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CHANGES IN THE TESTES FOLLOWING SPINAL CORD INJURY AND THE ATTENUATING EFFECTS OF LICOFELONE

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CHANGES IN THE TESTES FOLLOWING SPINAL CORD INJURY AND THE ATTENUATING EFFECTS OF LICOFELONE

by

Ryan Dean Fortune, B.S.

APPROVED:

________________________________________

David Loose, Ph.D.
Advisory Professor

________________________________________

Ruth Heidelberger, M.D., Ph.D.

________________________________________

Darren Boehning, Ph.D.

________________________________________

Michelle Hook, Ph.D.

________________________________________

Edgar T. Walters, Ph.D.

APPROVED:

________________________________________

Dean, The University of Texas

MD Anderson Cancer Center UTHealth Graduate School of Biomedical Science
CHANGES IN THE TESTES FOLLOWING SPINAL CORD INJURY AND THE ATTENUATING EFFECTS OF LICOFELONE

A DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by

Ryan Dean Fortune, B.S.
Houston, Texas
Date of Graduation August, 2018
Dedication

This dissertation is dedicated to Raymond Grill, PhD. You were my first mentor for this project, and most of the work was done in your lab. I wish you could be here to see the end my journey towards a doctorate. May you rest in peace.
Acknowledgements

I would especially like to thank David Loose, PhD, for taking me into his lab when my first lab moved across states. We are grateful for the U.S. Department of Defense’s generous funding and support (Award Number: W81XWH-12-1-0481). Thanks to Feng Li, PhD, the Metabolomics core, Advance Technology Cores of Baylor College of Medicine, for his proficient analysis of bile acids in the serum. We would also like to thank Darren Boehning, PhD, UTHealth, for his generosity in teaching and helping perform caspase assays on our samples; and Redwan Huq, Baylor College of Medicine, for his proficiency in flow cytometry, as well as his willingness to troubleshoot and dedication to proper science. We thank Ashley Hood and the rest of the Center for Clinical and Translational Sciences TL1 program for generous funding and support. We would like to thank Jeff Frost, PhD, UTHealth, for helping to teach proper lab techniques. Thanks to Rebecca Berdeaux, PhD, UTHealth, for generous use of lab equipment. Finally, thanks to Sarah Riosa and Alissa Poteete, MS, for their patience, support, and technical assistance.
Abstract:

CHANGES IN THE TESTES FOLLOWING SPINAL CORD INJURY AND THE ATTENUATING EFFECTS OF LICOFELONE

Ryan Dean Fortune, B.S.

Advisory Professor: David Loose, Ph.D.

Spinal cord injury is a devastating disease that researchers have had very limited success in treating. In addition to interrupted innervation, spinal cord injury causes pathologic changes in a multitude of organ systems. Male infertility is one such complication that is particularly devastating because the patient population is predominantly young men. Our lab has previously shown that the blood testis barrier breaks down after spinal cord injury. This dissertation shows the local metabolomic and mRNA changes that spinal cord injury causes within the testes using a Sprague Dawley rat model, including the elevation in eicosanoids, increased oxidative stress, chronically elevated unconjugated bile acids, altered immune cell populations, acutely decreased testosterone production, and acute elevations in lysolipids. In addition, we show promising and long lasting attenuation of many of these changes with early treatment with licofelone, a dual cox/lox inhibitor, including attenuation of bile acid levels, lysolipids, inflammation, and oxidative stress. This indicates a
promising avenue for future research into alleviating this symptom of a devastating injury.
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CHAPTER 1: INTRODUCTION

1.a History of Spinal Cord Injury

This first known recorded scientific document, the Edwin Smith Surgical Papyrus, describes spinal cord injury as “an ailment not to be treated”. (1, 2) 4,000 years later, US military doctrine still places spinal cord injury patients in the expectant category during mass casualty events. (3) Although many medical advances were made in the intervening time, they all help manage spinal cord injury instead of treating it. From Lord Nelson of the Napoleonic Wars, to General Patton of WWII fame, spinal cord injury claimed many lives throughout history before supportive treatment became medically possible and widespread (1). 1936 saw the opening of the first spinal cord injury hospital unit, which trail-blazed the supportive therapies and rehabilitative regimens that spinal cord injuries require. (1) The first major advancement in improving spinal cord injury long term outcomes was the advent of the “Model System” in the early 1970’s, which consolidated all of the different services that patients with spinal cord injury require into one comprehensive team. (1) The success of this system resulted in the establishment of the National Spinal Cord Injury Database that allows for analysis of spinal cord injury outcomes and the identification of issues unique to this patient population. (4) Today, spinal cord injury mortality is much lower than 40 years ago, especially in the first year of injury. This is largely due to better identification and treatment of medical issues specific to this patient population due to the establishment of the National Spinal Cord Injury Database. (5) However, we still have no treatment that can treat the spinal cord injury itself, and patients still struggle with the many aspects of their disease.
1.b General Organization of the Spinal Cord

To better understand the ramifications of spinal cord injury, it is helpful to understand what the spinal cord’s role in the central nervous system is. Put simply, the spinal cord is a bundle of both efferent and afferent nerves that acts as a highway of information between the brain and the rest of the body. Starting from the brainstem, the spinal cord extends down the vertebral column, protected by the spinal vertebrae. At each level, anterior and posterior nerve roots break off between the vertebrae to either side and merge to form spinal nerves. These nerves send out branches to innervate skin, muscles, and other tissues. This distribution is clearly demonstrated by the body’s dermatomes, which are the areas of skin innervated by each spinal nerve. The anterior nerve roots typically hold efferent motor nerves, innervating muscles and allowing for voluntary movement. The posterior nerve roots typically hold afferent sensory nerves, allowing for proprioception, temperature, pain, touch, and vibration sense. In the spinal cord, these nerves are bundled into different “tracts” that organize the cord. The pathways of these tracts vary depending on the nerve’s function.(6) For example, upper motor neuron cell bodies are located in the cerebral motor cortex in the brain, which send most of their axons down into contralateral side of the spinal cord in the lateral corticospinal tract, while some of them send them down the ipsilateral anterior corticospinal tract. Once at the level of the muscle that they innervate, these axons synapse with lower motor neuron cell bodies, which send their axons out to innervate the muscle fibers on the contralateral side of the originating cell body in the brain. Pain and temperature sensing nerves have their cell bodies in the dorsal nerve roots, extending axons both to the skin and into the spinal cord to synapse with another nerve
cell body on the ipsilateral side that extends its axon up one of the contralateral spinal thalamic tracts to the thalamus in the brain. Touch, proprioception, and vibration sensory nerves also reside in the dorsal roots, but send their axon up the ipsilateral spinal cord in the posterior columns up to synapse in the medulla before traveling up the contralateral side to the thalamus.

1.c Reflexes and the Autonomic Nervous System

In addition to these basic in and out signals, the spinal cord is also important for many involuntary actions. The most basic of these are the spinal reflexes. For example, the patellar reflex starts with afferent nerve endings in the quadriceps that lead past the neuron cell body in the dorsal root to synapse in the spinal cord with 2 different neurons. The first neuron sends an axon back to the quadriceps to contract the muscle. The second is an inhibitory interneuron that synapses again in the spinal cord to inhibit the contraction of the muscle opposing the quadriceps: the hamstrings. This reflex, like many, can be inhibited or exaggerated by conscious thought, indicating influence on these reflex arcs from the brain. The spinal cord is also capable of adapting these responses. Repeated noxious stimulation can lead to sensitization, meaning that the nerves are easier to activate. Repeated activation of spinal reflexes can lead to habituation, meaning that the reflexes are inhibited and the nerves require more stimulation to activate. This flexibility and adaptable learning goes beyond simple deep tendon reflexes and is involved in fine motor skill acquisition, athletic training, and training complex body movements and muscle memory. This constant change and the ability to form new or change the strength of current connections is known as plasticity.
This plasticity of the spinal cord allows for it to continue to adapt as the body grows and learns.(7)

**The spinal cord also plays a role in the autonomic nervous system, which is made up of the parasympathetic and sympathetic systems.** These complicated involuntary systems regulate the internal organs and viscera, including blood pressure, heart rate, the balance of vasoconstriction and dilation, and organ function. These organs are innervated by axons whose neuronal cell bodies are located in ganglia found throughout the body. These neurons are in turn innervated by preganglionic neurons whose cell bodies are located in the CNS. The sympathetic preganglionic neuronal cell bodies are located in the brainstem and all along the spinal cord, while the parasympathetic preganglionic neurons are mainly in the brainstem, with a few in the sacral spinal cord that help regulate bowel, bladder, and sexual function. Both systems’ preganglionic neurons receive input from multiple areas of the brain. The afferent neurons for these systems are one and the same, and are located in the dorsal root ganglia with their axons in the organs. These then travel up axons the spinal cord, branching and synapsing around their neuronal cell bodies in the dorsal horns. (6, 8)

1.d Non-Neuronal Cell Types

In addition to the different types of motor and sensory neurons, there are a host of cells that play a more supportive role. Although there are many more than briefly listed here, these are the major cell types that either play a major role in spinal cord injury or are relevant to this dissertation.
Astrocytes, a type of macroglia, are one of the most numerous cell types in the CNS. They help regulate homeostasis of synapses, vasoconstriction and dilation, flow of nutrients, and maintenance of the blood brain barrier. In damaged CNS tissue, astrocytes proliferate and can create a barrier in the form of a glial scar. (9)

Microglia are the CNS equivalent of peripheral macrophages. Macrophages are immune cells that specialize in phagocytosis. Phagocytosis is the process by which cells “devour” debris, foreign molecules, and microbes. Macrophages reside both in peripheral tissue as resident macrophages, or are mobilized and recruited to areas of inflammation or cell damage. These are distinct from neutrophils, which are the first responders of the body’s innate immune system. Neutrophils are phagocytic cells that are particularly effective against foreign microbes and are involved in inflammatory processes. (10)

Oligodendrocytes are the functional equivalent of Schwann cells within the CNS. They wrap their appendages around many surrounding neurons’ axons and compact in layers to form highly specialized layers of insulation known as myelin sheaths. These enable axons to transport their signal much more quickly than unmyelinated neurons. (11)

