of ischemia-reperfusion. Neurovascular injuries can result in the loss of neuromuscular function and chronic pain leading to substantial disability. Currently, the gold standard for preserving extremities is reinstitution of blood flow. In resource limited environments, immediate blood loss replacement is not always feasible thereby increasing the ischemic insult to the extremity via tourniquet use. The restoration of blood flow to ischemic tissue exacerbates the damage which can lead to long term injury to the warfighter and decreases their chance for survival. Ischemia-reperfusion injuries (IRI) are often accompanied by peripheral nerve injuries that cause long term injury and disability to the warfighter, thus leading to poor quality of life and a burden of health care costs. Current treatments for peripheral nerve injuries due to combat-related injuries remain limited and have significant room for improvement. Previous studies in *in vitro* systems and mammalian models indicate the biologic, Hydrogen Sulfide (H_2S), as a potential therapeutic to preserve neuromuscular function and promote nerve and muscle regeneration following traumatic injury. This study aimed to develop a rat model of IRI of the left hindlimb to evaluate the efficacy of H_2S in peripheral nerve regeneration and neuromuscular function. Validation of the IRI rat model was assessed via gait analysis, electrophysiological measurements, fluorescent imaging, and histological studies. Future work will use the developed rat model to test the hypothesis that ***hydrogen sulfide promotes peripheral nerve regeneration and preserves neuromuscular function following ischemia-reperfusion injury of the peripheral nerves in rats.*** Completion of this study will aid in the advancement of therapies that preserve peripheral nerve function and promote nerve regeneration.

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1.0 EXECUTIVE SUMMARY

In 2006, the military introduced an improvement in body armor which significantly decreased fatality rate at point of injury. Unfortunately, the face, head, and extremities continue to be vulnerable despite armor improvements; increased injury severity scores, primarily to the extremities and maxillofacial regions, have been reported. Blast, penetrative and blunt force injuries are also accompanied by severe **neurovascular**, **musculoskeletal** and pulmonary injuries as a result of ischemia-reperfusion. Initial trauma triggers a physiologic response to survive. The body begins shunting blood from less essential organs to the vital organs such as the heart and brain. The ensuing hemorrhage from the extremity increases stress on the body with an ensuing massive inflammatory response. Once the hemorrhage is controlled, the body enters shock and experiences significant oxygen deprivation. This deficit is what causes long term injury to the warfighter and decreases their chance for survival. A retrospective analysis of advanced resuscitative strategies and modern vascular techniques revealed successful limb salvage is achievable with excellent graft patency. In resource limited environments, immediate blood loss replacement is not always feasible, thereby increasing the ischemic insult to the extremity via tourniquet use. The restoration of blood flow to ischemic tissue excaberates the damage which can lead to long term injury to the warfighter and decreases their chance for survival. Ischemia-reperfusion injuries (IRI) are often accompanied by peripheral nerve injuries that cause long term injury and disability to the warfighter, thus leading to poor quality of life and a burden of health care costs. Current treatments for peripheral nerve injuries due to combat-related injuries remain limited and have significant room for improvement. Novel biotechnological interventions are needed to preserve extremity viability and save the warfighter's life. The discovery and development of novel therapeutics that preserve nerve function and promote nerve regeneration following traumatic injury, requires an adequate animal model to simulate military relevant neurovascular injury. Previous studies in *in vitro* systems and mammalian models indicate the biologic, Hydrogen Sulfide (H_2S), as a potential therapeutic to preserve neuromuscular function and promote nerve and muscle regeneration following traumatic injury. H_2S is endogenously generated and regulates cell survival and growth, metabolic activity, apoptosis, antioxidant response, and inflammatory pathways (Henderson, Singh et al. 2010, Henderson, Jimenez et al. 2011, Ball, Reiffel et al. 2013). It has a broad range of physiological and pathophysiological functions, and this is underscored by the protection it affords against IRI in heart, liver, kidney, and brain (Dinh, Hazel et al.). Administration of low concentrations of sodium hydrosulfide (NaHS) prior to reperfusion in mice protected against hindlimb IRI mediated cell apoptosis, and muscle tissue edema and necrosis . However, it is unknown whether this protective effect was extended to nerve regeneration and neuromuscular functional recovery. The rat animal model has been extensively used to study peripheral nerve injury and recovery . Peripheral nerve regeneration and neuromuscular function in rats can be evaluated with established methods including gait analysis, electrophysiological measurements, and histological studies. Therefore, this study aimed to develop a rat model simulating peripheral nerve injury induced by ischemia-reperfusion to the hindlimb. This model was chosen since 20% of total vascular injuries occur in the extremities in combat settings. Previous studies were used as a reference point for the development of our neurovascular injury model. This study aimed to develop a rat model of IRI of the left hindlimb with the purpose of evaluating the efficacy of **sodium hydrosulfide** in peripheral nerve regeneration and neuromuscular function. Validation of the IRI rat model was assessed via gait analysis, electrophysiological measurements, fluorescent imaging, and histological studies. Future work will utilize the developed rat model simulating peripheral nerve injury induced by IR in the hindlimb to test the hypothesis that ***sodium hydrosulfide promotes peripheral nerve regeneration and preserves neuromuscular function following ischemia-reperfusion injury of the peripheral nerves in rats.*** Completion of this study will aid in the advancement of therapies that preserve peripheral nerve function and promote nerve regeneration.

2.0 INTRODUCTION

In 2006, the military introduced an improvement in body armor which significantly decreased fatality rate at point of injury. Unfortunately, the face, head, and extremities continue to be vulnerable despite armor improvements; increased injury severity scores, primarily to the extremities and maxillofacial regions, have been reported. Blast, penetrative and blunt force injuries are also accompanied by severe **neurovascular**, **musculoskeletal,** and pulmonary injuries because of ischemia-reperfusion, with 20% of total vascular injuries occurring in the extremities in combat settings. In resource limited environments, immediate blood loss replacement is not always feasible, thereby increasing the ischemic insult to the extremity via tourniquet use. While tourniquet use saves lives, once a tourniquet is removed and blood flow is restored to the ischemic tissue, this can cause an ischemia-reperfusion injury (IRI) and exacerbate damage to the injured extremity. IRI can lead to long term injury to the warfighter and decreases their chance for survival. IRIs are often accompanied by peripheral nerve injuries (PNIs) that cause long term injury and disability to the warfighter, thus leading to poor quality of life and a burden of health care costs.

Peripheral Nerve Degeneration Following Injury

The peripheral nerve is composed of three fibrous connective tissues: the epineurium, perineurium, and endoneurium. The epineurium contains blood vessels, fibroblasts, macrophages, and surrounds the perineurium. The perineurium surrounds the nerve bundles and each individual nerve bundle is surrounded by the endoneurium. The endoneurium also harbors fibroblasts and macrophages in addition to both non-myelinating Schwann cells and the myelinating Schwann cells (SCs) that encase axons in their protective myelin sheath. When a peripheral nerve injury (PNI) is sustained, the degree of injury severity can vary and ranges from neurapraxia, to axonotmesis, and neurotmesis (Reviewed, (Lee and Wolfe 2000)).

Neurapraxia injuries are the least severe and consist of local myelin damage but preserve the axon continuity and do not lead to distal degeneration of the nerve (Reviewed, (Lee 2021)). Neurapraxia only takes weeks to months for complete recovery. Axonotmesis injuries have the most variation in their severity as they are defined by the loss of continuity of axons. Prognoses of axonotmesis are based on the amount of surrounding connective tissue also injured and cannot be further subclassified without histological assessment (Reviewed, (Lee and Wolfe 2000)). Neurotmesis injuries are the most severe and are not dependent on nerve trunk transection but demonstrate the same physiological disruption of end organ function(s), including motor, proprioception, touch, temperature, pain, and sympathetic (Reviewed,(Lee and Wolfe 2000)). End organ functional recovery of PNI denervation occurs in the reverse order; however, spontaneous recovery is minimal after sustaining complete nerve disruption (Reviewed,(Lee and Wolfe 2000)). Wallerian degeneration (WD) is the highly complex process of coordinated morphologic and biochemical changes that occur in response to peripheral nerve damage. WD is initiated by nerve severance and destruction of the axon's myelin sheath quickly follows (Carroll and Worley 2017). Although WD is rapidly initiated following nerve transection, the subsequent processes of chromatolysis, SC responses and formation of the bands of Büngner, myelin removal, and finally nerve regeneration that follows is estimated by the Mayo Clinic Health System to occur at a rate of 1 mm per day. Therefore, without effective nerve regeneration treatments the injured person can expect to live with life-long disability including loss of motor function and chronic neuropathy (i.e., numbness, weakness, and pain) (Reviewed,(Lee and Wolfe 2000)). Current treatment options range from non-surgical (observations, physiotherapy, and medications) and surgical methods (neurorrhaphy, nerve transfer and grafting, and use of artificial conduits.

Current Treatments for Nerve Injuries

Non-surgical treatments rely on the body's innate ability to repair damaged fibers over time. However, this can be problematic given the slow rate of nerve regeneration. Nerves regenerate at approximately 1 mm per day, which means that healing can take a long time, particularly when the damage covers a

significant length of the nerve. This slow rate of recovery can result in muscle atrophy due to lack of innervation, as well as prolonged periods of disability, which can be detrimental to a patient's quality of life.

Surgical treatments offer a more direct approach to report, yet they too can come with their own limitations. Procedures like neurorrhaphy, nerve transfer, and nerve grafting have inherent risks such as infection, bleeding, and the potential for further nerve damage. Furthermore, donor site morbidity and the availability of compatible nerves for grafting present significant issues. Even when surgical repair is successfully, incomplete reinnervation may occur, leading to ongoing treatment. Additionally, certain nerve injuries can result in neuromas causing pain and causing further nerve dysfunction. In some cases, PNIs can lead to chronic pain and other sensory disturbances, such as phantom limb sensation in amputees, significantly affecting the quality of life.

While existing treatment strategies for PNIs provide some management and facilitate recovery, they have substantial limitations. Given their dependency on the body's natural healing ability, the slow rate of nerve regeneration can lead to problems such as muscle atrophy and prolonged periods of disability. Given the limitations and challenges of current treatment strategies for PNIs, the necessity for novel therapeutic interventions become more pressing. These new interventions could help preserve the functionality of affected extremities and improve the quality of life for patients, especially in the context of combat-related injuries. In this regard Hydrogen Sulfide, H_2S has emerged as a promising potential therapeutic.

Hydrogen Sulfide in Mammals

Hydrogen sulfide (H_2S) is an endogenously generated, inorganic gasotransmitter that is generated from L-cysteine in mammals ((Chun, Eom et al. 2022)). There are four organ-specific enzymes responsible for the endogenous biosynthesis of H_2S: cysteine aminotransferase (CAT), cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfotransferase (3MST) ((Chun, Eom et al. 2022), Reviewed (Powell, Dillon et al. 2018), Reviewed,(Nicholson and Calvert 2010)). Endogenous H_2S is produced at low concentrations and can either be fast acting or stored in cells (Reviewed, (Wu, Wang et al. 2015)). Exogenous forms of H_2S are studied for optimal release rates (e.g., slow or hybrid) and concentrations in which the efficacy of H_2S therapeutics can be enhanced (Reviewed, (Wu, Wang et al. 2015)). H_2S plays many roles in the body as it has been identified in all organs and is capable of diffusing both intra- and intercellularly (Reviewed, (Nicholson and Calvert 2010), (Chun, Eom et al. 2022)). It has a broad range of physiological and pathophysiological functions including regulation of cell survival and growth, metabolic activity, apoptosis, antioxidant response, and inflammatory pathways. H_2S as a therapeutic has gained interest due to its protective role after IRI in the central and peripheral nervous systems, the cardiovascular system, hepatic system, renal system (i.e., kidney), pulmonary system, and the GI tract (Reviewed, (Chun, Eom et al. 2022)). H_2S is also involved in cellular processes such as: angiogenesis, apoptosis, cell cycle, inflammation, oxidative stress, synaptic transmission, and vascular tone ((Chun, Eom et al. 2022), and Reviewed,(Chun, Eom et al. 2022)).

Hydrogen Sulfide in the CNS and PNS

CBS produces the majority of H_2S present in the central nervous system (CNS), while CSE is the major producer within the peripheral nervous system (PNS). In the CNS, dysregulation of H_2S has been linked to neuropathic pain and its role in cognitive function has spearheaded efforts to further explore H_2S's protective neuromodulatory effects against cognitive diseases (e.g., Alzheimer's and Parkinson's disease) and disorders (10.1111/jnc.12854 and reviewed, (Sharif, Iqbal et al. 2023)). H_2S has also been extensively studied in myocardial IRI models where reduction of endogenous H_2S resulted in hypertension and exacerbated the effects of myocardial IRI, while administration of exogenous H_2S donors (e.g., NaHS, Na_2S, and CaS) resulted in a reduction of infarct size (Reviewed, 10.1016/j.phrs.2010.06.002 and reviewed, 10.1155/2015/186908). PNIs are defined by loss of sensory and motor function that may present

with lifelong symptoms of burning, numbness, sharp and shooting pain, tingling, and throbbing typically caused by trauma or compression (i.e., tourniquet use and IRI) (10.3390/ antiox12020294). PNIs commonly consist of mixed nerve injuries, in which all fibers are affected to varying degrees further complicating recovery as current treatments are subpar for severe injuries like neurotmesis, especially those with nerve transection(s) (10.3390/ antiox12020294 and reviewed, 10.5435/00124635-200007000-00005). Following peripheral nerve injuries (PNIs), Schwann cells are essential mediators of Wallerian degeneration (WD), the degeneration of axons distal to an injury (Ref).

H_2S Affects Schwann Cell Responses to Peripheral Nerve Injuries (PNIs)

Schwann cells (SCs) are neuroglial cells that recruit macrophages for clearance of cellular debris during Wallerian degeneration (WD). In the PNS, CSE and 3MST are present, and *in vivo* only injured nerves contain SCs with CSE upregulated (10.1111/jnc.12854). H_2S is a major regulator of Schwann cell dedifferentiation and their dynamics during WD after a PNI is sustained. The dedifferentiation and proliferation of SCs coordinate peripheral nerve degeneration by crushing and recycling captured myelin, creating a conducive microenvironment for reformation of the myelin sheaths around the degenerated axons of the regenerating peripheral nerve (10.3390/antiox11081606). Because SCs and successful WD are directly responsible for the functional outcome of nerve regeneration and repair, identifying successful means to regulate H_2S-mediated SC regulation is imperative for the restoration of the afflicted limb's functionality after PNIs.

In the PNS, *N*-Ethylmaleimide (NEM, an *N*-ethyl derivative of maleic acid) inhibit H_2S-producing enzymes, CSE and 3MST, mediating SC regulation (10.3390/antiox11081606). Specifically, NEM expression prevents demyelination and proliferation of dedifferentiated SCs which assist in the removal of myelin via autophagy (myelinophagy), and disrupts SC signaling of macrophages, inflammatory immune cells (e.g., neutrophils), and fibroblast recruitment distal to the site of injury (Reviewed, /10.3389/fnmol.2020.608442 and 10.3390/antiox11081606). Park *et al*. utilized NEM on sciatic nerve explants, unteasing H_2S signaling in SCs and identified its transcriptional regulatory role in denervated SCs during WD (10.1111/jnc.12854). Specifically, NEM-CSE mediated inhibition of H_2S signaling blocks SC myelin ovoid fragmentation, delaying axonal degeneration and subsequent PNI regeneration *in vivo* (10.1111/jnc.12932). However, H_2S production enhancers could not increase the rate of peripheral nerve degeneration to decrease the total time of PNI regeneration following traumatic injury (10.1111/jnc.12932). Furthermore, H_2S signaling-mediated SC regulation is essential for PNI regeneration because if SC remyelination is incomplete once the peripheral nerve axons have successfully reached the ending organ, then nerve regeneration is insufficient for successful recovery of end organ function (10.4103/1673-5374.147940 and 10.1111/jnc.12854).

