Background including importance of the proposed work:

Large animal model of spinal cord injury: A key to translation

People who have experienced an acute spinal cord injury (SCI) face a life-changing situation that can be not only devastating to the person with the injury but also to associated family and friends. Some of the significant challenges that people with acute SCI face are permanent loss of mobility, compromised bowel and bladder function, severe neuropathic pain, and intensive rehabilitation to adapt to alterations in daily living. SCI affects 232,000 to 316,000 persons in the United States and there are an estimated 12,000 new cases each year alone, with many more cases occurring worldwide (1). Many advancements and new understandings of spinal cord injury have occurred recently which have led to new possible treatments for SCI but despite this, there is still no definitive treatment to preserve or enhance neurological function after injury.

After the initial injury, the events of secondary damage play a significant role in the pathophysiology of SCI. In order to develop effective treatments, it is critical to understand the neurobiology of this secondary damage. To date, small animal models have been utilized to comprehensively study the pathobiology of SCI including secondary damage, as well as therapeutic targets; however, this is not always an ideal approach to long-term research for SCI treatments in humans. The vast majority of animals used to study SCI are rodents, and the injury models in mice and rats are already well characterized highly reproducible (2,3,4). The scientific community that studies SCI has recently established that a large animal model is essential in order to test both invasive and non-invasive treatment strategies. This large animal model provides a translational intermediary, which would more closely model SCI in the human population. In addition, it provides a model to test medical advancements and treatments increasing the likelihood of them being successful in human patients (5,6). Due to these translational needs, a porcine model of SCI was developed (7). The porcine model of SCI has many advantages including being cost effective compared to other large animals and, perhaps the most important advantage relating to the neurobiology of SCI, are the anatomical, physiological and genetic similarities to humans. As it pertains to a spinal cord injury model, swine have striking similarities to humans in their response to injury, cartilage repair, drug-binding sites and interactions in the nervous system, among other things (Kuzmuk et al. 2011). A porcine model of disease has been utilized in various other research paradigms due to these similarities. The swine also maintain bioenergetic and metabolism similarities as demonstrated with mitochondrial genetics, making the pig an important model for the study of SCI and the damaging effects of inflammation and oxidative stress.

Bioenergetics of SCI

A key component in the pathophysiology of SCI secondary injury is oxidative stress. Indeed, therapies that target oxidative stress have gained attention because of their robust protective effects in rodent models; however, none of these results have been tested in a porcine model (8). Early consequences of SCI are not only overt cell death via necrosis, but also disruption of Ca2+ homeostasis around the injury site that can lead to mitochondrial dysfunction, but also other metabolic changes associated with decreased oxygen consumption and apoptosis in designated parts of the lesion. An acute increase in reactive oxygen species (ROS) and cellular damage resulting from both ROS derived from dysfunctional mitochondria and influx of immune cells has been seen after the injury has occurred (10,11). Other studies have demonstrated that lipid peroxidation of both neurons and blood vessels near the site of injury significantly contribute to much of the damage during secondary injury (12). These studies have utilized treatments using anti-inflammatory drugs and antioxidants, such as
methylprednisolone and penicillamine methyl ester, which have resulted in compelling success in rodent models (13,14). Also, metabolic compounds that not only increase the cells ability to combat oxidative stress, but also can act as an alterative bioenergetic fuel have been shown to lead to improved behavioral indices and ultimately lead to a decreased lesion size in rats, which are promising results (15,16).

Despite their success in rodents, none of these treatment successes have been effectively translated from the lab to the clinic for use by patients with SCI. While there are innumerable reasons for the inadequate translation of findings from the rodent model to the human condition, variables such as the size of the animal, temporal window for targeting oxidative damage, basic metabolic parameters, pharmacokinetics and pharmacodynamics, and complexity of the immune response are large differences that prevent the seamless transition to patients with SCI. Because of these limitations in translating findings from rodents to patients, the overarching goal of this research is to provide a better understanding the alterations in oxidative stress and the immune response in a clinically-relevant porcine model of SCI. This study is one essential component in the task of ultimately translating anti-inflammatory and antioxidant therapies to human clinical trials. Thus, we believe there is an unmet need to characterize oxidative stress and inflammation in the porcine model of SCI in order to ultimately segue into treatments that can be translated to the clinical setting. We propose the following aims:

Specific Aims and Hypotheses

Aim 1: Test the hypothesis that T-10 SCI in the adult pig leads to an increase in inflammatory markers in the spinal cord compared to uninjured control (laminectomy only: LAM) animals. SCI induced immune activation is central to secondary damage. Inflammatory mediators play a dual role in clearing dead and dying cells as a result of the primary injury, infection prevention and also spreading of the lesion to compromise other parts of the spinal cord. Interrupting the inflammatory cascade has been demonstrated to be beneficial in rat models of SCI and has not been evaluated in this porcine model. Understanding the temporal succession of inflammatory events is critical to developing the most effective treatment strategies. Adult male Yucatan mini-pigs will receive a T10 contusion SCI and will be evaluated at acute and chronic time points, 48-hours and 6-weeks respectively. Both pro-inflammatory and anti-inflammatory factors will be measured and a temporal profile of overall immune responses will be constructed.