1.e Blood Brain Barrier

Most of these cell types have a functional equivalent that resides outside of the central nervous system. This is because the CNS is an immune privileged or modulated environment that is kept separate from the periphery by the blood brain barrier. The blood brain barrier (BBB) is a vascularly associated layer that controls the passage of
cells and molecules both into and out of the central nervous system. It consists of endothelial cells, pericytes, tight junctions, and astrocyte end-feet surrounding all of the vasculature that supplies the brain. (12) While made up of the same building blocks and is effectively an extension of the BBB to cover the spinal cord portion of the central nervous system, the blood spinal cord barrier and blood retinal barrier are both morphologically and functionally somewhat distinct. (13)

1.f Peripheral Immunity and Immune Tolerance

In addition to the immune cells within CNS described above, T cells are an immune cell types that are relevant to this pathologic process, briefly described here. “T-cell" is a relatively broad term that describes a population of cells that specialize in cellular immunity that are developed primarily in the thymus. This consists of multiple classes of T-cells that specialize in certain roles. For instance, memory t-cells are long lived cells that "remember" certain antigens the body has been exposed to in the past, allowing for the body to quickly mount a response if it encounters the antigen. Cytotoxic t-cells destroy cells of the body that have been infected with viruses or have mutated into tumor cells. Helper t-cells secrete cytokines that help activate or regulate the immune system. There are many more types of T-cells and other immune cells whose roles are beyond the scope of this manuscript. (14)

Immune tolerance is a process by which the body identifies and eliminates immune cells that are auto reactive, meaning that they react to antigen that is a part of the body instead of foreign origin. The immune system is trying to make itself aggressive against foreign invaders, while preventing the development of autoimmune
disease. This process actively occurs early in life. Female gonads, the ovaries, undergo meiosis while still in the womb. This means the proteins unique to the female oocytes are included in the immune tolerance process. Males, however, do not begin spermatogenesis until puberty, well after this specialized process occurs. (15, 16) It is then no surprise that spermatocytes, and their unique proteins, are highly antigenic, causing the body to create anti-sperm antibodies when exposed to the peripheral immune system.

1.g Epidemiology of Spinal Cord Injury

Over 12,000 patients with spinal cord injury are added per year to the United State National Spinal Cord Injury registry. This does not include those that did not survive their initial resuscitation. (17) According to NSCISC since 2015, 38.3% of SCI patients received their injuries in a motor vehicle accident. Although more women are more likely to be involved in a car accident and more likely to be injured in more serious collisions, men are disproportionally involved in more of the type of high speed accidents that can result in SCI, especially in the 6% from motorcycle accidents. (18) Another 13.8% of spinal cord injuries are caused by gunshot wounds. Again, men are far more likely to be the victim of violent crime than women. Another 8.2% are caused by sports, which, with the exception of horseback riding, also show a disproportionate number of males engaged in more risky events. The 31.6% caused by falls and the remaining small percentages are still overwhelmingly a male population, but with less self-evident reasons. These disparities in the mechanisms of SCI lead to a 78% male patient population, many of which are young enough to have not started to have a family of their own.
1. Pathophysiology of spinal cord injury

The immediate stage of spinal cord injury is somewhat self-explanatory, but is worth noting the specifics. First, the BSCB is locally and physically broken, allowing access to the peripheral immune system. Second, blood vessels are broken, causing an influx of blood directly into the spinal cord, along with all the proteins and immune cells that are normally kept separate. Third, this interruption of normal blood supply causes localized areas of hypoxia if there is not enough redundant blood supply. Finally, neuronal axons are abruptly severed or crushed, causing spinal shock, which is a full loss of all spinal reflexes for a period of time after injury, regardless of eventual outcome. (19)

Following this direct tissue damage is a release of pro-inflammatory and chemotactic signals. This recruits neutrophils to the injury area within 4 hours, which stay for around 5 days. Although necessary to clean up totally destroyed tissue, neutrophils cause secondary damage to surrounding tissue due to an overwhelming response of proteolytic and oxidative mediators. By 2 days, macrophages enter the area to stay for months, causing more secondary damage due to releasing pro-inflammatory cytokines and reactive oxygen species that cause oxidative damage. However, macrophages are also necessary for the cleanup of damaged tissue and some cytokines are neuroprotective and promote regrowth. Finally, T-cells enter the injury area at various times throughout the animal’s life, but their role in secondary damage remains controversial. (20) From days 5-14 post injury, astrocytes surround the injury site to try and corral the inflammatory process occurring. This glial scar becomes permanent, inhibiting axonal regrowth. Similarly, the area of injury becomes filled with
fibroblasts, which eventually shrinks down the area into a fibrotic scar. (21) The cord remains an oxidative and inflammatory environment well into the chronic phases of injury. (22) Progressive pathologic changes have been noted both rostral and caudal to the injury site, indicating a non-localized multistage disease process. (23) It has also been shown that SCI causes hyperexcitability, growth, and spontaneous activity in nociceptive neurons in the spinal cord. (24) Although much of these changes are pathologic, the plasticity of the CNS may have positive benefits as well. It has been shown that the brain undergoes reorganization and growth of new pathways following SCI, presumably as a method to attempt to regain function or better utilize what function remains. (25)

1.i Symptoms of spinal cord injury

There are many symptoms of spinal cord injury besides the obvious loss of sensory and motor function. The major breakthroughs in the treatment of spinal cord injury have been in treating the many symptoms that lead to increased morbidity and mortality.

Pain after spinal cord injury can come from many sources. These are classified into either nociceptive pain, which is pain coming from damage to tissues, or neuropathic pain, which is from defective nervous tissue or compressive radiculopathy. Pain at, above, or below the injury site is common, as is pain from muscle spasms or instability, and visceral pain from things such as kidney stones, gall stones, or bowel dysfunction. The pain that is most difficult to treat in most patients is neuropathic pain. This pain is treated with antidepressants like amitriptyline or anticonvulsants like
gabapentin with very mixed effectiveness, with a long list of medications to try if those should fail. (26) In many cases, this pain could be caused by continued inflammation and oxidative stress in the spinal cord. (22) However, there is also evidence of primary afferent nociceptor hyperactivity in dorsal roots contributing significantly to neuropathic pain. (27)

**Voluntary bladder control is lost following spinal cord injury.** Under normal circumstances, the bladder is under both autonomic and somatic control. The bladder has an inner sphincter, controlled by smooth muscles, and an outer sphincter, controlled by somatic innervation from the sacral nerve roots. The detrusor muscle, the muscle that constricts the bladder wall, is controlled by a parasympathetic reflex in the sacral spinal cord, while the sphincter is controlled by an autonomic center in the brain. When spinal cord injury occurs, the sphincter loses both somatic and autonomic innervation, but the detrusor muscle may retain depending on if the injury is suprasacral. This results not only in an inability to void, but can cause high pressures in the bladder, causing kidney damage. These effects necessitate either permanent Foley catheter, supra pubic catheter, or repeated straight catheter. If the patient retains enough upper limb mobility, straight catheterization is possible and is preferred by most patients. Because of this repeated or continued intrusion into a sterile environment, urinary tract infections are very common. Repeated urinary tract infections run the risk of developing antibiotic resistant bacteria, which further complicates this chronic condition. (28)

**Spasticity and atrophy develop below the lesion level after spinal cord injury.** When a muscle loses nerve innervation by its innervating lower motor neuron dying, it receives little neuronal signal and atrophies at an accelerated rate. The muscle
behaves in accordance with “lower motor neuron disease”. However, when the upper motor neuron, the neuron in the spinal cord that synapses with the lower motor neuron, dies or has its axon cut, the muscle behaves much differently. Upper motor neuron symptoms include heightened reflexes, rigidity, and spasticity. As stated above, lower motor neurons are also innervated by signals that originate in the periphery: reflex arcs. With the upper motor neuron gone, there is no longer inhibition from the brain to dampen that reflex, and the threshold to trigger it is lowered. A small feedback loop, noxious stimuli like heat, cold, or even bladder distention could then cause a spasm. Ironically, small amounts of muscle spasticity may help combat atrophy and osteoporosis, but when uncontrolled is immediately and negatively impactful on the patient’s daily life. While physical therapy is the first option, drugs like baclofen help immediately dampen this symptom. However, many physical medicine and rehabilitative medicine physicians specializing in spinal cord injury are hesitant to give doses large enough to completely eliminate the upper motor neuron symptoms and try to find a balance between long term effects and short term benefits. There are many electrical stimulatory treatments that aim to somewhat replace the inhibitory effects that stimulation from repeated stimulation. (29)

The rapid development of osteoporosis in long bones below the lesion level is one of the most medically striking aspects of spinal cord injury. This is so rapid that some patients can develop dangerous levels of hypercalcemia. It is well established that weight bearing exercise helps strengthen the skeletal system, even with just walking. However, the rapidity of this development indicates a different mechanism, possibly due to the loss of autonomic sympathetic innervation of
osteoblasts and vasculature. Increasing osteoporosis increases the risk of fracture. If this fracture goes unnoticed at the time of injury due to lack of sensation, this can result in infection, fat emboli, necrosis, and further tissue damage. This has traditionally been treated with bisphosphonates, but their efficacy is limited to the first year after injury.

(30)

**Loss of voluntary bowel control is one of the side effects of spinal cord injury that takes the longest to properly manage.** The bowels, like the bladder, are primarily innervated by the autonomic nervous system but with the external sphincter and abdominal muscles under voluntary control. The internal sphincter is controlled by a sacral reflex arc similar to the bladder. Bowel care after spinal cord injury has many crucial components and is one of the goals of rehabilitation that takes the longest to fine tune. The bowels also have a tendency to respond well to routine. Bowel training involves using suppositories, rectal stimulation to get the sphincters to dilate, medications like stool softeners or laxatives, and diet. Depending on the level of injury, patients can lose or retain control of either the abdominal muscles, the sacral reflexes, or both. Each of these combinations leads to a different pattern of bowel difficulties. Constipation, difficulty voiding, and incontinence are the hallmarks of the different patterns. (31)

**Gall bladder disease is yet another side effect of chronic spinal cord injury.** Because gall bladder disease is usually a clinical diagnosis, this can be a difficult disease to diagnose, which can make autonomic dysreflexia (see below) especially dangerous. Although there is a higher number of patient with SCI that develop gall stone disease compared to the general population, there is an even higher incidence of
biliary sludge. (32, 33) Biliary sludge is a unique sonographic finding that shows increased layering without acoustic shadowing. Biliary sludge can disappear after the cause has been removed, which is not currently possible in SCI. When chronic, it is associated with gall stones and pancreatitis. (34) This could be involved with spinal cord injury patients to develop metabolic syndrome. (35)

**Due to the disruption of autonomic innervation, the body has difficulty maintaining many physiologic processes** that are usually taken for granted. Blood pressure control and cardiovascular health are greatly linked with the autonomic nervous system, including vasoconstriction and dilation. It is also much more difficult for patients with spinal cord injury to regulate their body temperature. Without sympathetic innervation, patients do not sweat below the level of the lesion. Shivering is also similarly impeded. The body no longer vasoconstricts peripheral vessels in order to shunt blood internally to avoid heat loss. The loss of all of these temperature regulating mechanisms below the lesion level make it much more difficult for patients with spinal cord injury to tolerate hot or cold environments, requiring them to always dress in layers to be able to quickly change their body temperature.