H_2S is known to play a protective role in IRI induced local inflammation and can interact with molecules like myoglobin, hemoglobin (i.e., sulfhemoglobin), epidermal growth factor receptor, and vascular endothelial growth factor receptor 2 to improve vascular function and wound healing (Reviewed, 10.1155/2015/186908). NaHS (14 μmol/kg, i.p.) preconditioning injections 24-, 12-hours prior, and at initiation of reperfusion during IRI studies in mice produced anti-inflammatory effects (i.e., eNOS and p38 MAPK activation) that with further study could lead to implementation of H_2S as a therapeutic for preventing IRI tissue damage (Reviewed, 10.1016/j.phrs.2010.06.002). In PNIs of the sciatic nerve the gastrocnemius muscle becomes atrophied. Use of S-Propargyl-cysteine (SPRC, an endogenous H2S donor) when compared to NaHS and control animals in studies conducted by Xi *et al*., demonstrated accelerated functional recovery of the injured sciatic nerve in mice via CSE upregulation (10.3390/antiox12020294). Furthermore, angiogenesis was promoted, SC remyelination of axons produced more myelinated nerve fibers with thicker sheaths, and gastrocnemius muscle atrophy was alleviated (10.3390/antiox12020294). Moreover, the inhibition of H2S production affects SCs via downregulation of their various dedifferentiation markers (10.4103/1673-5374.147940).

The discovery and development of novel therapeutics that preserve nerve function and promote nerve regeneration following traumatic PNIs requires an adequate animal model to simulate military relevant neurovascular injury. Administration of low concentrations of sodium hydrosulfide (NaHS) prior to reperfusion in mice protected against hindlimb IRI mediated cell apoptosis, and muscle tissue edema and necrosis. However, it is unknown whether this protective effect was extended to nerve regeneration and neuromuscular functional recovery. Further research is needed to fully understand the mechanisms through which H_2S facilitates nerve regeneration, and to translate these findings into clinical application. While H_2S administration 20 minutes prior to reperfusion of hindlimb IRI illustrates H_2S therapeutics as a promising protectant against cellular damage sustained from PNIs, it remains to be studied if additional H_2S administration(s) during the reperfusion period can also prevent or decrease end organ dysfunction that commonly results from PNIs (Reviewed, (Wu, Wang et al. 2015)). Therefore, this study will test the hypothesis that ***hydrogen sulfide promotes peripheral nerve regeneration and preserves neuromuscular function following ischemia-reperfusion injury of the peripheral nerves in rats.*** To test out hypothesis, the study is categorized into three specific aims, each with its precise focus and methodology:

- ***Specific Aim 1:*** *Establish that hydrogen sulfide preserves neuromuscular function following ischemia-reperfusion injury (IRI) of the peripheral nerves.* This evaluation will be two-pronged, focusing on gait analysis and electrophysiological assessments.

  Gait analysis will be conducted using the CatWalk system, a comprehensive tool designed to capture multiple metrics associated with neuromuscular and peripheral nerve function. Baseline measurements will be taken prior to the injury which will establish each rat's normal gait parameters. After the injury, the gait metrics, including stride length and speed, stance width and base of support, swing speed and phase duration, and paw pressure and print will be monitored at days 3, 7, 14, and 28 post-reperfusion. Any progression or regression of these parameters over time can provide a quantitative measure of nerve regeneration and neuromuscular restoration, effectively evaluating the functional impact of the IRI and the efficacy of H_2S in promoting recovery. Significant deviations from baseline in gait parameters are expected, which should be ameliorated by H_2S treatment in injured animal.

  Electrophysiological measurements will supplement the gait analysis, offering insights into peripheral; nerve functional integrity in the IRI rat model. Two key measurements will be utilized: Compound Muscle Action Potential (CMAP) and Nerve Conduction Studies (NCS). CMAP reflects the number of functioning motor nerve fibers and their connectivity to the muscles, and changes in its amplitude can suggest a loss of functional motor nerve fibers or impaired neurotransmission due to IRI. Restoration of CMAP following H_2S would indicate successful nerve regeneration or improved neuromuscular junction functionality. Similarly, NCS measures the conduction velocity, the speed at which electrical impulses travel along a nerve. A decrease in conduction velocities post-IRI could point to demyelination or axonal damage, while restoration of NCV within normal range in rats given H_2S would indicate that there is nerve regeneration.

  This multi-faceted evaluation approach should establish the extent of IRI-induced neuromuscular dysfunction and demonstrates the potential therapeutic effects of H_2S, thereby advancing our understanding of nerve regeneration and neuromuscular restoration post-IRI.

- ***Specific Aim 2:*** *Evaluate H_2S impact on muscle atrophy, apoptosis, and nerve/muscle regeneration post-peripheral nerve IRI, using live in vivo imaging and histological, as well as gene/protein expression analyses.* This aim is designed to evaluate the impact of H_2S on muscle atrophy, apoptosis, and nerve/muscle regeneration after peripheral nerve IRI. This evaluation will be accomplished using a multi-faceted approach in combining *in vivo* imaging, histological analysis, and gene/protein expression studies. This aim is organized into two sub-aims:

Specific Aim 2a: *Visualize and evaluate the effects of hydrogen sulfide on blood flow, oxygen availability, muscle atrophy, and apoptosis in ischemia-reperfusion injury.* This subaims centers on the visualization and evaluation of H_2S on blood flow, oxygen availability, muscle atrophy, and apoptosis in the setting of IRI. These measurements will be obtained using a non-invasive IVIS imaging platform which utilizes bioluminescent and fluorescent imaging probes, such as IVISense Tomato Lectin 680 probe, IVISense Vascular NP 750 probe, IVISense Hypoxia CA IX 680 probe, and IVISense Annexin-V 750 probe. This enables real-time visualization of dynamic biological processes and states within living animals, enabling for longitudinal assessments of various aspects of IRI including blood flow, metabolic activity, oxygen activity, and muscle atrophy.

The data acquired data will be analyzed to validate the extent of ischemia-reperfusion injury to the hindlimb. It is expected that there will be observable differences between the injured and uninjured hindlimbs. The injured limb is expected to show decreased blood flow, altered metabolic activity, reduced oxygen availability, and increased muscle atrophy and apoptosis compared to the uninjured limb. These results will be used to supplement findings from other assessments and contribute to a comprehensive understanding of ischemia-reperfusion injury and the potential therapeutic effects of hydrogen sulfide.

Specific Aim 2b: *Investigate the impacts of hydrogen sulfide on nerve and muscle regeneration*. The first part of the study, histopathological changes in the sciatic nerve and hindlimb muscles following ischemia-reperfusion injury will be evaluated using H&E staining. Parameters such as the number and diameter of myelinated nerve fibers and the quantity of endoneurial microvessels will be assessed to understand the structural integrity and regeneration process of nerve fibers and the correlation between revascularization and nerve regeneration. Expected results include altered tissue architecture, presence of inflammation, altered nerve fiber characteristics, and a reduction in the density of microvessels in neurons, indicating impaired revascularization and possible nerve regeneration issues.

The second part of this aim focuses on the assessment of the regenerative capacity of skeletal muscle after ischemia-reperfusion injury. This will involve tracking the changes in hindlimb muscle weights over time and measuring biomarkers of myogenesis using RT-PCR and ELISAs. If H_2S enhances muscle regeneration, as hypothesized, results should include increased muscle weights, upregulated expression of key myogenesis biomarkers, and elevated levels of proteins related to myogenesis, indicating an enhanced regenerative response.

Overall, the results from these analyses will contribute to a comprehensive understanding of the degree of IRI and the potential effects of H_2S. This has the potential to lead to better strategies for managing IRI and promoting muscle and nerve regeneration.

- **Specific Aim 3:** *Elucidate the effects of H_2S on ischemia-induced cellular inflammation, metabolic dysfunction, and oxidative stress in rats.* This will include:

Specific Aim 3a: *Evaluate the degree of cellular inflammation in both the serum and muscle tissues, specifically within the gastrocnemius and tibial anterior muscle.* Muscle tissue homogenates and plasma samples will be analyzed for pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) concentrations using ELISAs. Cytokine levels will be evaluated at multiple time points, both pre-injury and post-injury (on days 3, 7, 14, and 28), to gain insight into the dynamics of cytokine expression over time in muscle tissues and plasma following reperfusion.

The anticipated results suggest that there will be increased levels of pro-inflammatory cytokines in both the local muscle tissues and systemic circulation in rats subjected to injury compared to the sham group, indicative of an inflammatory response. However, it is expected that rats treated with hydrogen

sulfide will have decreased levels of these cytokines, suggesting that the treatment mitigates the inflammatory response.

Specific Aim 3b: *Assess metabolic dysfunction & oxidative stress in hindlimb muscles and sciatic nerve through the application of bench assays.* These include tests for citrate synthase, calpain, myeloperoxidase, superoxide dismutase, calcium, glycogen, lactate, and SERCA, each indicating different aspects of cellular health and function.

Post-injury, expectations include reduced citrate synthase activity (indicating impaired mitochondrial function), increased calpain and myeloperoxidase activities (pointing to tissue damage and inflammation), altered superoxide dismutase activity (showing oxidative stress), disrupted calcium and glycogen levels, increased lactate (signifying hypoxia), and reduced SERCA activity (implying impaired muscle cell function). The study hypothesizes that treatment with hydrogen sulfide may alleviate these metabolic dysfunctions and lessen tissue damage linked to the injury.

The execution of our research project encountered an array of unexpected hurdles that interfered with our anticipated timeline, primarily due to the depletion of FY21 funds. We encountered significant setbacks at the start of our study, including delayed funding, the pending approval from the IACUC, and a prolonged process in assembling our technical team. Additionally, logistical issues pertaining to the scarcity of equipment and personnel restricted the volume of surgeries we could conduct daily and diminished our bench studies' efficiency. The situation was further compounded by ongoing renovations at CIRS and an untimely HVAC system failure, which created additional roadblocks. In the wake of these obstacles, our study was halted upon the conclusion of the model refinement study. However, the outcomes of the refinement study still proved to be invaluable, providing important insights into surmounting technical difficulties in future research endeavors.

In our forthcoming research, we will employ the developed rat model that replicates PNI initiated by IR in the hindlimb. This will be used to investigate our hypothesis: sodium hydrosulfide can stimulate peripheral nerve regeneration and maintain neuromuscular function following ischemia-reperfusion injury in rats' peripheral nerves. The successful execution of this study will contribute significantly to the progression of biotechnological treatments, potentially helping to preserve neurovascular and peripheral nerve function, as well as promoting nerve regeneration after traumatic injury in warfighters.

3.0 METHODS, ASSUMPTIONS AND PROCEDURES

3.1 Overall Study Design

The overall goal of this study is to test the hypothesis that ***hydrogen sulfide promotes peripheral nerve and muscle regeneration and preserves neuromuscular function following ischemia-reperfusion injury (IRI) of the peripheral nerves*** in a full pre-clinical study. The present investigation aims to employ an ischemia-reperfusion injury model of the sciatic nerve, to ascertain the protective benefits rendered by hydrogen sulfide. To address this hypothesis the study is divided into two phases:

Phase 1: Model Refinement Study. The primary goal of this protocol is to refine and reduce the number of animals required for a full pre-clinical study. Therefore, the parameters mentioned below are critical to measure for the evaluation and validation of the proposed animal model as these will indicate areas for improvement. For example, it is possible that the measured markers may indicate a faster rate of recovery than anticipated. If this is the case, we can either increase the duration of ischemia or reduce the reperfusion period depending on the results collectively. In the case that neuromuscular dysfunction is not present immediately following the IRI, the study will be paused to formulate a new surgical approach. The model developed here will be refined such that there is only one sham and one ischemic group to test therapeutics.

This study will validate and refine an ischemia reperfusion injury model of the sciatic nerve to determine the optimal ischemia duration and reperfusion time points for testing the overarching hypothesis. The animal groups used in this objective are outlined in Table 1. The sciatic nerve is made ischemic via the ligation of the major vessels supplying the left hindlimb (femoral) with microvascular clamps for either 2 or 4 hours. Tissue blood flow will be continuously measured in the hindlimb muscles with the OxyFlo sensor to validate ischemic insult and the reinstatement of blood flow following the surgery. The reperfusion injury will be induced through the release of the microvascular clamps and evaluated at the designated time points in a 28-day described in Table 1. To determine whether this is an adequate model of neurovascular injury via ischemia reperfusion, a sham group for all designated time points will be utilized. The sham group will serve as a control group to account for changes in the below (Part 2: Ischemia Reperfusion Injury Criteria) due to surgery rather than effects from the injury induced in rats. The sham procedure is further described in Section 3.3.

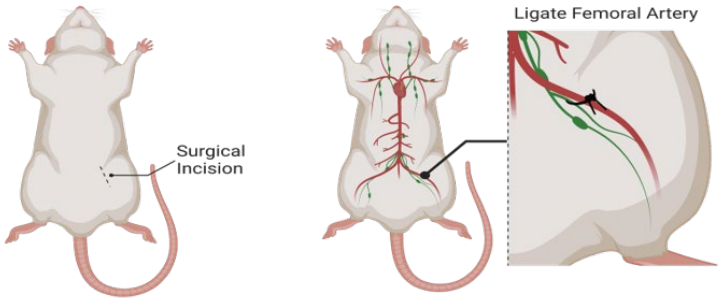

Figure 1

Ischemia Reperfusion Injury Criteria:

Part 1: Validation of Ischemia Reperfusion

- Blood flow to ligated major vessels is absent as determined by the OxyFlo sensor.
- Tissue oxygenation in adjacent muscles of the affected hindlimb is reduced compared to non-affected hindlimb muscles as determined by the OxyLite sensor.
- Blood flow is restored in the affected hindlimb as determined by the OxyFlo sensor.
- Tissue oxygenation in adjacent muscles of the affected hindlimb increases following restoration of blood flow as determined by the OxyLite sensor.

Part 2: Injury Determination (further described in Section 3.4 – 3.6)

- Abnormal gait compared to sham rats as assessed with CatWalk instrumentation and software.
- Abnormal electrophysiological measurements compared to sham rats in the affected hindlimb muscle as determined by changes in nerve conduction velocity and slow compound muscle action potential (CMAP).
- Abnormal histopathology of sciatic nerve and hindlimb muscles compared to sham rats as determined by H&E staining.