Aim 2: Test the hypothesis that there are increases in ROS production after SCI injury compared to LAM controls. Mitochondrial dysfunction and immune activation induced by SCI produce an oxidative environment that contributes to the pathophysiology of the injury. Studies targeting secondary oxidative stress have been effective in rodents; however, it is still unknown for a porcine model of SCI. A critical step in the translation of a novel therapeutics is the evaluation of the time course of ROS levels after SCI. In parallel to aim 1, adult male Yucatan mini-pigs will receive a T10 contusion SCI followed by evaluation of oxidative stress in the spinal cord. The results will enable a better understanding of the progression of oxidative damage, which will enable the use of antioxidant therapies to treat SCI at appropriate times.

Experimental Groups and Outcome Measures

a. Experimental groups: Animals will be randomly assigned into the following groups: (a). Uninjured laminectomy control (LAM); (b). T10 contusion SCI. At either an acute (48 hours) and chronic (6 weeks) time points. There will be n=6 animals/group/time point, for a total of 12 animals.
Methods

Surgical procedure:

In this study, we will use male and female Yucatan miniature pigs that will be anesthetized based on previous experiments (Lee et al. 2013). Urinary catheter is placed into the bladder, via cystotomy for postsurgical urine drainage, and mechanical ventilation is maintained on every pig. Standard procedures of monitoring heart rate, respiratory rate, blood pressure, body temperature and oxygen saturation are recorded during surgery. Hydration is maintained by IV Ringer’s solution. The animal is laid in a prone position and T10 is identified by careful palpation and visualization. A midline incision is made between T8 and T13 to expose the vertebrae using electrocautery. The dorsal vertebral processes are removed from T9 to T13, and a full laminectomy is performed on T10. Based on anatomic landmarks, two multi-axial screws are inserted into the pedicles of T12 and T13, and a rod is placed between the screws to hold the base of the apparatus. A stabilization arm is attached to the base is used to align the apparatus directly above the T10 vertebrae.

The weight drop apparatus consists of a guidance system, which is a track that allows the weight to slide down with the applied force of gravity and impact the exposed spinal cord. A 20g weight is attached to the guidance system at the desired height of 20cm. Just prior to the injury the animal is given succinylcholine at 1mg/kg after a 2 minute wait, to prevent spontaneous movement after the injury and subsequent destabilization of the device. To produce the injury, the weight is then dropped, leading to an impact of the exposed spinal cord. Following initial injury with the impacting weight, a 100g weight on top of impacting weight is added for an additional 5 minutes to compress of the spinal cord. Following the sustained compression, the apparatus is removed and the wound is closed in layers.

Following surgery, the animals are given acepromazine as needed to facilitate a calm recovery from surgery, since these animals awake from anesthesia unable to stand. Antibiotics are also administered to attenuate major infections. Animals are monitored frequently for either a 48-hour or 6-week period, depending on the testing metric. For the 6-week time-point, the urinary catheter is removed 7 – 10 days after SCI upon the ability to reflexively urinate. (Lee et al. 2013)

Assessment of inflammatory markers in the spinal cord tissue:

Animals will be humanely euthanized at either 48 hours (acute) or 6 weeks (chronic) after SCI. At each time point, spinal cord tissue will be extracted and rapidly dissected into three 1.5 cm sections as described (17). 1.5 cm sections of spinal cord will also be dissected 7mm rostral and caudal of the injury epicenter. Intracellular cytokine synthesis for TNF-α, IL-1β, IL-6, IL-17A, IFN-γ, and MCP-1, will be detected with a BD Biosciences intracellular cytokine staining kit as described [16-19]. Pro-inflammatory cytokine/chemokines will be detected with spinal cord protein lysates with a Luminex Beadlyte 21-plex multi-cytokine (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, GM-CSF, KC, IFN-γ, MCP-1, MIP-1β, TNF-α, RANTES, VEGF) system as described [13, 16]. This system allows for the highly sensitive detection of all of the above cytokines in a single assay and will be run in triplicates. Groups (Uninjured, LAM and T10 contusion) from each time-point (acute and chronic) will be analyzed by one-way analysis of variance (ANOVA).

Assessment of reactive oxygen species in the spinal cord tissue:

Using a subset of spinal cord tissue from the above experiment, oxidative stress will simultaneously be examined by flow cytometry as described (18-21) using the redox-sensitive dyes dichlorodihydrofluorescein diacetate, dihydroethidium, CellROX, and dihydrorhodamine. As with assessment of inflammatory markers, flow-cytometry data from groups (Uninjured, LAM and T10 contusion) at each time-point (acute and chronic) will be analyzed by one-way analysis of variance (ANOVA).
References