**Uncontrolled sympathetic activation can cause autonomic dysreflexia**, which is life threatening emergency characterized by flushing, sweating, and dangerously high blood pressure. It is due to an uncontrolled activation of the sympathetic portion of the autonomic nervous system due to a persistent, undetected noxious stimulus. Due to a lack of sensation below the lesion level, large, or even small, noxious stimuli can go unnoticed by the patient. This could be compared to how advanced diabetics with peripheral neuropathy can develop necrotic ulcers on their feet.
from having a rock in their shoe. A broken bone, a urinary tract infection, an abscess, or even simply having their clothes on too tight can cause a steady or sudden unopposed sympathetic activation. This can lead to dangerously high blood pressures and cerebral hemorrhage and even death. (36) Autonomic dysreflexia has been linked to similar processes as neuropathic pain and carries some overlap in their mechanism. (37)

**A spinal cord injury carries with it many changes to a patient’s social life,** and even more medical complications than are listed above. Many patients, even after rehab, are no longer able to perform the duties required of their old job. Many patients move in order to live in a wheelchair accessible house or to be closer to one of the major spinal cord injury rehabilitation centers. Patient’s relationships with loved ones can undergo a fundamental change, as shown by the higher incidence of divorce for spinal cord injury patients. Depression and suicidality of patients with spinal cord injury is also increased. (38, 39)

**1.j Testes biology**

The bulk of the testis consists of 400-500 convoluted seminiferous tubules. These tubules contain an inner lumen that connects all the way up to the epididymis. These tubules are surrounded by myoid peritubular cells that create a basal lamina between themselves and the Sertoli cells. The bulk of the seminiferous tubules are made up of these large Sertoli cells that regulate spermatogenesis. Each Sertoli cell reaches from the basal lamina at the periphery of the seminiferous tubule, to the lumen. Sertoli cells are sustentacular cells, meaning that they “sustain” developing sperm cells and provide all nutrients and signals for their development. Removing a spermatocyte
from the Sertoli cell it is attached to before the sperm is fully mature causes the sperm cell to undergo apoptosis. Immature sperm progenitor cells are on the periphery of these tubules. As you look inward towards the lumen you would find more and more mature spermatocytes until fully mature spermatids are released into the lumen to travel to the epididymis where they wait in preparation for ejaculation. The area outside of the seminiferous tubules is called the interstitium, which is where blood vessels, cells of Leydig, resident immune cells, and fibroblasts reside. (40)

**Puberty marks the beginning of spermatogenesis and the formation of the blood testes barrier.** Because the unique proteins that form the exterior of the sperm are not produced elsewhere in the body, and because spermatogenesis doesn’t occur until well after immune tolerance has occurred, the sperm are highly antigenic. This creates the need for the sperm, and even the majority of the spermatogenesis process, to be kept separated from the immune system. This is done by creating an immune privileged environment by the formation of the blood testes barrier, similar to how CNS keeps itself separated from the peripheral immune system. The blood testes barrier mainly consists of a tight junctional network between Sertoli cells near the periphery of the seminiferous tubules, a division which creates a basal and adluminal compartment. This physical barrier prevents unsanctioned migrations of cells, proteins, or toxins into the lumen of seminiferous tubules. This allows the Sertoli cells complete control over the spermatogenesis process.(40) The tunica propria, ectoplasmic specializations, gap junctions, and desmosomes also play a role in the BTB. (41)

**In addition, the testes use multiple methods to create an immune attenuated environment within the entirety of the testes, even outside the blood testes**
**barrier.** First, the testes are kept at a lower temperature than the rest of the body.(16) It is well established that the immune system can increase its aggressiveness and effectiveness by raising the body’s temperature, either with a systemic rise in temperature or fever or by a localized increase temperature as found with abscesses.(42) Sperm cell development is hindered by temperatures elevated to normal body temperatures.(43) Whether these traits evolved together or are simply happy coincidences is a much more difficult question to answer. Second, the Sertoli cells secrete signal mediators that attenuate immune responses. This was shown by transplanting Sertoli cells into peripheral tissue, which creates a localized immune attenuated area. (44) Finally, some cells outside the seminiferous tubules that reside in the interstitial space, like cells of Leydig and resident macrophages, are modified to regulate and suppress the other cells of the immune system.(45)

1.k Spermatogenesis

Each cross section of a seminiferous tubule will not have all the different stages of sperm cell development. If you look at a seminiferous tubule longitudinally, there is a clear, overlapping waves of spermatogenesis. This divides the seminiferous tubules into XII stages in rats, with each stage only having 3 to 4 different stages of sperm progenitor cells.(46) These cells are connected to the Sertoli cells by different types of junctions depending on their developmental stage. Because the immature sperm progenitor cells are located in the basal compartment, and the more mature sperm cells are in the adluminal compartment, this means that the sperms cells migrate across the BTB. This migration occurs at stage VIII, where the BTB is broken down and reformed layer by layer around the migrating cell. (41)
A single spermatogonium could theoretically develop into 4096 sperm, but usually only ~25% make it completely through the process. The remainder are “eaten” by the Sertoli cell after undergoing apoptosis. Spermatogenesis is a very delicate process. Many things can go wrong even in perfect conditions. By weeding out the majority of sperm cells, the body ensures a higher percentage of well-formed motile sperm in order to increase chances of successful fertilization.(41) Spermatogenesis is a testosterone dependent process. The cells of Leydig in the testes provide a steady amount of testosterone to the Sertoli cells. Sertoli cells will stop spermatogenesis and initiate apoptosis in many stages of sperm development in the absence of testosterone. (47)

Spermatogenesis is also sensitive to temperature, increased bile acids, oxidative stress, trauma, and auto-immune reactions. Heat injury will cause an injury localized to particular stages of the seminiferous tubule, creating alternating areas of apoptosis and normal tissue. (43) Increased bile acids also cause increased apoptosis but, in contrast, cause a wide spread breakdown of the blood testis barrier and germ cell sloughing. (48) Immune mediated infertility is characterized by an infiltration of active immune cells. (49) Sperm cells are more sensitive to different insults depending on their stage of development. (50)

1.1 Male Infertility in Spinal Cord Injury

Before the advent of the NSCID, less than 1% of men with SCI were able to father children. Now, we have in vitro fertilization (IVF) and intracytoplasmic sperm injection (ISCI), which has a success rate between 30 and 60% in patients with SCI.
However, both IVF and ISCI are prohibitively expensive for many patients, with no guarantee for success. (51-53) There are three possible sources of male infertility after spinal cord injury: erectile dysfunction, anejaculation, and low sperm quality. Patients with an intact parasympathetic sacral reflex arc (injury site of L2 or above) can achieve reflexive erections. (53) Otherwise, erectile dysfunction is treated the same way that a patient without SCI would: via oral medication or mechanical prostheses. (17, 53-57) Anejaculation is treatable with mechanical or electrical stimulatory devices, or extracted from the epididymis via needle in refractory cases. (53, 56, 58, 59) However, low sperm quality is more difficult to treat. In the general population, low sperm motility is accompanied by low sperm numbers, both of which contribute to infertility. In SCI, sperm counts are normal, but the sperm have low motility and increased fragility. A portion of this is due to changes to the seminal plasma, including inflammatory mediators, reactive oxygen species, and immune cell infiltrate. However, a large portion of these pathologic changes are intrinsic to the sperm cells themselves before they ever leave the testes, indicating pathologic changes in the testes. (60) This topic has not been well explored.

1.m Study Justification and Licofelone

Taking note of the fact that SCI causes a breakdown in the BSCB other than that of the injury site, increased permeability of the BBB, and a disruption of tight junctions in epithelial cell layers throughout multiple organ systems, our lab decided to evaluate the blood testes barrier after SCI. It was found that the blood testes barrier was disrupted as early as 72 hours and continued to be more permeable up to 10 months. Furthermore, there was evidence of elevation in phospholipase A2 and glutathione peroxidase,
enzymes that are important in inflammatory and oxidative pathologies. (61)

Phospholipase A2 cleaves phospholipids to release arachidonic acid, which can be further metabolized by lipoxygenases (LOX) or cyclooxygenases (COX) into prostaglandins and leukotrienes. (62) Blocking COX alone can result in increased amounts of LOX metabolites, which can further increase oxidative stress. (63) Licofelone is a dual COX/LOX inhibitor that has completed phase III clinical trials for the treatment of osteoarthritis. Licofelone shows much better gastric tolerability than that of selective COX inhibitors, which is important in a patient population that has gut motility issues like SCI. (64) It is also an analgesic, modulating mechanical hypersensitivity in the hindlimbs of rats with chronic SCI. (22) For these reasons, we believe that licofelone could have therapeutic benefits to the pathologic changes in the testes following spinal cord injury.

Although licofelone is continually described as a dual COX/LOX inhibitor, its mechanism of action is more nuanced. First, licofelone competitively inhibits 5-LOX, which is the main enzyme responsible for inflammatory leukotrienes. However, this inhibition is weak unless in the presence of 5-LO-activating protein (FLAP), which is normally present in physiologic conditions. (65) Second, licofelone is a strong and direct inhibitor of COX-1, but is a weak inhibitor of COX-2. However, licofelone strongly inhibits mPGES-1, which is the final enzyme of PGE-2 synthesis. PGE-2 is the main inflammatory effector metabolite of COX-2, making its suppression give the appearance of a strong COX-2 inhibition. Inhibiting the synthesis of PGE-2 directly, instead of all of the metabolites COX-2 produces, has been proposed to be more effective in treating pathologic inflammatory conditions. (66) Licofelone is absorbed in the intestines,
reaching peak concentrations after 2-4 hours and halving after 11 hours. (67, 68)
Licofelone’s halflife has not been fully evaluated due to multiple active metabolites, but has remained effective in other models up to 96 hours after dosing. (69) Licofelone’s metabolism has not been fully evaluated, but there is evidence of metabolism by hepatocytes, a large amount of fecal excretion, and a small amount excreted by the kidneys. (67, 68)
CHAPTER 2: MATERIALS AND METHODS

Eighty-eight male Sprague Dawley rats (200–250 g) were used for metabolomic and 69 were used for gene expression microarray studies for the data in chapter 3. The difference between these studies is that eight naïve rats were used for the acute time-points in the metabolomics experiments and 16 in the messenger RNA (mRNA) experiments. Sixty-six more of these rats were used for the data in chapter 4. They were divided into groups as indicated in Table 2 and Table 3. Naïve cohorts from the 24 h and 72 h groups were determined to be age-equivalent and were used as one group in the metabolomic studies.