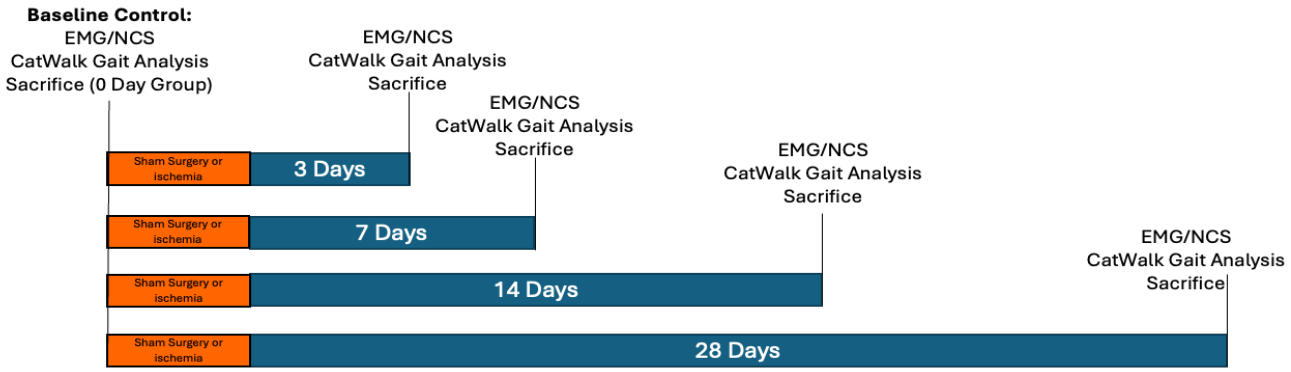

Figure 2

TABLE 1. **Phase 1:** Model Validation and Refinement Study Design

Group/Model (Total n=39)		Duration of Ischemia	Study Measurements* and Necropsies
1	Baseline Control, n=3	Sham	0 days
2	Control, n=3	Sham	3 days
3	Control, n=3	Sham	7 days
4	Control, n=3	Sham	14 days
5	Control, n=3	Sham	28 days
6	Exp, n=3	2 hr	3 days
7	Exp, n=3	2 hr	7 days
8	Exp, n=3	2 hr	14 days
9	Exp, n=3	2 hr	28 days
10	Exp, n=3	4 hr	3 days
11	Exp, n=3	4 hr	7 days
12	Exp, n=3	4 hr	14 days
13	Exp, n=3	4 hr	28 days

Phase 2: Preclinical Study. In this study, we aim to investigate the protective role of hydrogen sulfide (H_2S) in a validated and optimized IRI model of the sciatic nerve, developed during Phase 1. Using animal groups detailed in Table 2, we will simulate ischemia in the sciatic nerve through femoral vessel clamping for a duration of 4 hours. Continuous OxyFlo sensor measurements will be employed to validate ischemic conditions and the restoration of blood flow post-surgery. The subsequent reperfusion injury will be initiated by clamp release and evaluated at specified timepoints within a 28-day survival period (Table 2). A sham group will be implemented at all designated timepoints to serve as controls, accounting for surgical changes rather than injury-induced effects. For this study, control measures include the use of sham animals and vehicle control, ensuring an unbiased comparison for the observations derived from the experimental procedures.

TABLE 2. **Phase 2:** Preclinical Study Design

Group (Total n=78)		0 Days	3 Days	7 Days	14 Days	28 Days
1	Sham & Vehicle	n=6	n=6	n=6	n=6	n=6
2	4-hour IRI & Vehicle		n=6	n=6	n=6	n=6
3	4-hour IRI & H_2S		n=6	n=6	n=6	n=6

Sample Size Estimation: The required sample size was determined through a priori power analysis for a one-way ANOVA, assuming a large effect size (f) of 0.83, and a 2-sided test with a significance level (α) of 0.05. To achieve 82% power, six animals per group were calculated to be sufficient at the α level of 0.05. Consequently, the current study will include six animals per group.

3.2 Animals

Wistar Rat Selection: The utilization of Wistar rats in our study is strategically chosen to optimize the translatability and relevance of our findings to human pathophysiology, with a focus on the ischemia-reperfusion injury model. In studies modeling peripheral nerve injury and subsequent regeneration, Wistar rats have been found to be a pertinent model. Their peripheral nervous system exhibits notable parallels to that of humans in terms of complexity and regenerative responses. The use of this model will thus allow us to effectively test the efficacy of H_2S in peripheral nerve regeneration and neuromuscular function in a system that mirrors the human context.

A unique advantage of the Wistar rat model lies in its suitability for modeling ischemia-reperfusion injury. Extensive research has established the Wistar rat as a reliable model for this type of injury, particularly concerning the hindlimb, the region of focus in our study. Their anatomical characteristics and cardiovascular physiology render them highly responsive to the vascular manipulation required in simulating traumatic extremity vascular injury. The ability to execute precise and consistent surgical interventions in Wistar rats is another factor contributing to their choice. Their larger size relative to other rodent models permits more refined surgical procedures and, subsequently, greater control over the ischemia-reperfusion injury induction, thus minimizing experimental variability.

Moreover, the use of Wistar rats will enhance our assessment of neuromuscular function and peripheral nerve regeneration. Their capacity to perform reliably in gait analysis and the feasibility of obtaining high-quality electrophysiological measurements from these animals are pivotal for our study's methodologies. Wistar rats also naturally produce H_2S, the agent we are investigating. This offers a uniquely relevant model to explore the role of endogenously produced and exogenously administered H_2S in the context of peripheral nerve injury and regeneration.

Overall, the selection of Wistar rats as our model organism aligns with our study's scientific goals and maximizes the translational potential of our findings to the clinical setting, specifically targeting the improvement of outcomes following combat-related peripheral nerve injuries.

Age Group Justification: This study will employ the 100-130 day age group. The justification for use of this study hinges on two pivotal developmental milestones in Wistar rats:

First, neuromuscular maturity in the hindlimbs of Wistar rats is typically achieved by 90 days of age. A mature neuromuscular system is fundamental to our study because it provides a consistent baseline from which we can evaluate the effects of the experimental treatments. Employing younger rats, still undergoing growth and maturation, could introduce confounding factors. This is because the continual developmental changes could potentially mask the impacts of the interventions, thereby complicating our results. Therefore, selecting rats that have already achieved neuromuscular maturity helps to minimize such potential confounders, leading to more reliable and clear-cut interpretations.

The second factor lies in the fact that Wistar rats are generally full grown around the selected age range. This is crucial for our study as it pertains to muscle atrophy and injury assessment. In a scenario where rats are still growing, the natural enlargement in muscle size and strength could counterbalance or interfere with the observed impacts of muscle atrophy. Consequently, it might become challenging to distinguish between changes caused by normal growth and those instigated by our experimental manipulations. By employing mature rats, we can confidently attribute any noted muscle atrophy or injury to the experimental conditions, ruling out any effects of ongoing growth processes.

Thus, the synergistic contribution of these two factors - the attainment of neuromuscular maturity and the state of full growth - makes the 100-130 day age group optimal for this study. It provides a robust platform for a more controlled investigation of the phenomena being studied and increases the likelihood that the observed effects can be unambiguously linked to the experimental conditions. By

opting for this age group, we enhance the reliability and the external validity of our findings, thereby facilitating their comparison with other research outcomes in this domain.

Husbandry and Diet: All animals used in this study will be housed in the CIRS facilities. Animals are housed in a temperature-controlled room with a 12-hour light-dark cycle. Animals will be fed ad-libitum with a fixed formula diet (LabDiet, that closely align with nutritional requirements for long-term maintenance of rats). Fixed formula diet contains the same ingredients, in the exact same quantities, in every batch of diet to reduce experimental variability and avoids ingredients that are reported to have adverse confounding effects on experimental results (i.e.,fluorescent imaging studies) and always have access to water. They will be housed in accordance with the CIRS Operating Instructions governing animal housing (40V-013 - Feeding and Watering Schedules) and 40V-014 - Quarantine and Stabilization of Animals). Rats will be provided with gnawing (i.e. nylabones) and sheltering enrichment (i.e. Bio-Huts).

Pain Scoring: Using the scoring sheet from 1-13 (total score; Appendix A) the rats will be scored daily (adapted from Paster et al 2009). Scoring will be performed by suitably trained veterinary technicians and/or researchers. If a rat scores less than 5 it will be monitored twice daily until scores are equal or greater than 5 and the veterinarian informed. If a rat scores less than 3 on two consecutive days, then this is a threshold for euthanasia.

Euthanasia and Necropsy: Animals will be euthanized using IP Pentobarbital, 100 mg/kg (Euthanasia solution) in accordance with the AVMA Guidelines for the euthanasia of animals, 2020. An alternative method that can be used is isoflurane gas anesthesia overdose followed by bilateral thoracotomy as a confirmatory euthanasia method. Euthanasia will be performed by the attending veterinarian, veterinary technician, qualified surgical technician, or experienced/qualified research team member under direction of the attending veterinarian. Immediately after euthanasia, cessation of vital signs will be confirmed followed by immediate dissection/collection of tissue samples and disposal of the animal carcass. Following euthanasia, the sciatic nerve, bilateral anterior tibialis, soleus, and gastrocnemius are collected for histopathology, single fiber muscle testing, inflammatory assays, and metabolic assays at the designated groups study endpoint (Pre-ischemia (baseline), or 3, 7, 14, and 28 days after reperfusion). Furthermore, blood will be drawn post-euthanasia from vena cava to collect serum and plasma.

3.3 Ischemia Reperfusion Injury & Therapeutic Intervention

The overarching aim of this study is to empirically investigate whether hydrogen sulfide augments peripheral nerve and muscle regeneration and preserves neuromuscular function following IRI of the peripheral nerves in a comprehensive pre-clinical study setting. Specifically, we utilize an ischemia-reperfusion injury model of the sciatic nerve to ascertain the potential protective benefits conferred by hydrogen sulfide.

Methods: In our experimental approach, we induce IRI via a standard procedure detailed exhaustively in Appendix B. Succinctly, this involves a minor midline abdominal incision and a retroperitoneal dissection to make visible the major femoral vessels that supply the left hindlimb. Ischemia is then induced by the occlusion of these vessels for a duration of four hours utilizing micro-clips. The commencement of ischemia is verified by gauging the blood flow within the gastrocnemius muscle with the application of an OxyFlo probe. Following the ischemic interval, the micro-clips are carefully extracted and the incision site is sealed with sutures.

Sham procedure: In the sham procedure, analogous steps are followed, where the femoral artery, responsible for blood supply to the left hindlimb, is exposed for four hours while the subject is under anesthesia, but no occlusion is performed. Post this interval, the incision site is sealed with wound closure clips or through another appropriate closure technique.

Therapeutic Intervention: The experiment involves the administration of a single dose of 200 µL of phosphate buffered saline (PBS), encapsulating either 0 or 2 µg of sodium hydrosulfide (NaHS), administered via tail vein injection, 20 minutes prior to the reperfusion event. Consequently, the administered volume to each rat does not exceed 200 µL.

3.4 Specific Aim 1: Establish that hydrogen sulfide preserves neuromuscular function following ischemia-reperfusion injury (IRI) of the peripheral nerves.

To evaluate the preservation of neuromuscular function following ischemia reperfusion injury studies will be conducted at 3, 7, 14, and 28 days of reperfusion (post-ischemia) compared to sham at the designated end points and baseline measurements (pre-ischemia injury) using the following criteria:

Gait Analysis: To evaluate the preservation of neuromuscular function following ischemia-reperfusion injury, gait analysis will be conducted at 3, 7, 14, and 28 days of reperfusion compared to sham rats, as well as baseline measurements before the ischemia injury. Gait analysis will serve as an indicator of the severity of injury and the extent of neuromuscular function loss (Yu, Matloub et al. 2001, Bozkurt, Deumens et al. 2008, Dinh, Hazel et al. 2009, Heinzel, Langle et al. 2021). The Catwalk instrumentation and software will be used to assess gait abnormalities, following the protocol detailed in the provided SOP in Appendix C. The CatWalk instrument is a high-resolution automated gait analysis system equipped with a glass walkway embedded with sensors. It enables the capture of footprints as animals traverse the walkway, providing detailed information on various gait parameters. These parameters include stride length, stance duration, paw print area, and more, enabling a comprehensive assessment of locomotor function.

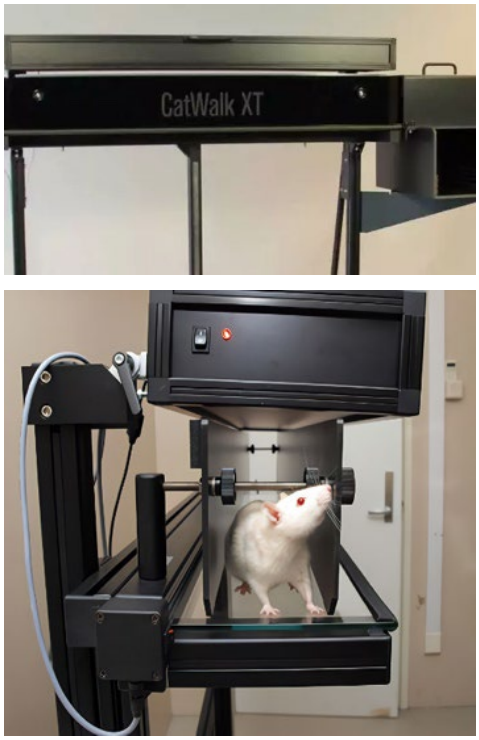

Figure 3

During the handling acclimation period, which spans 5 days, the rats will be familiarized with the experimental procedures. This acclimation includes daily handling sessions and CatWalk acclimation. The CatWalk acclimation process involves placing the rats on the CatWalk apparatus, providing treats as stimuli, and allowing the rats to explore the walkway. Over the course of the acclimation period, the number of runs completed by the rats will gradually increase.

To evaluate the severity of ischemia-reperfusion injury and the extent of neuromuscular function loss, gait analysis will be performed following the designated post-injury time points. The obtained gait data will be compared to baseline measurements and sham rats. The sciatic static index (SSI), derived from the footprints captured during CatWalk analysis, will be used as a quantitative measure to assess the recovery of function after sciatic injury. The SSI compares the stance width and base of support of the affected limb to that of the unaffected limb, with a lower SSI indicating a more severe impairment in neuromuscular function.

Anticipated Results: It is anticipated that rats subjected to hindlimb ischemia-reperfusion injury will exhibit gait abnormalities compared to sham rats, as indicated by alterations in various gait parameters measured by the CatWalk system. The severity of these abnormalities is expected to vary depending on the duration of reperfusion, with more pronounced impairments observed immediately after injury and potentially improving over time. The application of hydrogen sulfide as a therapeutic intervention during reperfusion is anticipated to mitigate the gait abnormalities associated with hindlimb ischemia-reperfusion injury. Rats treated with hydrogen sulfide are expected to show improvements in gait parameters compared to untreated rats, indicating a preservation or restoration of neuromuscular function.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in gait analysis using the CatWalk system include variations in animal behavior, the need for proper acclimation, and technical errors in data collection and analysis. To mitigate these issues, a thorough handling acclimation period will be implemented, ensuring the rats are accustomed to the experimental procedures. Strict adherence to the CatWalk acclimation protocol will also be followed. Furthermore, standardized data collection procedures and rigorous quality control measures will be employed to minimize technical errors and ensure reliable and meaningful results.

In conclusion, gait analysis using the CatWalk system is a valuable tool for evaluating the preservation of neuromuscular function following hindlimb ischemia-reperfusion injury. The anticipated results will provide insights into the severity of injury and the potential benefits of hydrogen sulfide as a therapeutic intervention. Mitigation strategies for potential pitfalls will be implemented to ensure the accuracy and reliability of the gait analysis data.