Immune cell infiltrate analysis

An additional 30 animals (n = 6/group) were used for immune cell identification and quantification via flow cytometry in three groups at 72 h (naïve, SCI, and sham) and in two groups at 1.5 years (naïve, SCI) after SCI at Baylor College of Medicine.

Spinal cord injury

All animals were handled in accordance with our Institutional Animal Care and Use Committee approved protocol. SCI rats received a spinal cord contusion injury at thoracic level 10 (T10) using the Infinite Horizons Spinal Impactor Device (Precision Systems Instrumentation) with 150 kdynes of force delivered over a 1-sec dwell time period. Sham-injured (sham) subjects were anesthetized and received a spinal laminectomy at T10, but did not receive a spinal cord contusion injury. All SCI and sham subjects received the following treatment during the post-operative period: 1) antibiotic (2.5 mg/kg Baytril) for a period of 10 days; 2) the opiate buprenorphine (0.025 mg/kg,
twice daily for a period of 5 days, then as needed); and 3) 1.5 cc of 0.9% saline, twice daily, for a period of 3 days to ensure hydration. SCI animals received twice-daily, manual bladder evacuations using the method of Crede as modified for rats for an anticipated period of 10–14 days, or until neurogenic bladder evacuation was established. Beginning on Day 1 post-surgery, SCI, and sham subjects were examined using the non-invasive, Basso, Beattie, Bresnahan open field locomotor test as described below. (70) Subjects that scored more than 2 on either Day 1 or 2 of the study were excluded as such a score indicates an insufficient level of spinal damage. All animals were fed ad libitum. Naïve animals received no other manipulations.

**Basso, Beattie, and Bresnahan scores (BBB scores)**

Animals were placed in a flat plastic field approximately 4 feet in diameter. Animals were carefully watched for 2 minutes as they tried to ambulate. Animals were given a score based upon the degree of movement of the hind limbs, including ankle, knee, and thigh movement, as well as more complex movements such as sweeping, stepping, and gait. Animals scoring more than a 2 on the first 2 days post injury were excluded from the experiment. A score of 0 represents no limb movement, 1 represents slight movement of one or two joints, and a 2 represents extensive movement of one joint or extensive movement of one joint and slight movement of another. (71) Sham animals were tested to ensure no deficits were present.

**Licofelone Dosing**

In addition to what was described above, all animals from chapter 4 were dosed via oral gavage with either licofelone suspended in Carboxymethylcellulose (CMC) or CMC
alone 3 hours after injury. Animals were then dosed once daily. Animals sacrificed on
day 90 received a total of 10 doses, while animals sacrificed at 72 hours received a total
of 4 doses, with the final dose given 1 hour before sacrifice. Vehicle treated animals
received CMC alone, while the remaining animals had licofelone suspended in the CMC
based on the animals weight. Animals were either given 10, 50, or 100 mg of licofelone
per kg of body weight.

End-stage tissue collection

At each of the time-points described above, animals were euthanized with 75 mg/kg of
Beuthanasia (390 mg/mL pentobarbital, 50 mg/mL phenytoin). Subjects were quickly
decapitated and the testes were collected, snap frozen in liquid nitrogen, and stored at
−80°C. The epididymides were not collected. A different set of SCI animals were injured
and euthanized using the same methods but were transcardially perfused with ice-cold
saline prior to perfusion; blood was collected transcardially into BD Microtainer tubes,
allowed to coagulate for 30 min, spun at 14,000 rpm for 10 min to separate out the
serum supernatant. Collected serum was used to determine serum levels of bile acids.

Flow cytometry

A single-cell suspension was prepared from rat testes using a 70-μm cell strainer (BD)
as described. Isolated cells were washed with ice-cold flow cytometry wash solution
(phosphate-buffered saline [PBS] + 2% goat serum +2% bovine serum albumin),
stained with fluorophore-conjugated antibodies (Table 1) or with ShK-F6CA, a
fluorophore-conjugated peptide selective for Kv1.3, a marker of activated effector-
memory T cells.
**Table 1:** Antibodies used for flow cytometry to identify immune cell populations.

<table>
<thead>
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<td>APC</td>
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<td>557030</td>
</tr>
<tr>
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<td>562108</td>
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<td>CD103</td>
<td>Alexa Fluor 647</td>
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<tr>
<td>Kv1.3</td>
<td></td>
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</table>

**Selection of antibodies used for flow experiments**

CD3 is part of the T cell receptor complex, expressed at the surface of only T lymphocytes. B220 is a 220 kDa isoform of CD45 expressed mainly at the surface of B cells in mouse and rat. (73) CD11b is a surface marker for monocytes, macrophages and granulocytes. (74) CD103 is expressed on the surface of most dendritic...
Ly6G is a surface protein predominantly expressed on neutrophils. CD161a (NK1.1) is a surface marker for natural killer lymphocytes. CD4 and CD8 are surface markers of helper T cells and cytotoxic T cells, respectively. Kv1.3 is a voltage-gated potassium channel whose expression on the cell surface is upregulated in C-C chemokine receptor type 7 effector memory T cells upon activation. CD62L (L-selectin) is expressed at the surface of naïve but not memory T cells. CD4, CD8, Kv1.3, and CD62L also are expressed by other cells; thus, the use of double staining with CD3 to ensure their expression was only studied on T cells.

Cells were washed and fixed in cold PBS + 1% paraformaldehyde. FACSCanto II or LSRFortessa flow cytometers (Becton Dickinson) with the FACSDiva software Data were used to acquire sample data within the Cytometry and Cell Sorting facility at Baylor College of Medicine, and analyzed using FlowJo software (Treestar). For each sample, doublet discrimination was performed on 30,000 acquired events.

**Metabolomic analysis**

After collection at each of the time-points listed above, the right testes were homogenized under liquid nitrogen. Samples from 88 animals were processed and metabolomics profiling was performed by Metabolon Inc. (Durham, NC). Briefly, an unbiased metabolomic profile of testis from SCI, sham, and age-matched control rats was performed using gas chromatography coupled to mass spectrometry (gas chromatography [GC/mass spectrometry [MS, Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization] and ultra–high performance liquid chromatography coupled to mass spectroscopy (ultra-
performance liquid chromatography [UPLC/MS, Waters ACQUITY ultra-performance liquid chromatography and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer]. At the time of this experiment, Metabolon’s metabolomics platform was capable of detecting more than 3,500 substances. Compounds found in the sample were cross-referenced to their library of compounds for identification. Analysis of variance (ANOVA) contrasts were used to identify biochemicals that differed significantly between experimental groups following log transformation and imputation of missing values, if any, with the minimum observed value for each compound. Metabolite outliers of more than 2 standard deviations away from the group mean were similarly excluded from analysis. Metabolites were filtered out of the dataset if they were not detectable in >60% of animals in the majority of groups or where the entire difference between sham and SCI group means was due to changes in 25% of animals or less, and subsequently excluded from analysis. Values were converted to a ratio to the mean of the sham group for graphing purposes.

**Serum bile acid**

Serum bile acids were measured using a method adapted from Woolbright and colleagues. (81) Rat serum samples were prepared using a methanol extraction procedure to facilitate the removal of serum proteins by centrifugation. This was done by mixing 30 μL of serum with 80 μL of methanol spiked with internal standards and briefly vortexing, then centrifuging at 15 000 × g for 20 min. The supernatant extracts were injected (5 μL) into a HPLC system (Agilent Technologies, Santa Clara, CA). Bile acid separation was achieved using a 1260 Infinity Binary LC System equipped with a
100 mm × 2.1 mm (C-18 BEH, Waters) column. The column temperature was maintained at 45°C and the flow rate was 0.3 mL/min with a gradient in a 25-min run. Gradients were run starting from 95% buffer A containing ammonia acetate (water/methanol, 80:20 v/v, pH = 8.4) and 5% buffer B containing ammonia acetate (acetonitrile/water 90:10 v/v, pH = 8.4) and to 75% A from 0–5.0 min; 75% A to 60% A from 5.0–10.5 min; 65% A to 5% A from 10.5 to 18 min; 5% A to 0% A from 18–22 min; 0% A was held for 1.0 min; 0% A to 95% A from 23–23.5 min; 95% A was held from 23.5–25 min to re-equilibrate the column. The HPLC eluate was analyzed in negative mode with electrospray ionization on a 6490 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) using the multiple reaction monitoring method. The drying gas temperature was set up at 250°C for positive mode and 290°C for negative mode, correspondingly. Drying and sheath gas flow maintained at 14 l/min and 12 l/min for both modes, respectively. Capillary voltage was set at 3500 V for positive mode and 3200 V for negative mode. The relative abundance of bile acids was calculated based on the peak area.

**Microarray analysis**

Microarray studies were performed in the UTHealth Microarray Core Facility under the direction of Dr. David Loose. The Microarray Core Facility provided experimental handling and support for all aspects of the Agilent microarray platform used in this study. The specific arrays used were the Agilent SurePrint G3 Rat GE 8x60K Microarrays. These arrays have 60,000 probes and a number of control probes for quality control assessment. We performed a total of 96 arrays on 12 groups as indicated in Table 1 with n = 8 for all groups. Following treatment, total RNA was extracted from
the testis (Qiagen, Hilden, Germany) and gene expression profiles examined by array. The arrays were pre-processed and captured image data for microarray features was accomplished with Agilent's SureScan and Feature Extraction software. Data were background corrected with the local background algorithms in the Feature Extractor software. The main software used for subsequent analysis was BRB Array tools (v 4.4). Data were normalized using quantile normalization, and transcripts that were differentially expressed identified by univariate analysis at $p < 0.01$ corrected for multiple testing (Bonferroni).

**Caspase-3 assay**

Caspase activity was performed on testis protein extract using the EnzChek caspase-3 assay kit #1, following the manufacturers protocol (Molecular Probes). This assay measures the increase in fluorescence generated by the cleavage of the aminomethylcoumarin (AMC)-labelled caspase-3 substrate Z-DEVD-AMC. The production of fluorescent substrate was monitored continuously every 5 min for approximately 2 h in a 96-well dish using an automated fluorescent plate reader with data acquisition software (LS55 luminescence spectrometer, PerkinElmer Instruments, Shelton, CT). The slope of the linear regression drawn through each time-point was used to determine change in fluorescence over time for each sample. A standard curve using known amounts of AMC was used to convert fluorescent values to specific catalytic activity. (82)

All assays were run under blinded conditions and all samples were randomized prior to analysis.
CHAPTER 3: CHANGES IN GENE EXPRESSION AND METABOLISM IN THE TESTES OF THE RAT FOLLOWING SPINAL CORD INJURY


3.a Introduction

Spinal cord injury (SCI) affects 12,000 new patients in the United States every year. (17, 53) Although the sensory and motor loss are the signature symptom, these represent only a fraction of the negative consequences of SCI. SCI elicits pathological changes to the gastrointestinal and urinary tracts, loss of bone density, muscle atrophy, neuropathic pain, autonomic dysfunction, sleep disturbances, and increased pulmonary complications. In addition to the physical ailments, patients with SCI commonly experience marital stress, job loss, depression, and other psychological and social issues semi-unique to each patient. One physical symptom that causes male patients much psychological stress, yet remains largely understudied, is the development of infertility.

More than 80% of patients with SCI are male, and the majority of those are young men of reproductive age who may not have had the opportunity to have a family yet, causing even more psychological stress. (17, 51) Reproductive assistance techniques have greatly improved in recent history: less than 1% of men with SCI in 1960 became
fathers via intercourse. This has improved in 2003 to an approximately 51% pregnancy rate in those who seek to reproduce including extensive medical assistance. However, such assistance is prohibitively expensive and unsuccessful in half of patients. (51, 52) Contrary to popular belief, sensory loss does not eliminate sexual capacity. Motor function loss can be circumvented and erectile dysfunction from neurological damage can be treatable via oral medication or mechanically in the majority of patients. (17, 53-57) Depending on the level of the lesion, even anejaculation can be treated with mechanical devices in the majority of patients. (56, 58, 59) However, infertility due to unfavorable sperm and ejaculate parameters are not so easily treated. Although a portion of the sperm's loss of motility after SCI appears to be from seminal plasma influence, a portion is intrinsic to the sperm itself. (60) This problem has remained largely unexplored.

Human and animal data show that SCI elicits whole–body, systemic changes that may contribute to the disturbances described above. (61, 84-86) These changes consist of both local and systemic inflammatory events, as well as altered immune function. (85, 86) In addition, it has long been established that SCI causes a breakdown of the blood–spinal cord barrier, which results in the influx of foreign substances and activated immune cells into traumatized spinal tissues. (87) SCI also has been shown to cause inflammation, loss of structural integrity, and immune cell activation/infiltration in tissues such as the uroepithelium of the bladder and the tissues of the lung. (86, 88, 89) In an earlier study, we showed that a clinically-relevant spinal contusion injury produced an early but sustained long-term disruption of the blood–testes barrier (BTB) in the testes of adult male Sprague-Dawley rats. (61) SCI-induced early/chronic BTB failure was
shown by dynamic contrast enhanced MRI, with subsequent tissue analysis demonstrating a loss of tight junction protein expression, immune cell infiltration, and the presence of normally-excluded serum proteins. (61) BTB leakage was sustained up to 10 months post-SCI, suggesting a long-term, potentially permanent functional deficit in testicular function following SCI. (61) The mechanism(s) underlying this sustained BTB pathology are unknown and are the focus of this present study. We performed a temporal metabolomic and microarray analysis of SCI-induced changes within the testis of male rats following spinal contusion injury from early acute (24 h) to chronic (3 months) post-SCI. We demonstrate that spinal contusion injury, the most common form of SCI, results in early but sustained biochemical, molecular, and cellular events as detected by metabolomics and gene expression analysis that may contribute to the condition of SCI-induced infertility in males. (70, 90)

Methods:

Eighty-eight male Sprague Dawley rats (200–250 g) were used for metabolomic and 69 were used for gene expression microarray studies. The difference between these studies is that eight naïve rats were used for the acute time-points in the metabolomics experiments and 16 in the messenger RNA (mRNA) experiments. They were divided into groups as indicated in Table 1. Naïve cohorts from the 24 h and 72 h groups were determined to be age-equivalent and were used as one group in the metabolomic studies.

For more complete methods, please see the methods chapter.
<table>
<thead>
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<th></th>
<th>24 Hours</th>
<th>72 Hours</th>
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<th>90 Days</th>
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<td>(8)</td>
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<tr>
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<tr>
<td>Naïve (N)</td>
<td>(4)</td>
<td>(4)</td>
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</table>

**Table 2:** Animal groups for metabolomics and array experiments in chapter 3.
3. Results:

**SCI-dependent cellular immune response: FACS:**

FACS of testicular tissue (Figure 1) showed that neutrophil counts were significantly increased at 72 hours in the acute SCI animals verses both sham and naïve animals (26.4% and 30.8% increase respectively, p<0.05), but are not elevated in the chronic 1.5 year post-surgery SCI animals. In the 1.5 year chronic SCI animal there is an elevation in T cells in the testes (41.6% increase, p<0.001) that was not seen at 72 hours. There is no significant change in T-cell phenotypes at either time point.

**Metabolomics:**

In the testes, 369 metabolites were detected by UPLC/MS and GC/MS. These metabolites were clustered by biochemical function into amino acid, peptide, carbohydrate, energy, lipid, nucleotide, cofactors and vitamins, and xenobiotic “super pathways” and clustered again into smaller “sub pathways” such as eicosanoids, lysolipids, glycolysis, etc. We took Metabolon’s initial ANOVA analysis as a screening tool to find sub pathways where over 30% of metabolites were significantly changed and subjected them to further analysis as described in the methods.

One of the pathways that was significantly differential between groups was eicosanoid metabolites (Figure 2). Of the 5 eicosanoids that were found in the testes in the metabolomic profiling, all 5 were significantly different at 24 hours after SCI compared to sham by ANOVA with Bonferroni correction for multiple comparisons in the testes. Three of the five metabolites were significantly different when comparing SCI to naïve. The SCI mean fold change compared to sham ranged from 1.6 to 1.828. PGD2
remained significantly elevated at 72 hours; at no other time points were there significant differences in eicosanoid metabolites.

**Testicular lysolipids, phospholipids that by metabolism have lost one acyl chain, were also significantly different after SCI.** After filtering as described in the methods, thirty-eight lysolipids were detected in the testes: 11 of PC origin, 14 of PE origin, 6 of PI origin, 4 of PS origin, and 3 of PG origin. Nine of 11 lysolipids (Figure 3) of PC origin were significant by ANOVA with Bonferroni correction for multiple comparisons in SCI rats compared to sham at 72 hours post-surgery (the remaining 2 were significant by student’s t-test). The increase in lysolipid levels at 72 hours post-SCI ranged from 3.587 to 10.04. None of the lysolipids of other classes were significantly changed. Lysolipid changes at other time points were negligible.

**Five selected metabolites that mark oxidative stress were also measured.** (Figure 4) Oxidized glutathione is unchanged at all time points, but the protective reduced glutathione is decreased at 24 hours compared to sham animals. Carnosine and Anserine are unchanged by ANOVA but are significantly different between SCI and sham by students t-test at 24 hours (p=0.033729 and 0.026128), 72 hours (p=0.024144 and 0.047352), and 28 days (p=0.006919 and 0.026611) post-surgery. 13-HODE + 9-HODE is elevated compared to sham at 24 hours.

**Four unconjugated bile acids were detected in the metabolomics profiling (Figure 5) and all 4 were elevated compared to sham (3/4 compared to naïve) at 28 and 90 days post injury.** The SCI mean fold change from sham mean ranged from 2.238 to
4.352. Bile acid changes at earlier time points were negligible. In addition, 21 bile acids were measured in the serum of 90 day post-surgery rats by a separate mass spectrometric analysis, but only 1 (GLCA) was found to be statistically different from sham by ANOVA (data not shown). Serum levels of bile acids showed a significant correlation (Figure 6) to testis levels of bile acids ($R^2 = 0.7975$), which is even more pronounced when separated into SCI and sham groups ($R^2 = 0.9506$ and 0.9567 respectively). Interestingly, the difference between sham and SCI bile acid concentrations in the serum versus the difference between sham and SCI bile acid concentrations in the teste had an even higher correlation ($R^2 = 0.9845$). There was a correlation ($R^2$) value of 0.7975 between plasma levels and testes levels, but no statistical differences in unconjugated bile acids in the serum.

**mRNA**

We were interested in determining what changes occurred within the testis at the RNA level after SCI and therefore performed microarray experiments at the same time-points that were done in the metabolomic experiments. After analyzing these data for significantly changed transcripts in BRB Array tools, Ingenuity IPA software was used to cluster mRNA into pathways. Some pathways were changed in a rather incoherent pattern with conflicting activation and suppression of several transcripts, such as immune function genes at all time-points, while others were single gene changes whose significance was suspect. Overall, there were 267 transcripts significantly changed at 24 h after SCI, compared with sham, 252 transcripts at 72 h after SCI, 507 transcripts 28 days after SCI, and 392 transcripts 90 days after SCI. Here we present the major pathways affected by SCI.
The 24 h post-SCI animals had decreased expression of eight transcripts directly involved in the testosterone production pathway (Figure 7), including the rate limiting step of cholesterol transport into mitochondria (STAR) and the rate limiting enzyme (CYP11A1). (91-93) The SCI mean fold change from sham mean ranged from 0.2882 to 0.5190.

The mRNA array at 72 hours shows that 8 different transcripts involved in DNA Function and cell cycle were decreased post SCI (Figure 8). The SCI mean fold change from sham mean ranged from 0.5214 to 0.8242.