Electrophysiological Measurements: To evaluate neuromuscular functional recovery following ischemia-reperfusion injury, electrophysiological measurements will be performed on the affected hindlimb muscle compared to sham rats. Changes in nerve conduction velocity (NCV) and slow compound muscle action potential (CMAP) will be assessed at the designated time points outlined in Table 1.

Real-time data collection methodology: Electrophysiological measurements will be conducted following the protocol detailed in the provided SOP in Appendix D. In brief, electrophysiological measurements will be conducted on the left sciatic nerve of isoflurane-anesthetized animals placed on a heating pad, and recorded using an AD Instrument to measure CMAP and NCV. To measure CMAP, a single 0.5 ms duration nerve stimulation will be applied. A needle electrode will be inserted intramuscularly at the sciatic notch (in the left gluteal region) to stimulate the sciatic nerve, while the recording electrode will be positioned in the central region of the left gastrocnemius. The current will be gradually increased until the maximum CMAP reading is obtained and recorded.

To measure NCV, a single 0.5 ms duration pulse will be used to stimulate the sciatic nerve at two sites: first at the left sciatic notch (proximal site) and then in the gastrocnemius (distal site). The recording electrode will be placed in the plantar of the foot. NCV will be calculated as the distance between the stimulation sites (proximal - distal, measured in meters) divided by the difference between the latency of the proximal and distal sites (measured in seconds).

Controls: To ensure the validity of the measurements, appropriate controls will be included. Internal controls will involve stimulating the right sciatic nerve, which is not directly affected by the ischemia-reperfusion injury. This will provide a reference point for comparing the measurements obtained from the affected hindlimb muscle. Sham rats, which have not undergone the ischemia-reperfusion procedure, will serve as additional controls for comparison.

Anticipated Results: Abnormal electrophysiological measurements, including changes in NCV and slow CMAP, are expected in the affected hindlimb muscle of rats subjected to hindlimb ischemia-reperfusion injury compared to sham rats. These measurements serve as indicators of neuromuscular dysfunction following the injury. The severity of these abnormalities is anticipated to vary depending on the duration of reperfusion, with more pronounced impairments expected immediately after injury and potentially improving over time. It is anticipated that the administration of hydrogen sulfide during the reperfusion phase will mitigate the abnormal electrophysiological measurements observed in the affected hindlimb muscle. Rats treated with hydrogen sulfide are expected to exhibit improved NCV and CMAP compared to untreated rats, indicating a preservation or restoration of neuromuscular function.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in electrophysiological assessments include variations in animal physiology and technical errors during data collection and analysis. To mitigate these issues, strict adherence to standardized protocols will be followed during the measurements. Careful attention will be given to electrode placement and stimulation parameters to ensure accurate and reliable recordings. Rigorous quality control measures will be employed, including repeated measurements and comparison to sham rats. Any unexpected results or discrepancies will be thoroughly investigated, and if necessary, additional measurements will be conducted to ensure the validity of the data.

Electrophysiological assessments of NCV and CMAP are valuable tools for evaluating neuromuscular functional recovery following hindlimb ischemia-reperfusion injury. The anticipated results will provide insights into the severity of injury and the potential benefits of hydrogen sulfide as a therapeutic intervention. By implementing stringent protocols and quality control measures, accurate and reliable data will be obtained to enhance our understanding of the effects of ischemia-reperfusion injury on neuromuscular function and the potential therapeutic effects of hydrogen sulfide.

Preservation of mechanical properties of skeletal muscle: To quantify the mechanical properties of injured skeletal muscle and assess the potential amelioration of musculoskeletal dysfunction following IRI, an isolated muscle test system with a dual-mode muscle lever will be employed. This assessment will utilize single fibers isolated from tissues collected at 0 days (pre-injury) and post-injury on days 3, 7, 14, and 28 using the extensively detailed SOP provided in Appendix J and K.

The following parameters will be measured to evaluate the mechanical properties of the skeletal muscle and are extensively detailed in the SOPs provided in Appendix K:

1. Muscle Resting Length: The resting length of the muscle will be measured to determine its initial position and baseline characteristics.
2. Resting Force: The resting force of the muscle will be measured to assess the baseline force generated by the muscle at rest.
3. Stimulation: Electrical stimulation will be applied to the muscle to evaluate its contractile response and assess its functional capacity.
4. Fatigue: The muscle will be subjected to repeated contractions to assess its fatigue resistance and endurance capacity.
5. Stiffness: The stiffness of the muscle will be quantified to evaluate its resistance to deformation under an applied force.
6. Force Frequency: The force-frequency relationship of the muscle will be assessed to determine its ability to generate force at different stimulation frequencies.

7. Twitch: The twitch response of the muscle will be measured to evaluate its contractile properties and responsiveness to electrical stimulation.

Anticipated Results: It is anticipated that rats subjected to hindlimb ischemia-reperfusion injury will exhibit impaired mechanical properties compared to sham-injured rats. This may include reduced muscle force generation, decreased fatigue resistance, altered stiffness, and altered force-frequency relationship. If the administration of NaHS ameliorates musculoskeletal dysfunction following ischemia-reperfusion injury, it is hypothesized that rats treated with NaHS will exhibit mechanical properties comparable to those of sham-injured rats. This would indicate a preservation of the mechanical function of the skeletal muscle.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in the assessment of mechanical properties include variations in muscle preparation, electrode placement, and measurement techniques. To mitigate these issues, standardized protocols and procedures will be followed for muscle preparation and electrode placement. Careful attention will be given to ensure consistent and accurate measurements of the mechanical parameters. Additionally, calibration and quality control measures will be implemented to validate the accuracy and reliability of the measurements. Expert supervision and training will be provided to the personnel performing the tests to minimize technical errors.

The assessment of mechanical properties using an isolated muscle test system will provide valuable insights into the preservation or restoration of musculoskeletal function following ischemia-reperfusion injury. The anticipated results will contribute to our understanding of the effects of NaHS administration and its potential as a therapeutic intervention. By adhering to standardized protocols and implementing rigorous quality control measures, we aim to obtain reliable and meaningful data on the mechanical properties of the injured skeletal muscle.

3.5 Specific Aim 2a: Visualize and evaluate the effects of hydrogen sulfide on blood flow, oxygen availability, muscle atrophy, and apoptosis in ischemia-reperfusion injury.

***In vivo* imaging**: *In vivo* imaging using bioluminescent and fluorescent imaging probes will be conducted to evaluate the extent of IRI immediately after surgery and at the endpoint of the study. *In vivo* imaging using the IVIS imaging platform will be conducted to evaluate the extent IRI in the hindlimb. The IVIS imaging system is a non-invasive imaging platform that utilizes bioluminescent and fluorescent imaging techniques to visualize and quantify various biological processes. The IVIS imaging platform consists of a highly sensitive camera equipped with advanced optics and filters that allow for the detection of bioluminescent and fluorescent signals emitted by imaging probes (Figure 4). This system provides real-time imaging capabilities, enabling the visualization of dynamic processes in living animals. To perform the imaging, animals will be administered specific bioluminescent and fluorescent imaging probes, as described previously. The emitted signals from these probes will be captured by the IVIS camera and converted into high-resolution images. The acquired images will then undergo analysis using specialized software to quantify and visualize the targeted biological processes.

The IVIS imaging platform offers several advantages for assessing ischemia-reperfusion injury. It provides a non-invasive and longitudinal assessment, allowing for repeated imaging of the same animal over time. The high sensitivity of the system enables the detection of low levels of signals, providing detailed information about the extent of injury and the efficacy of therapeutic interventions. Additionally, the system offers a wide range of imaging modes and versatile probes,

enabling the evaluation of multiple aspects of IRI, including blood flow, oxygen availability, muscle atrophy, and apoptosis.

The selected probes will enable the visualization of key aspects related to IRI, including blood flow, oxygen availability, muscle atrophy, and apoptosis. The following imaging probes will be utilized along with the detailed SOP described in Appendix E:

1. **Blood Flow:** The IVISense Tomato Lectin 680 probe (NEV10060) will be used to visualize blood flow in the hindlimb. This probe binds to lectin, which enables the visualization of the vascular network and perfusion status.

2. **Metabolism:** The IVISense Vascular NP 750 probe (NEV10150) will be employed to assess the metabolic activity in the hindlimb muscles. This probe provides information about the metabolic state and tissue viability.

3. **Oxygen Availability:** The IVISense Hypoxia CA IX 680 probe (NEV11070) will be utilized to evaluate oxygen availability in the hindlimb. This probe specifically binds to carbonic anhydrase IX, a marker of tissue hypoxia, allowing for the visualization of areas with limited oxygen supply.

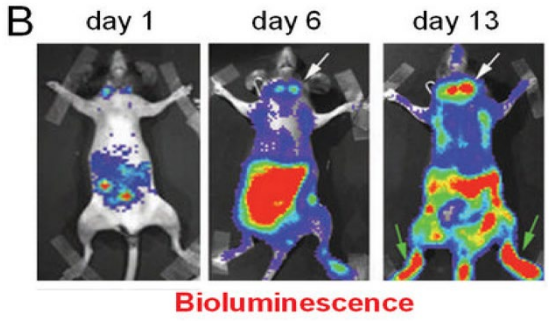

Figure 4

4. **Muscular Atrophy (Apoptosis):** To assess muscle atrophy and apoptosis, the IVISense Annexin-V 750 probe (NEV11053) will be used. This probe binds to phosphatidylserine, a membrane marker exposed during the early stages of apoptosis, enabling the visualization of apoptotic cells in the hindlimb muscles.

Acquired images will be subsequently analyzed to validate the extent of IRI to the left hindlimb, compared to the uninjured right hindlimb. These images will provide quantitative and qualitative data on blood flow, metabolism, oxygen availability, and muscle atrophy, offering valuable insights into the pathophysiological changes associated with IRI.

Anticipated Results: The use of *in vivo* imaging with the selected probes is expected to reveal distinct differences between the left hindlimb, which underwent ischemia-reperfusion injury, and the uninjured right hindlimb. Decreased blood flow, altered metabolic activity, reduced oxygen availability, and increased muscular atrophy and apoptosis are anticipated in the injured hindlimb compared to the control.

The IVIS imaging results will complement and further validate the findings from other evaluations, such as gait analysis, electrophysiological assessments, and histopathological examinations. They will contribute to a comprehensive understanding of the extent of IRI and the potential therapeutic effects of hydrogen sulfide.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in IVIS *in vivo* imaging include variations in probe distribution, background noise, and technical limitations. To mitigate

these issues, standardized protocols for probe administration and imaging will be followed. Careful calibration and optimization of imaging parameters will be conducted to minimize background noise and ensure accurate visualization. Positive and negative controls will be used to validate the imaging results. Additionally, technical expertise and thorough quality control measures will be implemented to maximize the reliability and validity of the acquired images.

In conclusion, IVIS *in vivo* imaging using bioluminescent and fluorescent imaging probes provides a non-invasive and dynamic assessment of ischemia-reperfusion injury in the hindlimb. The anticipated results will yield valuable information about blood flow, metabolism, oxygen availability, and muscular atrophy, aiding in the evaluation of IRI severity and the potential effects of hydrogen sulfide as a therapeutic intervention. By adhering to standardized protocols and implementing stringent quality control measures, we aim to obtain reliable and meaningful data to enhance our understanding of the pathophysiological changes associated with IRI.

3.6 Specific Aim 2b: Investigate the impacts of hydrogen sulfide on nerve and muscle regeneration.

Histopathological Assessment: To evaluate the histopathological changes in the sciatic nerve and hindlimb muscles following IRI, H&E staining will be performed. This assessment will involve the examination of tissue sections under a microscope to identify abnormal histopathological features compared to sham rats. Specifically, the following parameters will be analyzed:

1. **Morphometric Analysis of Myelinated Nerve Fibers:** Morphometric analysis will be conducted to evaluate the number and diameter of myelinated nerve fibers in the sciatic nerve and hindlimb muscles. This analysis will provide insights into the structural integrity and regeneration of nerve fibers following the ischemia-reperfusion injury.

2. **Evaluation of Endoneurial Microvessels:** The number of endoneurial microvessels in the sciatic and tibial neurons will be assessed as a marker of nerve regeneration. This evaluation will involve quantifying the density of microvessels within the endoneurium, providing valuable information on the revascularization process and its association with nerve regeneration.

Anticipated Results: In comparison to sham rats, we anticipate observing abnormal histopathological findings in the sciatic nerve and hindlimb muscles following the ischemia-reperfusion injury. These abnormalities may include changes in tissue architecture, presence of inflammatory infiltrates, and altered myelinated nerve fiber characteristics. The severity of these histopathological changes is expected to vary depending on the duration of reperfusion.

Regarding the morphometric analysis of myelinated nerve fibers, we anticipate a decrease in the number and diameter of myelinated nerve fibers in the affected tissues compared to sham rats. This reduction in myelinated nerve fibers reflects the degenerative changes caused by the injury.

The evaluation of endoneurial microvessels is expected to reveal a decreased density of microvessels in the sciatic and tibial neurons of rats subjected to ischemia-reperfusion injury. This reduction suggests impaired revascularization and may correlate with compromised nerve regeneration in the affected limbs.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in histopathological assessment include tissue processing artifacts, staining inconsistencies, and subjective interpretation. To mitigate these issues, standardized protocols for tissue fixation, processing, and staining will be strictly followed. Quality control measures, such as using appropriate positive and negative controls, will be implemented to ensure reliable staining results. Blinded assessments by

experienced histopathologists will be conducted to minimize subjective bias and ensure accurate interpretation of the histopathological features.

Histopathological assessment using H&E staining is a crucial method for evaluating the structural changes and regenerative potential in the sciatic nerve and hindlimb muscles following ischemia-reperfusion injury. The anticipated results will provide valuable insights into the histopathological alterations associated with the injury and the potential effects of hydrogen sulfide as a therapeutic intervention. By adhering to standardized protocols and implementing rigorous quality control measures, we aim to obtain reliable data to advance our understanding of the histopathological changes and regenerative processes occurring in the affected tissues.

Regenerative Capacity Evaluation: To assess the regenerative capacity of skeletal muscle following ischemia-reperfusion injury, the following evaluations will be performed:

1. Hindlimb Muscle Weights: Hindlimb muscle weights will be recorded at two time points: 0 days (pre-injury) and post-injury at 3, 7, 14, and 28 days. Changes in muscle weights over time will provide insights into the regenerative capacity of the skeletal muscles.

2. Biomarkers of Myogenesis: To evaluate the process of myogenesis, biomarkers will be measured using reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assays (ELISAs) in skeletal muscle tissue. Muscle samples will be collected at 0 days (pre-injury) and post-injury at 3, 7, 14, and 28 days.

The specific methodologies for conducting these assays are extensively detailed in the SOPs provided in Appendix H & I. These protocols will ensure standardized and accurate measurements for the evaluation of skeletal muscle regenerative capacity.

Anticipated Results: If our hypothesis is correct, and NaHS administration enhances the regenerative capacity of skeletal muscle following ischemia-reperfusion injury, we expect to observe the following:

- Increased hindlimb muscle weights compared to the untreated group, indicating enhanced muscle regeneration.
- Upregulated expression of key biomarkers involved in myogenesis, as measured by RT-PCR, suggesting an increase in muscle regeneration and repair.
- Elevated levels of specific proteins associated with myogenesis, measured by ELISAs, indicating an enhanced regenerative response.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls for assessing regenerative capacity may include sample variability, technical errors, and interferences. To mitigate these issues, strict adherence to the provided SOPs will be followed. Quality control measures, including appropriate positive and negative controls, will be employed to ensure accurate and reliable data. Standardized sample collection and handling procedures will be implemented to minimize variability. Any unexpected results or discrepancies will be thoroughly investigated, and if necessary, assays will be repeated to ensure data validity.

The evaluation of regenerative capacity in skeletal muscle following ischemia-reperfusion injury involves assessing hindlimb muscle weights and measuring biomarkers of myogenesis using RT-PCR and ELISAs. The anticipated results will provide insights into the regenerative response of skeletal muscle and the potential enhancement of regenerative capacity through NaHS administration. By implementing rigorous methodologies and quality control measures, we aim to generate reliable data to advance our understanding of skeletal muscle regeneration and the therapeutic potential of hydrogen sulfide.

3.7 Specific Aim 3a: Evaluate the degree of cellular inflammation in both the serum and muscle tissues, specifically within the gastrocnemius and tibial anterior muscle.

Muscle Tissue and Plasma Cytokine Array: Muscle tissue homogenates and plasma samples will be collected to assess the levels of pro-inflammatory cytokines within the gastrocnemius and tibial anterior muscles, as well as in the systemic circulation. ELISAs will be performed to quantify the concentrations of key pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β). These cytokines are known to play crucial roles in the inflammatory response. The evaluation of cytokine levels will be conducted at two time points: 0 days (pre-injury) and post-injury on days 3, 7, 14, and 28. This longitudinal assessment will provide insights into the dynamic changes in cytokine expression within the muscle tissues and plasma over the course of reperfusion following hindlimb ischemia. The detailed protocol for muscle tissue and plasma cytokine analysis is provided in the SOPs available in Appendix L & W. These SOPs outline the specific steps for tissue collection, homogenization, plasma separation, and ELISA procedures, ensuring standardized and reproducible measurements of cytokine levels in both tissue homogenates and plasma samples.

Anticipated Results: The assessment of cytokine levels in muscle tissue homogenates and plasma samples at different time points will provide insights into the temporal profile of the local and systemic inflammatory response within the gastrocnemius and tibial anterior muscles following hindlimb ischemia-reperfusion injury. It is anticipated that rats subjected to injury will display increased levels of pro-inflammatory cytokines compared to sham rats at various post-injury time points, indicating an inflammatory response in both the local muscle tissues and the systemic circulation. On the other hand, rats treated with hydrogen sulfide are expected to exhibit decreased levels of pro-inflammatory cytokines, suggesting a mitigated inflammatory response due to the therapeutic intervention.

Potential Pitfalls for the Assays and Mitigation Plan: Potential pitfalls in the cytokine analysis include variations in tissue processing, sample preparation, plasma separation, and ELISA procedures. To mitigate these issues, standardized protocols and procedures outlined in the SOPs will be followed for tissue collection, homogenization, plasma separation, and cytokine quantification at each time point. Quality control measures will be implemented to ensure the accuracy and reliability of the ELISA results. Furthermore, positive and negative controls will be included in the assay to validate the specificity and sensitivity of the cytokine measurements.

The analysis of pro-inflammatory cytokine levels in muscle tissue homogenates and plasma samples at multiple time points will provide valuable insights into the temporal dynamics of the local and systemic inflammatory response following hindlimb ischemia-reperfusion injury. The anticipated results, guided by the detailed protocol in the SOPs, will contribute to our understanding of the temporal profile of inflammation within the targeted muscles and the potential therapeutic effects of hydrogen sulfide. By adhering to standardized protocols and implementing rigorous quality control measures, we aim to obtain reliable and meaningful data on cytokine levels in the muscle tissues and plasma at each time point.

3.8 Specific Aim 3b: Assess metabolic dysfunction and oxidative stress in hindlimb musclse and sciatic nerve through the application of bench assays.

The goal of this specific aim is to evaluate metabolic dysfunction in hindlimb muscles and the sciatic nerve following IRI using a series of bench assays (Figure 5). These assays have been

selected based on their relevance to the metabolic processes implicated in injury and their potential as indicators of tissue damage and therapeutic efficacy. By employing these assays, we aim to gain a comprehensive understanding of the metabolic alterations associated with hindlimb ischemia-reperfusion injury and the potential benefits of H_2S as a therapeutic intervention. The specific methods employed for conducting these assays are extensively detailed in the SOPs provided in Appendix L-V. The SOPs outline step-by-step instructions for sample preparation, reagent handling, assay procedures, data acquisition, and analysis for each assay. By adhering to these well-documented procedures, we ensure consistency, accuracy, and reproducibility in our experimental approach.

Citrate Synthase Assay: Citrate synthase is an enzyme responsible for the conversion of oxaloacetate and acetyl-CoA into citrate in the mitochondrial tricarboxylic acid cycle (Vigelso, Andersen et al. 2014). The readout of this assay is the measurement of citrate synthase activity, which reflects mitochondrial energy metabolism (Blomstrand, Radegran et al. 1997, Larsen, Nielsen et al. 2012, Vigelso, Andersen et al. 2014). Ischemia-reperfusion injury is known to impair mitochondrial function (Kuznetsov, Javadov et al. 2019). Therefore, it is anticipated that the activity of citrate synthase, a marker of mitochondrial energy metabolism, will be reduced following injury.

Calpain Activity Assay: Calpain is a calcium-dependent protease involved in tissue damage and inflammation. The readout of this assay is the quantification of calpain activity, indicating the extent of proteolytic degradation and tissue injury (Smith, Lecker et al. 2008, Pandurangan and Hwang 2012). Increased calpain activity is associated with tissue damage and inflammation (Ji, Su et al. 2016, Hyatt and Powers 2020). Thus, it is expected that calpain activity will be elevated following ischemia-reperfusion injury.

Myeloperoxidase (MPO) Activity Assay: MPO is an enzyme released by activated neutrophils and is an indicator of inflammation and oxidative stress (Khan, Alsahli et al. 2018). The readout of this assay is the measurement of MPO activity, reflecting the degree of neutrophil infiltration and tissue inflammation. Ischemia-reperfusion injury is characterized by the infiltration of inflammatory cells, leading to increased MPO activity. Therefore, it is anticipated that myeloperoxidase activity will be elevated following injury.

Superoxide Dismutase (SOD) Activity Assay: SOD is an antioxidant enzyme responsible for the conversion of superoxide radicals into hydrogen peroxide (Wang, Branicky et al. 2018). The readout of this assay is the quantification of superoxide dismutase activity, indicating the capacity to scavenge reactive oxygen species. Ischemia-reperfusion injury generates reactive oxygen species, which can be scavenged by SOD (Granger and Kvietys 2015). It is expected that SOD activity will be altered as a result of oxidative stress during injury.

Calcium Level Assay: Calcium plays a crucial role in cellular signaling and homeostasis (Bootman and Bultynck 2020). The readout of this assay is the measurement of calcium levels, reflecting alterations in calcium handling and potential calcium overload. Ischemia-reperfusion injury disrupts calcium homeostasis, leading to calcium overload (Kristian and Siesjo 1998). It is anticipated that calcium levels will be increased following injury.

Glycogen Level Assay: Glycogen is a storage form of glucose and an essential energy source in tissues (Zhang, Ma et al. 2021). The readout of this assay is the quantification of glycogen levels, indicating the depletion or preservation of glycogen stores. Ischemia-reperfusion injury can deplete glycogen stores due to energy imbalance (Bailey, Radda et al. 1982, Tian, Zhao et al.

2023). Thus, it is expected that glycogen levels will be reduced in hindlimb muscles and the sciatic nerve.

Lactate Level Assay: Lactate is a byproduct of anaerobic metabolism and indicates tissue hypoxia (Lee 2021). The readout of this assay is the measurement of lactate levels, reflecting the degree of anaerobic metabolism and tissue oxygenation status. Ischemia-reperfusion injury induces anaerobic metabolism, resulting in lactate accumulation (Kalogeris, Baines et al. 2012). Hence, it is anticipated that lactate levels will be elevated following injury.

Sarcoplasmic reticulum calcium ATPase (SERCA) Assay: SERCA is responsible for calcium reuptake into the sarcoplasmic reticulum in muscle cells (Yu, Matloub et al. 2001). The readout of this assay is the quantification of SERCA activity, indicating the efficiency of calcium handling and sarcoplasmic reticulum function. Ischemia-reperfusion injury can impair SERCA activity (Li, Ma et al. 2020, Colomer-Saucedo, Darcy et al. 2023). Therefore, it is expected that SERCA activity will be decreased in hindlimb muscles.

Assay	Category	Objective/Purpose
Citrate Synthase Assay	Enzymatic Assay	Assess metabolic changes in response to IRI.
Calpain Activity Assay	Enzymatic Assay	Skeletal muscle remodeling.
MPO Activity	Enzymatic Assay	Involved in ischemia reperfusion injury.
SOD Activity	Enzymatic Assay	Oxidative stress state. Altered following ischemia reperfusion injury.
Calcium	Intracellular Level Assay	Loss of calcium homeostasis following ischemia reperfusion injury.
Glycogen	Intracellular Level Assay	Assess metabolic changes in response to IRI.
Lactate	Intracellular Level Assay	Assess degree of hypoxia and metabolic stress in response to IRI.
SERCA	Enzymatic Assay	Responsible for transporting calcium (Ca(2+)) from the cytosol into the lumen of the sarcoplasmic reticulum (SR) following muscular contraction. The Ca(2+) sequestering activity of SERCA facilitates muscular relaxation in both cardiac and skeletal muscle.

Figure 5

Anticipated Results: Hydrogen sulfide has been shown to possess cytoprotective, anti-inflammatory, and vasodilatory properties. It is anticipated that the application of hydrogen sulfide as a therapeutic intervention during reperfusion will mitigate metabolic dysfunction and tissue damage associated with hindlimb ischemia-reperfusion injury. Specifically, it is expected that hydrogen sulfide treatment will restore or preserve the activity of citrate synthase, reduce calpain and myeloperoxidase activities, enhance superoxide dismutase activity, regulate calcium levels, maintain glycogen stores, attenuate lactate accumulation, and improve SERCA activity.

Potential Pitfalls for the Assays and Mitigation Plan: Several potential pitfalls may arise during the execution of these assays, including technical errors, interferences, and sample variability. To mitigate these issues, rigorous quality control measures will be implemented, including proper assay standardization, appropriate positive and negative controls, and repeated sample analyses. Careful attention will be given to sample handling, storage, and preparation to minimize variability. Any unexpected results or discrepancies will be thoroughly investigated, and if necessary, assays will be repeated to ensure accuracy and reliability of the data.

The application of bench assays targeting various metabolic parameters will allow for the comprehensive assessment of metabolic dysfunction in hindlimb muscles and the sciatic nerve following ischemia-reperfusion injury. These assays will provide valuable insights into the underlying mechanisms of injury, the potential benefits of hydrogen sulfide as a therapeutic intervention, and the overall metabolic status of the tissues. By addressing potential pitfalls and employing rigorous quality control measures, we aim to generate reliable and meaningful data to advance our understanding of hindlimb ischemia-reperfusion injury and inform the development of effective therapeutic strategies.

3.9 Statistical Analysis:

Continuous outcome variables will be assessed for normality by the Shapiro-Wilks test. Normally distributed continuous outcomes will be presented as mean and standard deviation, and one-way analysis of variance (ANOVA) testing with a Tukey's post hoc test will be performed to determine significant differences among the groups. Otherwise, non-normally distributed outcomes will be presented as median and interquartile range (IQR), and Kruskal-Wallis Test (a non-parametric method) with Dwass-Steel-Chritchlow-Fligner (DSCF) pairwise comparisons. GraphPad Prism will be used for statistical analyses.

4.0 MAJOR EVENTS/MILESTONES/SUCCESS

In preparation for the execution of this project,

- **Personnel Onboarded**
  - Lab Manager, March 2022
  - Clinical Molecular Technician, May 2022
  - Biomedical Associate, June 2022
  - ORISE Post-doctoral Fellow, June 2022
- **Kick Off Meeting**:
  - PI and Co-PI had an internal kickoff meeting, November 2021
  - Team kickoff meeting July 2022
  - CIRS kickoff meeting, September 2022
- **IACUC Approval:**
  - **Model Refinement (FWH20220036AR):** Submitted December 2022 and approved on July 22, 2022
    - Amendment #1: Change of Procedure to include IVIS imaging
      - Submitted October 24, 2022 and approved on November 7, 2022
    - Amendment #2: Add Personnel
      - Submitted October 26, 2022 and approved on November 7, 2022
    - Amendmnet #3: Add Personnel and request 10 additional animals to improve closing procedure
      - Submitted March 20, 2023 and approved on May 12, 2023 (NOA sent May 15, 2023 but contingent on the completion of suture training with Attending Vet, additional AALAS training, and 1:1 meeting with AV – was all completed June 1, 2023)
    - Amendment #4: Change of Procedure (move study groups) and request 5 additional animals.
      - Submitted June 26, 2023 and retracted on July 11, 2023
  - **Preclinical Study (FWH20230045AR):** Reviewed Feb 2023, approval contingent on the completion of Model Refinement Amendment #3. Withdrew June 13, 2023.

- **ST IPR:** March 2023
  - Presenter: Shauna Hill Ph.D.
  - Attendees: Lt Col Thomas Percival, Diana del Monaco, Ph.D., Shelly Tavish, and Ed Chagoy
- **All experimental procedures completed:**
  - Model Refinement Animal Surgeries March, 2023
- **Data Analysis:** August 2023
- **Dissemination of Results:** N/A, Disseminated results internally

5.0 RISK ASSESSMENT

5.1 Risk Analysis:

FY22Q3 – Delays in funding availability: The project experienced a delay in the availability of funds, which were initially scheduled to be accessible earlier. It was not until March 2022 that the necessary funds were finally made available.
Risk: This delay in funding availability presented a risk of postponing the start of crucial project activities, potentially hindering our ability to stay on schedule and within budget.
Impact: The lack of funding until March 2022 affected the project's timeline. Activities that should have been initiated earlier had to be postponed, causing a domino effect on the project schedule.
Mitigation: To handle this challenge, we adjusted our project plan and schedules, redistributing tasks to make the most effective use of the delayed funds. In the future, we'll be strengthening our financial planning and risk management strategies to minimize the chance of similar issues occurring.

Delays in IACUC Approval and Technical Team Hiring:
Risk: Delays in obtaining IACUC approval and hiring the technical team have caused significant setbacks in starting the model refinement study.
Impact: Delayed timeline and potential impact on achieving program goals and objectives.
Mitigation: The animal surgeries will be extended into OY2 to ensure completion, as agreed upon with Mr. Barnicott at CIRS.

Equipment Availability:
Risk: Not all required equipment is currently in-house, contributing to delays.
Impact: Potential reduction in the number of surgeries that can be performed per day and increased reliance on existing equipment.
Mitigation: If the anesthesia monitoring equipment is not received by January 2023, alternative equipment in-house will be used, resulting in a reduced number of surgeries per day (from 4 to 1).

Overall, the delays in IACUC approval and technical team hiring, along with equipment availability, have impacted the timeline and efficiency of the model refinement study. Mitigation measures are in place to ensure completion, but adjustments and compromises may need to be made to accommodate the delays and equipment limitations.