In addition to the mixed suppression and induction of immune function genes we see at all time points, the mRNA array data from 28 days and 90 days show a chaotic mix of genes involved in chemotaxis.
**Figure 1:** Flow assisted cell sorting (FACS) of testes tissue for immune cell phenotypes:

*: \( p < 0.05 \), **: \( p < 0.001 \)

**Aa:** Neutrophils were elevated 72 hours post SCI compared to sham and naïve (26.4% and 30.8% respectively, \( p < 0.05 \)).

**Ab:** T-cell phenotypes were unchanged after injury.

**Ba:** T-cells were elevated 1.5 years post SCI (41.6%, \( p < 0.001 \)).

**Bb:** T-cell phenotypes were unchanged after injury.
Figure 2: Metabolomic assessment of eicosanoids in the testes at 24 hours post-spinal cord injury
**Figure 2:** Metabolomic assessment of eicosanoids in the testis at 24 hours post-surgery:

Whisker plots illustrate the median (horizontal bar), the 25th to 75th percentile (box) and the minimum to maximum value (whiskers). All values were scaled to set the sham mean equal to 1 (horizontal dotted line). *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001.

12-HETE in SCI animals is elevated (ANOVA: p=0.0044) compared to both sham (p≤0.01) and naïve (p≤0.05) with an SCI mean fold change from sham mean of 1.6.

15-HETE in SCI rats is elevated (ANOVA: p=0.0050) compared to sham (p≤0.01) with an SCI mean fold change from sham mean of 1.655.

5-HETE in SCI rats is elevated (ANOVA: p=0.0023) compared to sham (p≤0.01) with an SCI mean fold change from sham mean of 1.828.

PGE2 in SCI rats is elevated (ANOVA: p=0.0147) compared to sham (p≤0.01) and naïve (p≤0.05) with an SCI mean fold change from sham mean of 1.661.

PGD2 in SCI rats is elevated (ANOVA: p=0.0198) compared to sham and naïve (p≤0.05) with an SCI mean fold change from sham mean of 1.703.
Figure 3: Metabolomic assessment of lysolipids at 72 hours post-surgery.
Figure 3: Metabolomic assessment of lysolipids at 72 hours post-surgery:

Legend is the same as Figure 2.

Compared to sham, lysolipids that were elevated after SCI were

1: 1-palmitoylglycerophosphocholine (p≤0.01, ANOVA: p=0.0055, mean fold change: 8.118),
2: 2-palmitoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0113, mean fold change: 5.021),
3: 1-stearoylglycerophosphocholine (p≤0.01, ANOVA p=0.0067, mean fold change: 7.889),
4: 1-oleoylglycerophosphocholine (p≤0.01, ANOVA: p=0.0043, mean fold change: 5.929),
5: 2-oleoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0092, mean fold change: 7.841),
6: 1-linoleoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0316, mean fold change: 3.587),
7: 2-linoleoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0362, mean fold change: 6.869),
8: 1-arachidonoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0388, mean fold change: 4.694),
9: 2-arachidonoylglycerophosphocholine (p≤0.05, ANOVA: p=0.0207, mean fold change: 10.04).

Compared to naïve, lysolipids elevated after SCI were

3: 1-stearoylglycerophosphocholine (p≤0.05),
4: 1-oleoylglycerophosphocholine (p≤0.05), and
5: 2-oleoylglycerophosphocholine (p≤0.05).
Figure 4: Metabolomic assessment of oxidative stress markers at 24 hours post-surgery:

Legend is the same as Figure 2.

**Oxidized glutathione** was not changed at 24 hours.

**Reduced glutathione** was suppressed after SCI compared to sham \((p \leq 0.01\), ANOVA: \(p=0.0064\), mean fold change: 0.3376).

Carnosine and anserine were unchanged at 24 hours \((\text{ANOVA: } p=0.0966\) and \(p=0.0786\), respectively).

**13-HODE + 9-HODE** was elevated after SCI compared to sham \((p \leq 0.05\), ANOVA \(p=0.0337\), mean fold change: 1.550).
**Figure 5:** Metabolomic assessment of unconjugated bile acids in chronic SCI.
**Figure 5:** Metabolomic assessment of unconjugated bile acids in the chronic stages of injury.

Legend is the same as Figure 2.

**Bile Acids 28 days after injury:**

**Cholate** in SCI rats was elevated (ANOVA: $p=0.0003$) compared to sham ($p \leq 0.01$, mean fold change: 2.238) and naïve ($p \leq 0.001$).

**Chenodeoxycholate** in SCI rats was elevated (ANOVA: $p=0.0016$) compared to sham ($p \leq 0.05$, mean fold change: 2.844) and naïve ($p \leq 0.01$).

**B-Muricholate** in SCI rats was elevated (ANOVA: $p=0.0096$) compared to sham ($p \leq 0.05$, mean fold change: 2.534) and naïve ($p \leq 0.05$).

**a-Muricholate** in SCI rats was elevated (ANOVA: $p=0.0001$) compared to sham ($p \leq 0.01$, mean fold change: 2.815) and naïve ($p \leq 0.001$)

**Bile acids 90 days after injury:**

**Cholate** in SCI rats was elevated (ANOVA: $p=0.0001$) compared to sham ($p \leq 0.001$, mean fold change: 3.760) and naïve ($p \leq 0.01$).

**Chenodeoxycholate** in SCI rats was elevated (ANOVA: $p=0.0002$) compared to sham ($p \leq 0.001$, mean fold change: 3.836) and naïve ($p \leq 0.001$).

**B-Muricholate** in SCI rats was elevated (ANOVA: $p=0.0303$) compared to sham ($p \leq 0.05$, mean fold change: 2.420).

**a-Muricholate** in SCI rats was elevated (ANOVA: $p=0.0010$) compared to sham ($p \leq 0.01$, mean fold change: 4.352) and naïve ($p \leq 0.01$).
**Figure 6:** Correlation between levels of bile acids in the testes and levels of bile acids in the serum at 90 days post-surgery for both SCI and sham animals combined ($R^2 = 0.7975$). Separating the measurements into SCI and sham groups increased the correlation ($R^2 = 0.9506$ and 0.9567 respectively). Taking the difference between sham and SCI bile acid concentrations in the serum versus the difference between sham and SCI bile acid concentrations in the testes had an even higher correlation ($R^2 = 0.9845$).
Figure 7: Array assessment of the expression of steroidogenesis genes' mRNA transcripts at 24 hours post-surgery.
Figure 7: Array assessment of the expression of steroidogenesis genes’ mRNA transcripts at 24 hours post-surgery:

Legend is the same as Figure 2.

**CYP11a1** mRNA expression is suppressed in sham animals (sham vs naïve: p≤0.0001), and even more suppressed in SCI animals (SCI vs sham: p≤0.01, SCI vs naïve: p≤0.0001) with a mean fold change vs sham of .4486 (ANOVA: p<0.0001).

**CYP17a1** mRNA expression is suppressed in sham animals (sham vs naïve: p≤0.01), and even more suppressed in SCI animals (SCI vs sham: p≤0.01, SCI vs naïve: p≤0.0001) with a mean fold change vs sham of 0.3878 (ANOVA: p<0.0001).

**HSD3B2** mRNA expression is not suppressed in sham animals but is suppressed in SCI animals (SCI vs sham: p≤0.001, SCI vs naïve: p≤0.0001) with a mean fold change vs sham of 0.3214 (ANOVA: p<0.0001).

**HSD3B4** mRNA expression is suppressed in both sham animals (sham vs naïve: p≤0.01) and SCI animals (SCI vs naïve: p≤0.0001) with a mean fold change vs naive of .2882 (ANOVA: p<0.0001).

**SCARB1** mRNA expression is not suppressed in sham animals but is suppressed in SCI animals (SCI vs naïve: p≤0.001) with a mean fold change vs naive of 0.3653 (ANOVA: p=0.0012).

**SCARB1 alt** (alternative transcript) mRNA expression is suppressed in sham animals (sham vs naïve: p≤0.05), and even more suppressed in SCI animals (SCI vs sham: p≤0.01, SCI vs naïve: p≤0.0001) with a mean fold change vs sham of 0.5190 (ANOVA: p<0.0001).
\textbf{STAR alt} mRNA expression is suppressed in sham animals (sham vs naïve: $p \leq 0.0001$), and even more suppressed in SCI animals (SCI vs sham: $p \leq 0.0001$, SCI vs naïve: $p \leq 0.0001$) with a mean fold change vs sham of 0.4382 (ANOVA: $p < 0.0001$).

\textbf{STAR} mRNA expression is suppressed in both sham animals (sham vs naïve: $p \leq 0.0001$) and SCI animals (SCI vs naïve: $p \leq 0.0001$) with a mean fold change vs naïve of 0.3082 (ANOVA: $p < 0.0001$).
**Figure 8:** Array assessment of the expression of cell cycle and DNA function genes' mRNA transcripts at 72 hours post-surgery.
**Figure 8:** Array assessment of the expression of cell cycle and DNA function genes’ mRNA transcripts at 72 hours post-surgery:

Legend is the same as Figure 2.

**CEP290** mRNA expression is altered between groups (ANOVA: p=0.0341) but the multiple comparison test failed to differentiate between groups.

**TAF1D** mRNA expression is elevated in sham animals (sham vs naïve: p≤0.0001), and suppressed in SCI animals (SCI vs sham: p≤0.0001, SCI vs naïve: p≤0.001) with a mean fold change vs sham of 0.5214 (ANOVA: p<0.0001).

**IWS1** mRNA expression is elevated in sham animals (sham vs naïve: p≤0.0001), and less elevated in SCI animals (SCI vs sham: p≤0.01, SCI vs naïve: p≤0.0001) with a mean fold change vs sham of 0.6554 (ANOVA: p<0.0001).

**CCNC** mRNA expression is suppressed in SCI animals compared to sham (sham vs naïve: p≤0.001) with a mean fold change vs sham of 0.8242 (ANOVA: p=0.0014).

**MTRR** mRNA expression is elevated in sham animals (sham vs naïve: p≤0.01), but is not in SCI animals (SCI vs sham: p≤0.001) with a mean fold change vs sham of 0.5730 (ANOVA: p=0.0003).

**MTRR alt** mRNA expression is elevated in sham animals (sham vs naïve: p≤0.01), but is not in SCI animals (SCI vs sham: p≤0.01) with a mean fold change vs sham of 0.6093 (ANOVA: p=0.0022).