FY23Q1 – Limited Personnel for Surgery Days:
Risk: The availability of personnel for surgery days is limited with the contract technical team.

Impact: The nature of the surgery requiring dedicated personnel per patient can lead to resource constraints and potential delays in the progression of bench studies.
Mitigation: The team needs to ensure that sufficient personnel are allocated for each surgery, including a surgeon, first assistant, and anesthesia supervisors. Adequate planning and coordination are necessary to minimize the impact on the team and the progression of bench studies.
Room assignment too small – can only support a single surgery at a time

Time-Intensive Anesthesia Monitoring:
Risk: Anesthesia monitoring for this protocol is a time-intensive process, requiring dedicated personnel per patient.
Impact: The need for multiple personnel during surgery can strain resources and potentially affect the efficiency of the team and bench studies.
Mitigation: Careful scheduling and coordination should be implemented to ensure that the required personnel, including anesthesia supervisors, are available for each surgery day. This will help maintain the pace of the studies and optimize resource utilization.

Overall, the limited availability of personnel for surgery days, coupled with the time-intensive nature of anesthesia monitoring, poses challenges to the team's efficiency and the progression of bench studies. Effective planning, scheduling, and coordination are crucial to mitigate these risks and ensure the smooth execution of surgeries and ongoing bench studies.

Electrical support:

FY23Q2 – CIRS Renovations, Restricted Personnel Availability, and Limited Time for Data Collection:
Risk: The ongoing CIRS renovations, restricted personnel availability, and limited time for data collection have imposed constraints on the study design.
Impact: These constraints require adjustments to the original study design, including the reduction in the number of animals, elimination of certain time points and histopathological studies, and modification of tissue harvesting protocols.
Mitigation: By making these adjustments, the study can still proceed within the given limitations, allowing for evaluation of neuromuscular function, positive response to H2S treatment, and assessment of protective effects at the 48-hour time point. These modifications will enable the successful completion of the investigation.

Overall, the constraints posed by CIRS renovations, restricted personnel availability, and limited time for data collection have necessitated modifications to the preclinical study design. By implementing these adjustments, the study can still provide valuable insights into the protective benefits of hydrogen sulfide on peripheral nerve and muscle regeneration and neuromuscular function preservation following IRI, despite the challenges faced.

FY23Q3 – Facility HVAC:
Risk: The HVAC system failure has resulted in a halt of all animal studies.
Impact: Delayed progress and potential negative impact on research outcomes.
Mitigation: Resolution of the HVAC system issue is projected by August 2023.

CIRS Renovations:

Risk: The team had to repeatedly move in and out of rooms for the painting and flooring project.
Impact: Disruption of work, loss of productivity, and potential damage to equipment or samples during relocation.
Mitigation: Accommodating the renovations by packing, organizing, and temporarily suspending work.

Amendment Approval Delay for MR Study:
Risk: The amendment for the MR study took three months to approve, causing significant setbacks.
Impact: Delayed timeline, potential loss of funding, and discontinuation of the study.
Mitigation: The research team made efforts to improve the suturing technique under the guidance of the AV, but the IACUC board required amendment approval before allowing further studies. Despite confidence from the research team and AV, the study will be discontinued due to timeline constraints.

5.2 Technical Challenges:

Long-term Anesthesia:
One of the significant technical challenges encountered during this research was the effective management of long-term anesthesia in rats. The primary issue was that the use of room air oxygen alone did not suffice in maintaining the vitality of the animals throughout the duration of the surgical procedures. This lack of sufficient oxygen during anesthesia proved detrimental, leading to an alarming mortality rate, with 6 out of 10 rats not surviving the surgery. To mitigate this critical issue, we made a strategic modification to our protocol by introducing oxygen tanks to supplement room air oxygen. This intervention significantly enhanced the survival rate of the animals, dramatically reducing the mortality rate to just 1 out of 14 rats, a remarkable improvement from the previous rate. This result demonstrated the efficacy of the added oxygen supply in supporting the survival of rats during long-term anesthesia and surgery.

Other adjustments made to the model throughout the course of this study also contributed to the improved outcome. First, we ensured a waiting period of a full ten minutes for the administration of Buprenorphine premedication, a modification which allowed for more effective pain management during surgery. Second, we modulated the anesthetic regimen by decreasing the level of Isoflurane while simultaneously increasing room airflow during the induction period. This adjustment was implemented to balance the anesthesia depth and minimize potential complications.

Third, to improve patient monitoring during these delicate procedures, we assigned a dedicated individual to each animal. This person's task was to closely observe and immediately respond to any changes in the animal's physiological parameters, which greatly enhanced the monitoring accuracy and reaction time during anesthesia.

Lastly, we enhanced the quality of heart rate readings by incorporating SomnoSuite, an advanced low-flow anesthesia system with an integrated digital pulse oximetry module. This tool allowed for optimized and accurate tracking of the rat's heart rate during the procedure, providing valuable real-time data for immediate intervention when necessary.

Overall, these modifications not only contributed to improved animal survival but also highlighted the importance of continual model refinement in experimental research. Our study

reinforces the need to adapt and improve anesthesia protocols and methods to overcome technical challenges and achieve desired outcomes in scientific investigations involving animal models.

Dietary Concerns:
In conducting research of the highest integrity, it is paramount to delineate the origin of any issues encountered. It's crucial to note that the dietary challenges during this study arose not from researcher oversight, but rather from unforeseen circumstances at the facility level. Several weeks prior to the initiation of the model refinement study, our team was informed by the CIRS ordering coordinator that the standard rodent feed was unavailable. Consequently, a vet-approved, standard laboratory grade substitute was sourced and introduced for the duration of the study.

The assumption that this replacement feed would adhere to the fixed formula diet typically used for long-term maintenance of rodents was an oversight. This experience underscores the importance of meticulously scrutinizing all changes to research protocols, even those that seem minor, as they can substantially impact the outcomes of a study. The procurement issues led to a substitute food with variable nutrient profiles being used. This inconsistency could dramatically affect the metabolism of the rats, potentially contributing to the skin issues observed during the study. Additionally, the nutrient variation in the non-research feed, which varied depending on the source, could affect ischemia reperfusion injury, leading to complications such as body weight variability.

Notably, during the study, the rats continued to gain weight, leading to apparent obesity and fat infiltration in the hindlimb muscles. This adverse physical change not only impacted the overall health of the rats but also posed significant challenges for electrophysiological measurements. The substitute diet utilized in our model refinement study also had inherent issues. Firstly, it followed the least cost formulation approach, which, while common in the commercial feed industry, is not suitable for research animals. Secondly, the substitute diet contained alfalfa meal and high levels of fat and protein. These ingredients, although suitable for pet rats, "show rats," and laboratory breeding, can negatively affect research outcomes. For instance, the presence of alfalfa meal could interfere with the accuracy of fluorescent imaging studies, and high protein content could lead to overeating and disrupt metabolic signaling.
In response to these challenges, we have identified specific dietary requirements to be implemented for the subsequent preclinical study:

1. **Lower Protein Content**: The diet should contain 19% protein or less, which closely aligns with the nutritional requirements for the long-term maintenance of rats. A higher protein content could lead to overeating and alterations in metabolic signaling. This may impact the efficacy of H2S, a signaling molecule that acts upstream to metabolic and antioxidant pathways.

2. **Fixed Formula Diet**: The diet needs to be a fixed formula, meaning that it contains the same ingredients, in the exact same quantities, in every batch. This consistency is crucial to reduce experimental variability.

3. **Absence of Alfalfa Meal and Soybean Oil**: The diet should not contain alfalfa meal, which can have adverse confounding effects on experimental results for fluorescent imaging studies, especially in regions of interest. Moreover, soybean oil should be avoided as it can lead to differences in body composition (weight, adiposity), glucose and insulin homeostasis, bone

density, and blood pressure. Its presence can affect the efficiency of the surgeries and the accuracy of EMG readings, while also modulating immunological responses.

It is important to note that research conducted in rodent models fed diets containing soybean meal may not translate effectively to human populations due to differences in consumption levels and metabolism.

In conclusion, these challenges emphasize the pivotal role of a consistent and appropriate diet in animal research, impacting not only the wellbeing of the animals but also the reliability and validity of experimental results. This situation also highlights the importance of vigilance and proactive management of any changes, even minor ones, within the research environment.

Continual Growth:
Another technical challenge we confronted in the model refinement study involves the continual growth of the rats in our care. This factor complicated our experimental proceedings, particularly during surgical procedures, as well as the analysis of outcomes. Even though previous literature indicates that rats of the breed we were using should have reached full growth by the age we performed surgeries, we observed acontinual increase in adiposity, particularly at the site of injury.

This unexpected growth phenomenon had two primary implications for our study. First, the increased adiposity made the surgical procedures more complex. Our surgeons had to navigate and move the fat pad away from the femoral artery, thereby increasing the procedural complexity and potentially influencing the surgery's efficiency and success.

Secondly, this growth trend potentially influenced our experimental outcomes. The breed of rats used in our study, Wistar rats, are generally considered fully grown around the selected age range. This characteristic is particularly relevant to our study, which revolves around muscle atrophy and injury assessment. In a situation where rats continue to grow, the natural enlargement of muscle size and strength could potentially counterbalance or obscure the observed impacts of muscle atrophy. Therefore, distinguishing between changes caused by normal growth and those instigated by our experimental manipulations becomes challenging.

The use of fully mature rats in such studies is imperative. Any noted muscle atrophy or injury can then confidently be attributed to the experimental conditions, ruling out any effects of ongoing growth processes. The issue of continued growth of rats, potentially due to age or diet, is an essential consideration for future studies. We aim to address this in the upcoming preclinical study by ensuring stringent control over diet and age of the rats to minimize any confounding effects of growth on the experimental outcomes.

In response to the growth challenge encountered, we have identified strategic adjustments to be incorporated in our upcoming preclinical study. Our observations during the model refinement study informed these changes.

We decided to switch from the original Wistar rat breed to the Wistar Han breed. This breed was chosen primarily because it averages 75-100 grams smaller than the Wistar breed used in the model refinement study. The smaller size of the Wistar Han rats, combined with a change in age group, should address the issues associated with continual growth and adiposity that complicated our surgical procedures and potentially confounded our results.

Furthermore, we adjusted the age group of our rat subjects from 90-120 days to 100-130 days. This change was made to ensure that we use fully grown rats, as literature suggests that Wistar rats should have completed their growth by this age range. The aim is to prevent the natural enlargement of muscle size and strength due to growth from counterbalancing or obscuring the impacts of muscle atrophy. By employing fully mature rats, we can confidently attribute any noted muscle atrophy or injury to the experimental conditions, ruling out any effects of ongoing growth processes.

Together, these modifications aim to ensure a consistent, fully grown subject population, thereby enhancing the reliability and validity of our preclinical study outcomes.

Suture Reopening and Incision Care:
During our model refinement study, a notable challenge encountered was the reopening of sutures post-surgery. Out of 19 surgeries, we documented 23 instances of suture reopening. This outcome not only affected our data consistency but was also a matter of concern regarding animal welfare. Initial steps were taken by consulting with our attending veterinarian to enhance the suturing technique. A significant change was the implementation of the single interrupted suturing technique coupled with the application of dermabond to the incision. This modification led to an improved outcome, with only three out of ten animals experiencing reopening of the surgical site post-operation, a marked improvement from our earlier observations.

However, a careful review of animal records and post-operative behavior indicated that the primary cause of incision reopening was not necessarily the suturing technique. Instead, self-injurying behavior exhibited by the rats emerged as the main culprit. We observed a higher incidence of suture reopening in rats that underwent four hours of IRI. This led to the hypothesis that the wearing off of pain medication between 48-72 hours post-surgery might increase irritation and the awareness of injury, consequently inducing self-injurying.

In anticipation of the preclinical study, several steps will be used to refine our protocol further, aiming to reduce the occurrence of self-injurying and improve incision care. The following measures are proposed based on our findings from the model refinement study:

- Preemptive analgesia: To further alleviate potential pain during the post-operative recovery period, preemptive analgesia will be administered. This will be carried out in consultation with the attending veterinarian to ensure the appropriate dosage and frequency.

- Application of EMLA cream: Prior to surgery, the research team will apply EMLA cream (a eutectic mixture of lidocaine and prilocaine) topically to the incision site. This local anesthetic aims to alleviate pain and irritation, potentially deterring the rats from self-barbering.

- Injection of lidocaine: In conjunction with EMLA cream, lidocaine will be injected locally at the incision site to provide additional pain relief during and after the surgical procedure.

- Post-operative monitoring: Rats will be monitored diligently for signs of self-barbering, discomfort, or distress for seven post-operative days. All observations will be systematically recorded, and any concerning behaviors or symptoms will be promptly reported to the attending veterinarian.

- Veterinary intervention: Based on observed behavior, the attending veterinarian may adjust the analgesic regimen or implement other interventions to alleviate any pain and discomfort. Such interventions could include increasing the frequency of EMLA cream application or the administration of additional analgesics.

By implementing these changes, we aim to effectively reduce the reopening of sutures and improve overall post-operative care, further committing to the welfare of the animals in our study. We believe that these measures will both enhance the reliability of our data and maintain our commitment to the principles of the 3Rs in animal research.

6.0 TRANSITION PLAN

6.1 Military Relevance:

This research holds substantial military relevance due to its focus on addressing peripheral nerve injuries—a common and debilitating consequence of combat-related injuries. The study aims to translate laboratory findings into real-world therapeutic strategies, targeting the unique and often challenging conditions faced by military personnel in the field. One of the primary goals of this study is to mitigate the effects of IRI, prevalent in battlefield conditions and associated with a high incidence of peripheral nerve damage. To this end, the study explores the therapeutic potential of H_2S, a compound known to regulate vital cell functions and survival. Implementing the administration of sodium hydrosulfide (NaHS), an H_2S donor, as an intervention strategy presents a novel approach to managing peripheral nerve injuries.

While traditional military medical interventions for extremity vascular injuries have predominantly centered on controlling hemorrhage and replacing blood loss, this approach often exacerbates ischemic insult to the extremity, particularly when immediate blood loss replacement is not feasible in resource-constrained military environments. Therefore, the need to develop preventative therapies that specifically address peripheral nerve injuries associated with such interventions is critical.

In the broader military context, the study's outcomes promise significant advantages. Effective therapeutic strategies resulting from this research could markedly improve the survival rates and long-term health outcomes of wounded warfighters. Moreover, by reducing the incidence of long-term disability and the associated need for continuous care and rehabilitation, these strategies could lead to considerable cost benefits for the military healthcare system. Importantly, any proposed therapeutic intervention must not only prove effective but must also be practical for use in combat or field situations. This necessitates that such therapies be easily administered, stable under varied conditions, and require minimal equipment or specialized training. Given its potential efficacy and practicality, the focus on NaHS in this research presents a promising step in this direction.

Finally, the study aligns directly with the Joint Capabilities Integration and Development System (JCIDS) topic area: OSD(HA). 2015DCR.C3-MRD-142, which underscores the need for regenerating or restoring certain tissues and functions in combat casualty care. This project, supported by DHA RESTORAL funding, is therefore a proactive response to a recognized military health requirement. In conclusion, this research holds significant potential to drive the development of effective, practical, and cost-efficient therapeutic strategies, enhancing the preservation of peripheral nerve function and promoting nerve regeneration among injured warfighters.

6.2 Transition Strategy:

In light of the critical impact of traumatic injury to peripheral nerves on the warfighter, our research aims to address this issue by investigating the therapeutic potential of Hydrogen Sulfide (H_2S) in promoting nerve regeneration and ameliorating neuromuscular dysfunction. Upon successful completion of the award, we have developed a comprehensive transition plan to move our research outcomes towards the next phase of development, with the ultimate goal of delivering effective therapies for peripheral nerve injuries. The goal is to move from the preclinical stage in rats to clinical trials through the evaluation in a swine animal model that simulates traumatic vascular injury in battlespaces. The transition plan is as follows:

1. **Preclinical Study in Rats (TRL-3):** The initial stage involves conducting a preclinical study in rats, exploring the therapeutic potential of Hydrogen Sulfide (H2S) in peripheral nerve regeneration. This stage includes experimental design, treatment administration, data collection, and analysis. The outcomes of this study will provide valuable insights into the effectiveness and safety of H2S in peripheral nerve regeneration, advancing the project to TRL-4.

2. **Experimental Validation and Refinement (TRL-4):** Building upon the preclinical study in rats, the next stage focuses on experimental validation and refinement. This involves replicating the results in a controlled setting, further optimizing the treatment protocols, and validating the reproducibility of the outcomes. The experiments will be designed to address any limitations or gaps identified during the TRL-3 phase. Successful completion of this stage will advance the project to TRL-5.

3. **Evaluation in Swine Animal Model (TRL-5):** Upon achieving TRL-5, the project will transition to the evaluation stage in a swine animal model that simulates traumatic vascular injury in battlespaces. This model closely mimics the conditions encountered in combat scenarios, providing a relevant testing environment. The primary objective of this stage is to assess the effectiveness and safety of H2S therapy in a larger animal model. The outcomes will be critical in determining the therapy's suitability for combat deployment use.

4. **Knowledge Product Development and Refinement (KRL-5 to KRL-7):** Concurrently with the TRL progression, the project will also undergo knowledge product development and refinement. This involves generating scientific publications, developing clinical practice guidelines, provider training materials, and patient brochures, among other clinical support tools. The knowledge products will be continuously refined and updated based on new findings and feedback from stakeholders.

5. **Advancement to Clinical Trials:** The successful evaluation in the swine animal model will pave the way for advancing the H_2S therapy to clinical trials. These trials will involve human subjects and aim to establish the safety, efficacy, and potential therapeutic benefits of H_2S in peripheral nerve regeneration. We will work closely with regulatory authorities and obtain the necessary approvals to initiate Phase I clinical trials, focusing on patient safety and preliminary effectiveness.

6. **Collaboration and Partnerships:** Throughout the transition process, collaboration and partnerships will play a vital role. We will engage with stakeholders in the military, medical, and research communities to foster relationships and secure the necessary resources for continued development and implementation. This includes collaborating with relevant DoD advanced

developers, industry partners, and potential funding agencies to ensure a smooth transition from animal models to clinical trials.

7. Risk Analysis: To ensure a successful transition, a comprehensive risk analysis will be conducted, considering factors such as cost, timeline, regulatory requirements, and manufacturability. This analysis will help identify potential challenges and develop appropriate mitigation strategies to address them effectively.

By combining the findings from the preclinical study in rats with the transition plan outlined above, we aim to advance the H_2S therapy from the preclinical stage to evaluation in a swine animal model and, eventually, to clinical trials. This strategic approach, involving battlespace-relevant evaluation and collaboration with key stakeholders, will contribute to the translation of the therapy into practical clinical applications and improve treatments for peripheral nerve injuries in combat scenarios.

7.0 RESULTS

Phase 1: Model Refinement Study

Validation of IRI:
Phase 1 of our study was centered around the validation of an IRI model in rats, with an overarching aim to examine the potential ameliorative effects of hydrogen sulfide in subsequent phases of the study. The methodological approach involved both technological quantification and visual substantiation of occlusion and reperfusion events. The effectiveness of the IRI model was primarily confirmed through the real-time detection of blood perfusion and tissue oxygenation, utilizing OxyFlo and OxyLite sensors respectively.

In line with the ischemia validation criteria, we observed a pronounced decrease in blood perfusion following arterial occlusion. Initially, blood flow was quantified at approximately 2800 blood perfusion units (BPUs) in the adjacent hindlimb muscle (Figure 6). This value plummeted to a stark average of 300 BPUs upon occlusion, thus evidencing a drastic 90% reduction in blood flow (Figure 6). Such a substantial decrease confirmed successful induction of ischemia in the rat model, an outcome that was further supported by direct visual observation of femoral artery occlusion.

Reperfusion of the ischemic tissue was similarly assessed, both qualitatively and quantitatively. Upon release of the occlusion, blood perfusion levels were seen to rebound to baseline measures as determined by the OxyFlo sensor. This restoration of blood flow concurred with the typical course of an IRI, and thereby affirmed the appropriateness of our rodent model for emulating this pathology.

Complementing the blood flow assessment, tissue oxygenation was also evaluated as an additional validation parameter. We recorded an average tissue oxygen tension of about 22 mm Hg pO_2 before occlusion. However, following the occlusion, tissue pO_2 fell within the range of 8-15 mm Hg. This observed decrease in tissue oxygenation, although not as pronounced as anticipated, further substantiates the successful establishment of ischemia in our model.

Challenges were encountered during the study related to the precision and stability of the oxygen tension measurements. Notably, the OxyLite sensor was highly sensitive to movement, and issues arose regarding the insertion of the probe into the tissue without causing collateral damage. Such obstacles potentially

introduced variability in our measurements, a factor that will be carefully addressed in the ensuing phases of the research.

Despite these complexities, the preponderance of the results robustly supports the validation of the IRI model in rats. This provides a foundation for the subsequent investigation of the therapeutic effects of hydrogen sulfide on IRI.

Body Weight:
In this study, we investigated the effects of hindlimb ischemia reperfusion injury (IRI) on body weight changes in rats over a 28-day period. We observed that regardless of the duration of IRI (2 or 4 hours) or the presence of sham operation (4 hours), there was an initial decline in body weight across all groups in the immediate post-operative period (Figure 7).

The data was expressed as a percentage change from baseline (Day 0), which facilitated the comparison of relative body weight alterations amongst different groups and time points. Over the initial 7 days following surgery, a decline in body weight was apparent, likely attributed to post-surgical stress and recovery (Figure 7). Interestingly, the sham-operated group also exhibited this trend, suggesting the incision and exposure of the femoral artery itself may have substantial effects on body weight, even in the absence of induced IRI (Figure 7). This underscores the importance of including a sham-operated group in studies like this for comprehensive data interpretation.

Following the initial decline, there was a notable rebound in body weight. By the end of the 28-day period, all animal groups had exhibited a significant increase in body weight relative to baseline, reflecting an overall trend of weight gain over time in housing (Figure 7). The 4-hour IRI group demonstrated a greater reduction in body weight compared to the other groups, implying a possible heightened response to IRI (Figure 7).

This study also emphasized the importance of providing diet gel recovery cups post-operatively, as these may help mitigate initial weight loss and support overall recovery. Despite their use in many other research facilities, these were not deemed necessary by the attending veterinarian at our facility (CIRS), which represented a technical challenge in our study.

It is important to note that the small sample size (n=2-3) precluded the possibility of performing reliable statistical analysis on the body weight data collected. Therefore, further research with larger sample sizes is needed to statistically confirm these trends and explore the underlying mechanisms driving these weight changes in response to IRI and the surgical process.

Pain Scoring:
Pain scoring was performed daily for all groups in this study, using an adapted version of the scoring sheet from Paster et al. (2009). The scoring system, ranging from 1-13, allowed for a comprehensive assessment of the rats' post-operative health and comfort levels. All scoring procedures were conducted by the trained research team.

In our scoring system, a higher score is indicative of better health and less pain. Therefore, a score of 13 would represent an animal in optimal health, experiencing no pain, while a lower score would suggest the presence of discomfort or health issues. It's important to note that a score below 3 on two consecutive days was considered a threshold for euthanasia, to ensure animal welfare. Rats scoring less than 5 were closely

monitored twice daily until their scores equaled or exceeded 5, and the attending veterinarian was informed in such instances.

Across the study duration, most rats maintained a relatively high pain score of 10 and above, suggesting that the animals were in good health and experiencing minimal pain (Figure 8). An exception to this trend was observed in the 2-hour IRI group on post-operative day 1, where the average pain score dropped to 9 (Figure 8). This still represents a relatively good health and comfort state, although slightly lower than other groups at that particular time point.

The reasons for this slightly lower pain score in the 2-hour IRI group at the early post-operative stage are not immediately clear and warrant further investigation. Factors such as the intensity and duration of the ischemic injury, surgical technique, and individual variations in health response may play a role.
The relatively high scores across all groups, including the IRI groups, might suggest that the severity of the induced IRI was not sufficient to cause significant discomfort or health issues as reflected by the pain scores. This indicates the need for further optimization of the experimental model to better simulate the pathophysiological conditions associated with IRI.

Despite the observed variations in pain scores, all animals maintained a score above the threshold for euthanasia, demonstrating that their well-being was appropriately managed during the study period. These findings emphasize the significance of effective post-operative health monitoring and care in experimental surgery studies, which can also impact other observed physiological parameters such as body weight.

Muscle Atrophy:
Muscle atrophy was evaluated in this study using a combination of methods, including histology, IVIS *in vivo* imaging, and measurement of wet tissue weights at necropsy. However, due to unforeseen issues detailed in the risk assessment section, histology and IVIS imaging could not be performed. Therefore, muscle atrophy evaluation was based solely on wet tissue weights.

Given the observed increase in body weight over time in housing, the data was expressed as a percentage of tissue weight to total body weight, to account for the potential influence of body weight fluctuations on tissue weight measurements. Despite the rats being ostensibly fully grown at the selected age range, they continued to grow, suggesting that they may not have been fully developed. As discussed earlier in the report, this posed a potential challenge in distinguishing between muscle growth due to natural development and muscle atrophy induced by our experimental conditions.

In the 2-hour IRI group, there was an overall trend of reduced weight of the gastrocnemius muscle compared to day 0 across 29 days of recovery (0 days; 0.540% ± 0.024, 3 days; 0.563% ± 0.043, 14 days; 0.523% ± 0.098, 29 days; 0.409% ± 0.306) (Table 3). A similar trend was observed in the 4-hour IRI group across all hindlimb muscles collected (tibialis anterior, soleus, and gastrocnemius) (Table 3). These trends suggest that our surgical IRI model induces muscle atrophy, as reflected by the reduction in muscle tissue weights.

However, it is important to note that the small sample size (n=0-3) in this study precludes the possibility of performing statistical analysis on the collected data. Therefore, while the observed trends are suggestive of muscle atrophy induced by IRI, further research with larger sample sizes is needed to statistically confirm these trends and to provide a more robust assessment of the effects of IRI on muscle atrophy.

Gait/Motor Function:

In this phase of our research, we evaluated the potential impact of IRI on gait and motor function in our rodent model, utilizing the CatWalk Noldus system. Despite a small sample size, which may have affected statistical significance, observable trends indicated alterations in gait mechanics.

Our analysis revealed significant changes in body speed, defined as the speed calculated by dividing the distance traveled by the animal's body from one initial contact of a paw to the next by the time taken to travel that distance (Figure 9). Specifically, a notable reduction in body speed was evident in both the 2-hour and 4-hour IRI groups within three days post-surgery compared to the sham operated rats. By day 28, the 2-hour IRI group exhibited a recovery to normal body speed (as defined by the sham group), however, the 4-hour IRI group did not manifest a similar recovery. This observation suggests a more prolonged and potentially severe impact on motor function following a longer period of ischemia.

We also investigated the base of support length, defined as the average width between either the front paws or the hind paws (Figure 9). We noted an initial increase in both front and hind paw base of support in both IRI groups post-surgery. For the 2-hour IRI group, front paw base of support normalized by day 7 and hind paw base of support normalized by day 14. However, for the 4-hour IRI group, only the front paw base of support returned to normal by day 14, while the hind paw base of support remained altered. This increase in base of support width, particularly in the hind paws in the 4-hour IRI group, may be indicative of an adaptive motor response to maintain balance and stability in the face of compromised muscular control.

Moreover, our analysis showed a reduction in diagonal phase dispersion in the 4-hour IRI group at days 3, 7, and 14 post-operation compared to the sham group (Figure 9). This trend was not found in the 2-hour IRI group. Phase dispersion is an important measure of coordination between the limbs during locomotion, and these results could suggest an impairment in the inter-limb coordination after a longer period of ischemia.

In terms of paw contact parameters, there was a significant increase in both front and hind paw maximum contact % three days post-surgery in both the 2-hour and 4-hour IRI groups compared to the sham operated rats. However, this parameter was restored to normal by day 7 (Figure 9). An increased paw contact time could signify a compensatory mechanism to maintain stability, which seems to be temporary and recoverable, at least in our study conditions.
Given the limited sample size of 1-3 rats per group, it was challenging to determine other statistically significant abnormalities in gait and motor function. However, the trends identified across different parameters provide valuable insights into the potential influence of IRI duration on locomotor recovery in our rat model. Future studies should look to confirm these findings with a larger sample size to provide more robust evidence.

Electrophysiological Measurements:
In the refinement phase of our study, we incorporated electrophysiological measurements to evaluate neuromuscular functional recovery following IRI. These measurements were focused on the changes in slow compound muscle action potential (CMAP), an aggregate of the electrical responses from multiple muscle fibers innervated by the sciatic nerve. By electrically stimulating the sciatic nerve, we induced action potentials that propagated to the gastrocnemius muscle. The CMAP reading is a reflection of the overall electrical activity of the muscle fibers and serves as an indicator of the functional integrity of the nerve, the neuromuscular junction, and the muscle itself.

Technical issues confined our study to only slow CMAP, owing to interference from multiple devices utilizing a shared electrical outlet. To prevent such issues in future studies, we plan to conduct electrophysiological measurements in a room with several outlets, thereby minimizing electrical interference from other equipment such as the SomnoSuite. However, these changes will necessitate the discontinuation of body temperature monitoring during these procedures due to associated probe interference.

The subsets of our model subjected to these electrophysiological measurements were the 4-hour IRI groups at both 14 and 28 days, as well as the 4-hour sham group at 28 days (Figure 10). We observed a significant trend in our results: the CMAP readings from the 4-hour IRI groups exhibited abnormalities when compared to the 4-hour sham group. Particularly, there was a notable discrepancy in the time taken to detect an electrical response across these groups. While responses in the sham groups were evident as early as 1 ms after stimulation, detection of a response in the 4-hour IRI groups occasionally required up to 10 ms. This delay is indicative of impaired nerve conduction or potential axonal damage caused by the prolonged ischemia.

Despite the preliminary nature of these studies, our findings strongly suggest that our surgically-induced IRI model engenders observable neuromuscular functional abnormalities. These abnormalities are characterized by altered nerve conduction velocity, as detected by CMAP measurements. These results not only underscore the potential of CMAP as a sensitive and reliable indicator of neuromuscular function post-injury, but they also highlight the need for further studies. These future investigations, aided by a more refined protocol free of technical interferences, will be instrumental in substantiating these findings and exploring their implications in greater depth.