**ALKBH8** mRNA expression is elevated in sham animals (sham vs naïve: p≤0.01), but is not in SCI animals (SCI vs sham: p≤0.001) with a mean fold change vs sham of 0.5452 (ANOVA: p=0.0002).
**IFT74** mRNA expression is suppressed in SCI animals (SCI vs sham: p≤0.05, SCI vs naïve: p≤0.01) with a mean fold change vs sham of 0.6086 (ANOVA: p=0.0009).

**ZNF519** mRNA expression is suppressed in SCI animals (SCI vs sham: p≤0.01, SCI vs naïve: p≤0.01) with a mean fold change vs sham of 0.5451 (ANOVA: p=0.0014).

**HELQ** mRNA expression is elevated in SCI animals (SCI vs naïve: p≤0.05) with a mean fold change vs naive of 2.169 (ANOVA: p=0.0182).
3.c Discussion:

Little is known regarding the cascade of pathological events initiated in the testes following SCI. In order to begin deriving a greater understanding of these acute and long-term pathological changes that negatively impact fertility, we elected to take a broad, multifaceted approach examining immunological, metabolomics, and mRNA expression studies. These studies were driven by prior studies that demonstrated that SCI caused significant activation of the immune system,(85) changes in hepatic function(84) and globally altered gene expression.(94-96) The amount of data generated via these methods is immense, encompassing multiple pathways and systems at multiple time-points.

Metabolomics is a recent tool that permits the quantitative assessment of large numbers of biochemicals or metabolites from many different pathways in order to get an unbiased snapshot of a tissue's metabolic profile. We analyzed our results focusing on multi-metabolite networks that could identify broad shifts in the metabolic state in the testis after SCI.

The greatest changes in the testicular metabolome observed at 24 h post-SCI were: 1) the establishment of an environment favoring both inflammatory and pro-oxidative conditions, and 2) a significant reduction in testosterone production. Inflammatory conditions appear to be favored via activation of two canonical pathways for arachidonic acid metabolism (cyclooxygenase [COX and lipoxygenase), which produce prostaglandins (PGE2/D2) and leukotriene precursors (some HETEs).(97) Oxidative stress is evident by a decrease in reduced glutathione, which is protective. An increase in the ratio of reduced glutathione to oxidized glutathione is recognized as a well-
established marker of oxidative stress. (98) 13-HODE +9-HODE, which together are markers for oxidative stress of free radical-mediated oxidation, is also increased at 24 h, although these can also be made by COX. (99) These metabolites, and the consistent decrease in antioxidants anserine and carnosine, point toward at least a mild oxidative insult in the testes at 24 h. Both oxidative stress and inflammation have been shown to be associated with SCI-dependent male infertility. (100)

The decrease in the mRNA of the enzymes responsible for testosterone production shows that low testosterone in acute SCI patients is at least partly due to changes at the transcriptional level. This could indicate that Leydig cells are under stress and are unresponsive, but we think it is equally likely that SCI causes a shock to the entire central nervous system (CNS), reducing the amount of luteinizing hormone or gonadotropin-releasing hormone released into the system at the level of the CNS, which would lead to less stimulation of the testosterone production pathway. Because previous studies have been contradictory, endocrine profiles after SCI are poorly understood; however, any disrupted testosterone production would reduce fertility. (53, 97, 101) These acute changes likely further develop or lay the groundwork for the alterations that follow.

At 72 h post-SCI, our data indicate increased cell death, debris, and catabolism within the testes. In the healthy testis, apoptosis occurs continuously at higher rates than most of the body as part of normal spermatogenesis. (102) These apoptotic sperm cells are quickly absorbed by the supportive sustentacular Sertoli cells in a non-pathologic manner. Our lab has previously shown extensive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and disorganization in segments of the
seminiferous tubules 72 h after SCI.(61) In such an environment, sperm production is not expected to continue at normal rates, if at all. Increased cell death within the seminiferous tubules, which would coincide with a cessation or slowing of spermatogenesis, would account for the coherent but small in magnitude decrease of so many mRNAs related to cell cycle and DNA function as seen in Figure 8. In support of this, at 72 h we also see an increase in nine lysophosphatidylcholines (LPCs). In apoptotic cells, caspase-3 activates calcium independent phospholipase A2, which catalyzes PCs into LPCs.(103-105) LPC has been shown to be released by apoptotic cells to attract phagocytic cells and to induce pro-apoptotic pathways.(106, 107) This leads us to speculate that the increase in neutrophils at this same time-point may be caused by this increase in LPC acting as a chemo attractant.

All these data point to an SCI-induced enhanced apoptotic environment within the testes. However, direct caspase activity assays (data not shown) show no change in caspase 3 activity in the testes 24 or 72 h after injury, indicating that overall apoptosis rates in the whole testis is not significantly elevated. However, previous data shows significant TUNEL-positive staining in a portion of the seminiferous tubules in a cross-section of the testes at 72 h after SCI.(61) This leads us to believe that a global insult to the testes occurs after SCI, but that only the seminiferous tubules in the developmental stages most sensitive to damage are affected enough to induce apoptosis, similar to what has been described in testis heat stress injury.(43, 106, 108) Heat injury induces apoptosis in seminiferous tubules at early (I–IV) and late (XII–XIV) developmental stages of spermatogenesis, while segments of seminiferous tubule at other developmental stages showed little damage.(43) Further study into which seminiferous
tubule and spermatogenesis developmental stages are most affected is needed. These pathologic processes are further compounded by an immune response, including a neutrophilic infiltrate. Because the testis is a uniquely immune privileged environment, many cell types are attenuated or suppressed when they would be highly activated and increased in other tissue. Any change in immune cells in the testis first must overcome the suppressive effect of the Sertoli cells, which modulate immune function in order to prevent autoimmunity against the antigens in sperm cells that appear after the process of tolerance. Whether these neutrophils were present earlier and contributed to the stronger inflammatory and oxidative environment seen at 24 h or whether they were recruited via chemotaxis to the testes by the increase in lysolipids seen at 72 h is a question that requires further study. In summary, we postulate that SCI causes cell death in localized segments of the seminiferous tubules and a decrease in the overall rate of spermatogenesis throughout the testis.

There is strong evidence of bile acid metabolism changes in SCI. Regardless of SCI lesion level, patients with SCI have a greatly increased risk of developing biliary sludge as early as 3 months and gallstones as a late secondary complication, despite some studies showing voiding time and contractility in the gall bladder being within normal levels in late injury. This, together with our data, suggests a more fundamental change in the bile acid metabolism than just decreased mechanical function. We found that all four unconjugated bile acids detected above background in the testes are elevated at 28 and 90 days after injury. Because the enzymes needed to create bile acids are not present in the testis, this suggests a hepatic change in bile acid metabolism and provides a strong case to further investigate into bile acid metabolism changes after
SCI. Indeed, bile acid levels in the serum correlated (Figure 6) with bile acid levels in the testis. Serum bile acids were increased in the serum of SCI rats but these changes did not reach statistical significance as occurred in the testis. This suggests the sequestration and trapping of bile acids in the damaged testis after SCI. Bile acids have direct cytotoxic activity due to detergent action and elevated bile acids have been shown to reduce male fertility via farnesoid X receptor alpha (FXR-α) and TGR5 (G-protein-coupled bile acid receptor 1; GPBAR1) receptor signaling in mice, causing sloughing of sperm cells, spermatid apoptosis, and a breakdown of the BTB, which suggests another mechanism by which the testis is unable to heal the BTB in chronic SCI.(33, 48, 109, 110) Of note, tauroursodeoxycholic acid, recently shown to be protective against apoptosis in the cord in SCI, was only detected at low levels in two of the 88 animals.(111, 112) Further study is needed to determine the complete etiology of bile acid alterations both systemically and within the testes to determine how altered bile acid metabolism shapes male fertility throughout the chronic phase of SCI.

A previous study at 72 h and 10 months post-SCI showed a permeable BTB and presence of immune cells, but overall normal histology in the testes at 10 months post-SCI.(61) These acute immune cell populations were not shown to be elevated in our data, suggesting that the histological changes were not changes in overall testes immune cell population, but rather were localized changes due to localized damage as discussed above. Either there is a similar localized change in our 1.5 year animals, or there is an evolving immune cell population that changed in the 8 months between those two time-points. This slowly and subtly changing pathology could explain the uncoordinated mRNA data we have seen in the 28 day and 90 day time-points.
Additionally, the testes, particularly the seminiferous tubules in the presence of a failed blood–testes barrier would be a target for immune activity/auto-antibody production in response to highly antigenic sperm and sperm progenitor cells. (61)

In summary, male infertility caused by SCI is a multifaceted problem. At 24 h post-SCI, we observed increased inflammation, oxidative stress, and reduced steroidogenesis, which have all been shown to cause infertility. At 72 h post-SCI, we see evidence of a pathologic apoptotic environment that affects localized segments of the seminiferous tubules, similar to that seen with increased scrotal temperature, and a neutrophilic infiltrate, which is necessary for ischemia reperfusion induced apoptosis, both of which can be expected to contribute to reduced fertility. (113) Increased levels of bile acids, such as seen at 28 and 90 days, have been shown to interfere with spermatogenesis. Finally, chronic immune responses in the testes, like the increased T-cell numbers observed at 1.5 years post-SCI, have also been shown to be detrimental to male fertility. Interestingly, an increase in T lymphocyte numbers points to a long-term activation of the adaptive immune response, suggesting the possibility of an autoimmune response to testis components secondary to SCI. All six of these processes can cause infertility. What remains to be seen are which ones, if prevented or treated, can preserve or restore male fertility.
CHAPTER 4: LICOFELONE ATTENUATES PATHOLOGIC CHANGES TO THE TESTES AFTER SPINAL CORD INJURY IN THE RAT


4.a Introduction

In the United States, Spinal cord injury (SCI) has an annual incidence of over 12,000 individuals. In addition to immediate sensory and motor loss due to central nervous system damage, patients’ experience symptoms in virtually every other biological system. One symptom that is particularly devastating to the 80% male patient population is male infertility. Although treatments for erectile dysfunction and anejaculation have been successful in many patients, infertility after spinal cord injury is primarily due to a decrease in sperm motility due to both toxic seminal plasma and intrinsic sperm fragility. In a previous study on this topic, our lab has shown drastic early increases in eicosanoids, oxidative stress, lysolipids, and other markers unfavorable for spermatogenesis in the testes using a rat model. Although this presents obvious pharmacologic targets, previous attempts to find pharmacological aids have failed despite promising preliminary data. Treatments for SCI still largely revolve around supportive care and early physical rehabilitation. Corticosteroids, prostaglandin
inhibitors, and other medications have all failed to improve patient outcomes, although some are still sporadically used in hospitals throughout the country.\textsuperscript{(114)} It is our belief that the eventual successful treatment in SCI is not going to be found narrowing in on a singular target, but by addressing multiple pathological pathways. Licofelone is a dual Cox/Lox inhibitor that targets 2 pathways of arachidonic acid metabolism that have both been implicated in pathological processes after SCI. By blocking both pathways, our data shows that early administration of this treatment can have long lasting effects.