Phase 2: Preclinical Study

Our research project faced a multitude of unforeseen challenges that disrupted its execution within the projected timeline following the exhaustion of FY21 funds. During this endeavor, we experienced several delays including funding availability, approval from the IACUC, and the hiring of a technical team, all of which significantly hindered the start of our study. We also grappled with logistical constraints such as equipment availability and limited personnel, which affected the number of surgeries we could perform per day and the efficiency of our bench studies. Moreover, the ongoing renovations at CIRS and the failure of the HVAC system added further obstacles to our progress.

These challenges have given us valuable insights into the myriad factors that can impact a research project's timeline and success. As we move forward, we aim to apply these learnings to future studies and strengthen our planning and risk management strategies.

If new funding is procured for this research study, we plan to execute future studies at UT Health, an institution that offers a conducive environment for complex research projects. We believe that by conducting our studies at UT Health, we can mitigate many of the logistical and infrastructural challenges that we faced in this project. The team at UT Health has a robust track record of successful research, and we are confident that our collaboration will yield positive results. Although our journey so far has been marked by challenges, we remain dedicated to our goal of advancing scientific knowledge in this field.

8.0 CONCLUSION/DISCUSSION

This research was aimed at refining and validating an ischemia-reperfusion injury model of the sciatic nerve as a foundational step in anticipation of testing the hypothesis that ***hydrogen sulfide promotes peripheral nerve and muscle regeneration and preserves neuromuscular function following ischemia-***

reperfusion injury (IRI) of the peripheral nerves in a full pre-clinical study. However, the completion of the project faced numerous challenges, leading to adjustments in the study design and timelines, impacting the ultimate outcomes.

The research was significantly hindered by delays in funding availability, IACUC approval, and the hiring of the technical team, thus affecting the project's schedule and the commencement of the model refinement study. These setbacks necessitated the redistribution of tasks, extension of animal surgeries, and compromise on the efficiency of the study. Further complications arose due to limited availability of essential equipment and personnel on surgery days, as well as the time-intensive nature of anesthesia monitoring. This necessitated a reduction in the number of surgeries performed daily and a reevaluation of resource allocation, particularly with respect to the contract technical team.

A significant constraint that emerged was the ongoing renovations at the CIRS facility, which led to restricted personnel availability and limited time for data collection. Consequently, the study design had to be revised, including reducing the number of animals, eliminating certain time points and histopathological studies, and modifying tissue harvesting protocols for the full study. Additionally, the HVAC system failure in the facility led to the suspension of all animal studies, and repeated relocation due to renovation activities disrupted workflow, leading to potential productivity loss. Lastly, the delay in approval for the MR study amendment has further setback the project, culminating in the discontinuation of the study due to timeline constraints.

Despite these setbacks, the research team made considerable strides towards refining the IRI model, which holds promise for future investigations. However, due to the mentioned constraints, the research team was unable to proceed to the next phase of testing the protective benefits of hydrogen sulfide on peripheral nerve and muscle regeneration. However, critical lessons were drawn from the model refinement study that will inform future research efforts in this field:

The refinement study highlighted the need for effective management of long-term anesthesia in rats. Given the issue of maintaining animal vitality throughout surgical procedures, future studies must prioritize strategic modification of their anesthesia protocols. This includes supplementing room air with additional oxygen, administering preemptive analgesia for effective pain management, modulating the anesthetic regimen, assigning dedicated personnel for continuous monitoring of individual animals, and using advanced tools such as SomnoSuite for optimized and accurate tracking of vital signs.

Unforeseen circumstances regarding diet variability underscored the importance of consistency and appropriateness of diet in animal research. Future studies must ensure the use of a fixed formula diet with a lower protein content, absence of alfalfa meal and soybean oil, and that aligns with the nutritional requirements of rats. This ensures the minimization of experimental variability and adverse effects on research outcomes. The continual growth of rats, particularly at the site of injury, was a critical technical challenge. Future research efforts need to account for this factor during the selection of rat breeds and age groups for the study. Choosing fully mature rats and maintaining stringent control over their diet minimizes the confounding effects of growth on experimental outcomes.

Suture reopening post-surgery emerged as a significant challenge during the refinement study, mostly attributed to self-injurious behavior by the rats. Future research efforts need to incorporate improved techniques for incision care. This includes refining suturing techniques, administering preemptive analgesia, applying topical local anesthetics to incision sites, and intensifying post-operative monitoring.

Overall, despite the discontinuation of the study, the refinement research offers valuable insights into overcoming technical challenges in future research efforts. The learnings drawn from managing long-term anesthesia, dietary consistency, rat growth, and suture care emphasize the need for continual model refinement in experimental research. The insights gained will inform the approach for future studies

aiming to test the protective benefits of H_2S in a similar animal model, thereby aiding in the promotion of peripheral nerve and muscle regeneration following IRI.

9.0 DELIVERABLES

9.1 Publications:

Review Article #1: therapeutic potential of H_2S in military relevant traumatic injuries
Review Article #2: animal models of traumatic neuromuscular injury

9.2 Presentations: N/A

10.0 COST

Total funding for this study: $945,750.00

11.0 REFERENCES

12.0 STUDY FIGURES AND TABLES

Figure 6: Oxylit & OxyFlo traces, exporting values

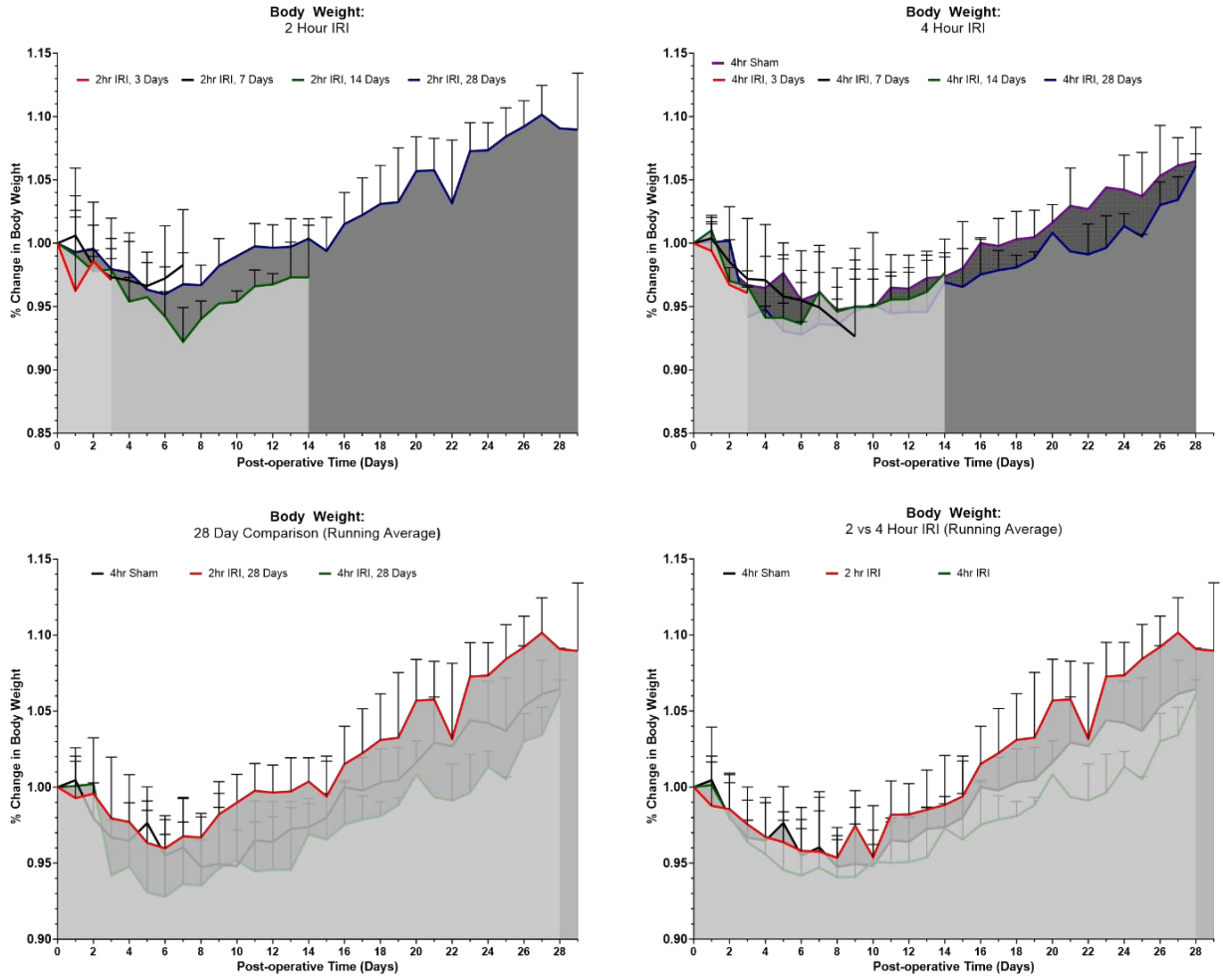

Figure 7

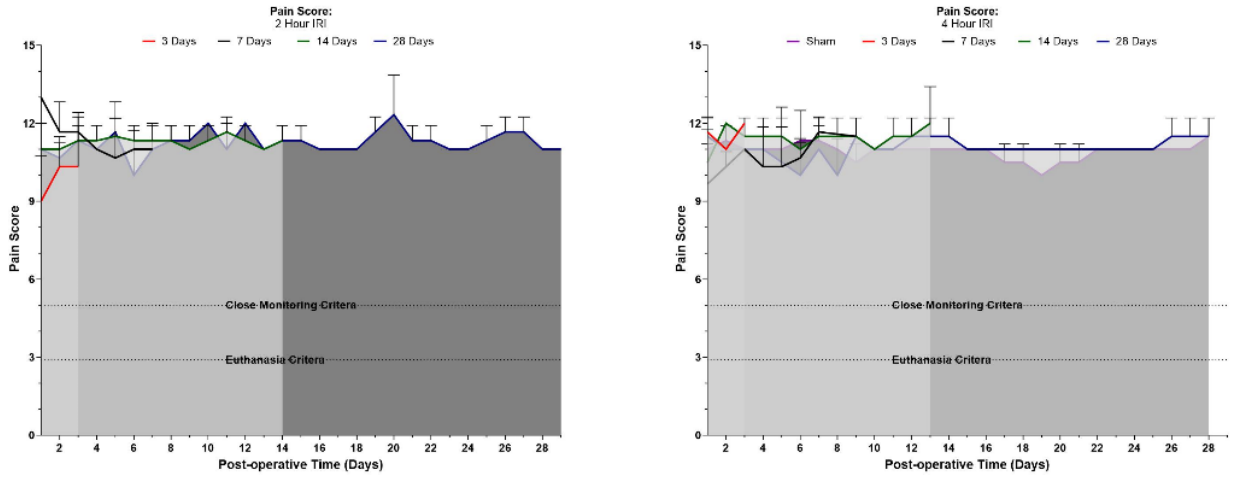

Figure 8

Figure 9: CatWalk Results, SSI analysis in effect
Figure 10: Electrophysiological Measurements, exporting traces

Table 3	0 Day (n=3)	2 Hour IRI			4hr Sham, 28 Days (n=1-2)	4 Hour IRI			
		3 Days (n=3)	14 Days (n=3)	29 Days (n=1-3)		3 Days (n=2-3)	7 Days (n=2-3)	14 Days (n=0-2)	28 Days (n=0-2)
Sciatic Nerve	0.009% ± 0.003	0.009% ± 0.002	0.010% ± 0.002	0.02%	0.01%	0.011% ± 0.001	0.009% ± 0.002	0.01%	-
Bilateral Anterior Tibialis	0.175% ± 0.017	0.180% ± 0.015	0.206% ± 0.021	0.200% ± 0.025	0.194% ± 0.012	0.180% ± 0.026	0.182% ± 0.008	0.296% ± 0.081	0.179% ± 0.001
Soleus	0.050% ± 0.005	0.051% ± 0.005	0.053% ± 0.002	0.052% ± 0.009	0.050% ± 0.006	0.048% ± 0.003	0.050% ± 0.010	0.047% ± 0.050	0.046% ± 0.006
Gastrocnemius	0.540% ± 0.024	0.563% ± 0.043	0.523% ± 0.098	0.409% ± 0.306	0.588% ± 0.064	0.560% ± 0.026	0.487% ± 0.098	0.565% ± 0.008	0.565% ± 0.045

13.0 LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS:

59th MDW	59th Medical Wing
AALAS	American Association for Laboratory Animal Science
AD Instrument	Brand name for a type of biomedical research equipment
α	Alpha, used as a symbol for significance level in statistical testing
ANOVA	Analysis of Variance
AV	Attending Veterinarian
AVMA	American Veterinary Medical Association
BPU	Blood Perfusion Units
CIDS	Joint Capabilities Integration and Development System
CIRS	Clinical Investigator Research Support
CMAP	Compound Muscle Action Potential
Co-PI	Co-Principal Investigator
CA IX	Carbonic Anhydrase IX
DHA	Defense Health Agency
Day 0	Baseline day or start of the experiment/study period
DSCF	Dwass-Steel-Chritchlow-Fligner method
ELISA	Enzyme-Linked Immunosorbent Assay
FAD	Funding Authorization Document
FWH20220036AR	A specific model refinement project identifier for CIRS IACUC
FWH20230045AR	A specific preclinical study project identifier for CIRS IACUC
FY	Fiscal Year
H_2S	Hydrogen Sulfide
H&E	Hematoxylin and Eosin
HVAC	Heating, Ventilation, and Air Conditioning
IACUC	Institutional Animal Care and Use Committee
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IP	Intraperitoneal
IR	Ischemia-Reperfusion
IRI	Ischemia-Reperfusion Injuries
IPR	In-Progress Review
IVIS	In Vivo Imaging System
KRL-5	Knowledge Readiness Level 5
KRL-7	Knowledge Readiness Level 7
mm	Millimeter
mm Hg	Millimeters of Mercury, a unit of pressure
ms	Millisecond
MPO	Myeloperoxidase
µg	Microgram
µL	Microliter
N/A	Not Applicable
NaHS	Sodium Hydrosulfide
NCV	Nerve Conduction Velocities
NEV10060	IVISense Tomato Lectin 680 probe
NEV10150	IVISense Vascular NP 750 probe
NEV11053	IVISense Annexin-V 750 probe
NEV11070	IVISense Hypoxia CA IX 680 probe

NOA	Notice of Action
OxyFlo	A brand name for a type of blood perfusion sensor
OxyLite	A brand name for a type of tissue oxygenation sensor
ORISE	Oak Ridge Institute for Science and Education
OSD(HA)	Office of the Secretary of Defense (Health Affairs)
PBS	Phosphate Buffered Saline
PI	Principal Investigator
PNI	Peripheral Nerve Injury
pO_2	Partial Pressure of Oxygen
RT-PCR	Real-Time Polymerase Chain Reaction
SERCA	Sarcoplasmic Reticulum Calcium-ATPase
SOP	Standard Operating Procedure
SOD	Superoxide Dismutase
SSI	Sciatic Static Index
ST	Science & Technology
TNF-α	Tumor Necrosis Factor alpha
TRL-3	Technology Readiness Level 3
TRL-4	Technology Readiness Level 4
TRL-5	Technology Readiness Level 5
UT Health	University of Texas Health Science Center
°	Degree symbol
µ	Micro symbol