**Methods**

Sixty-six male Sprague Dawley rats, 200-150 g, were used for metabolomic and gene expression microarray studies. They were divided into the groups as indicated in Table 3.

The metabolomics data from these animals were originally meant to be compared with the animals from Chapter 1, but the machines used were re-optimized between these studies. We set the mean value of the injured vehicle animals in this chapter to be equal to the mean value of the injured animals from chapter 1. This allows us to observe relative changes due to the effects of licofelone, but should not be taken as a robust statistical merging of the data.

For more complete methods, please see the methods chapter.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Vehicle</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
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<tr>
<td>72 Hours</td>
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<td>90 Days</td>
<td>8</td>
<td>11</td>
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**Table 3:** Animal groups for metabolomics and array experiments in chapter 4.
4.b Results

**Metabolomics:**

In the testes, 505 metabolites were detected by UPLC/MS and GC/MS. Compared to respective injured vehicle group, at 72 hours there were 92 metabolites that reached a p-value of less than .05 in the 10 mg/kg Licofelone group, 100 in the 50 mg/kg licofelone group, and 93 in the 100 mg/kg licofelone group. In the 90 day groups, there were 62 in the 10 mg/kg licofelone group, 58 in the 50 mg/kg licofelone group, and 18 in the 100 mg/kg licofelone group. Similar to our last study in this field, these metabolites were clustered by biochemical function into amino acid, peptide, carbohydrate, energy, lipid, nucleotide, cofactors and vitamins, and xenobiotic “super pathways” and clustered again into smaller “sub pathways” such as eicosanoids, lysolipids, glycolysis, etc. We took Metabolon’s initial ANOVA analysis as a screening tool to find sub pathways where over 30% of metabolites were significantly changed and subjected them to further analysis as described in the methods.(83)

**Similar to our first paper, one of the pathways that was most significantly differential between groups were eicosanoid metabolites** (Figure 9). Of the 4 eicosanoids that were found in the testes in the metabolomic profiling, 3 (5-HETE, 15-HETE, and PGE2) were significantly different at both 72 hours and 90 days after SCI with 10 mg/kg licofelone compared to injured vehicle by ANOVA with Bonferroni correction for multiple comparisons in the testes. PGE2 was significantly changed by treatment with 50 mg/kg licofelone at 72 hours. 15-HETE was also significantly changed by treatment with 50mg/kg licofelone at 90 days. The mean fold change for significant
differences between injured vehicle and treatment with licofelone ranged from 0.2957 to 0.6967.

Some of the testicular lysolipids were also significantly decreased by treatment with licofelone. Of the 9 that were elevated in chapter one, 4 lysolipids (Figure 10) of PC origin were decreased significantly by ANOVA with Bonferroni correction for multiple comparisons in rats treated with 50 mg/kg licofelone compared to injured vehicle at 72 hours post-surgery. The mean fold change for significant differences between injured vehicle and treatment with licofelone ranged from 0.3611 to 0.6278.

Multiple metabolites that are related to oxidative stress were also measured to be significantly changed. (Figure 11) Oxidized glutathione is decreased at both time points when treated with 50 mg/kg licofelone, while the protective reduced glutathione is increased in treated animals compared to injured vehicle animals. The precursors for glutathione, cysteine, n-acetylcyesteine, and cysteinylglycine, were all elevated with treatment at 72 hours after injury, particularly with 10 and 50 mg/kg licofelone doses. The antioxidant ascorbate was elevated with treatment with all doses at 72 hours, and with 10 mg/kg licofelone at 90 days. 4-hydroxy-nonenal-glutathione as well as 13-HODE + 9-HODE were decreased by treatment with licofelone, particularly by 10 and 50 mg/kg doses. The mean fold change for a significant decrease between injured vehicle and treatment with licofelone ranged from 0.223 to 0.7029. The mean fold change for a significant increase between injured vehicle and treatment with licofelone ranged from 1.268 to 3.396.
Of the 4 unconjugated bile acids that were elevated in the previous chapter, all four were decreased 90 days after injury with the treatment of 10 mg/kg of licofelone. (Figure 12) Chenodeoxycholate was the only bile acid to also decrease with treatment of 50 mg/kg licofelone. At 72 hours, b-muricholate and chenodeoxycholate both decreased from treatment with 10 mg/kg licofelone, and increased from treatment with 100 mg/kg licofelone. The mean fold change for a significant decrease between injured vehicle and treatment with licofelone ranged from 0.2506 to 0.6208. The mean fold change for a significant increase between injured vehicle and treatment with licofelone ranged from 1.194 to 1.684.
Figure 9a: Attenuation of eicosanoid production after treatment with licofelone in spinal cord injury.
Figure 9b: Attenuation of eicosanoid production in the testes after treatment with licofelone in spinal cord injury.
**Figure 9:** Attenuation of eicosanoid production in the testes after treatment with licofelone in spinal cord injury.

Whisker plots illustrate the median (horizontal bar), the 25th to 75th percentile (box) and the minimum to maximum value (whiskers). Scaled values were to set the vehicle mean equal to 1 (horizontal dotted line). *:p≤0.05, **:p≤0.01, ***:p≤0.001, ****:p≤0.0001.

**PGE2** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0111) with doses of 10 mg/kg (p= 0.0095, mean fold change=0.4974 )and 50 mg/kg (p=0.0119, mean fold change=0.5306 ) but not 100 mg/kg (p=0.00795 , mean fold change=0.6532), as well as at 90 days (ANOVA: p=0.0018) in SCI animals with doses of 10 mg/kg (p= 0.0021, mean fold change= 0.2957).

**5-HETE** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0445) in SCI animals with doses of 10 mg/kg (p= 0.0201, mean fold change=0.4813 ) and 50 mg/kg (p= 0.0267, mean fold change=0.6967), as well as at 90 days (ANOVA: p=0.0021) with doses of 10 mg/kg (p= 0.0012, mean fold change= 0.4813) and 50 mg/kg (p=0.0478, mean fold change=0.6832).

**12-HETE** is not statistically changed by treatment with licofelone. At 90 days, 10 mg/kg of licofelone treatment approaches significance at p=0.0681.

**15-HETE** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0226) in SCI animals with doses of 10 mg/kg (p= 0.0200, mean fold change=0.5645 ) and 50 mg/kg (p=0.0384, mean fold change=0.6471 ) as well as at 90 days (ANOVA: p=0.0010) with doses of 10 mg/kg (p= .0021, mean fold change=0.5148 )and 50 mg/kg (p=0.0325, mean fold change=0.6219).
Figure 10: Attenuation of lysolipid production in the testes after treatment with licofelone in spinal cord injury.
**Figure 10**: Attenuation of lysolipid production in the testes after treatment with licofelone in spinal cord injury.

Legend is the same as Figure 9.

2-palmitoylglycerophosphocholine is decreased by treatment with licofelone at 72 hours (ANOVA: $p=0.0192$) in SCI animals with doses of 50 mg/kg ($p=0.0185$, mean fold change=0.3611).

1-oleoylglycerophosphocholine is decreased by treatment with licofelone at 72 hours (ANOVA: $p=0.0019$) in SCI animals with doses of 10 mg/kg ($p=0.00089$, mean fold change=0.4953) 50 mg/kg ($p=0.0093$, mean fold change=0.4441) and 100 mg/kg ($p=0.0278$, mean fold change=0.6222).

1-stearoylglycerophosphocholine is decreased by treatment with licofelone at 72 hours (ANOVA: $p=0.0061$) in SCI animals with doses of 50 mg/kg ($p=0.0110$, mean fold change=0.3676).

2-oleoylglycerophosphocholine is decreased by treatment with licofelone at 72 hours (ANOVA: $p=0.0303$) in SCI animals with doses of 50 mg/kg ($p=0.0314$, mean fold change=0.4916) and approaches significance at 10 mg/kg ($p=0.0859$, mean fold change=0.6278).
Figure 11a: Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.
**Figure 11a:** Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.

Legend is the same as Figure 9.

**13-HODE+9-HODE** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0003) in SCI animals with doses of 10 mg/kg (p=0.0045, mean fold change=0.4835) 50 mg/kg (p=0.0005, mean fold change=0.4618) and 100 mg/kg (p=0.0076, mean fold change=0.5563) as well as at 90 days (ANOVA: p=0.0070) with doses of 10 mg/kg (p=0.0094, mean fold change=0.5038) and 50 mg/kg (p=0.0351, mean fold change=0.5497).

**4-hydroxy-nonenal-glutathione** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0054) in SCI animals with doses of 10 mg/kg (p=0.0111, mean fold change=0.253) 50 mg/kg (p=0.0082, mean fold change=0.262) and approached significance with 100 mg/kg (p=0.0927, mean fold change=0.502) as well as at 90 days (ANOVA: p=0.0061) with doses of 10 mg/kg (p=0.0122, mean fold change=0.229) and 50 mg/kg (p=0.0380, mean fold change=0.223).

**N-acetylcysteine** is increased by treatment with licofelone at 72 hours (ANOVA: p=0.0171) in SCI animals with doses of 10 mg/kg (p=0.0139, mean fold change=2.899) 50 mg/kg (p=0.0087, mean fold change=2.740) and approaches significance with 100 mg/kg (p=0.0981, mean fold change=1.824) as well significantly increased at 90 days (ANOVA: p=0.0322) with doses of 10 mg/kg (p=0.0155, mean fold change=1.925).
**Figure 11b:** Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.
Figure 11b: Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.

Legend is the same as Figure 9.

Cysteinylglycine is increased by treatment with licofelone at 72 hours (ANOVA: p=0.0077) in SCI animals with doses of 10 mg/kg (p=0.0081, mean fold change=3.000) 50 mg/kg (p=0.0041, mean fold change=2.950) and 100 mg/kg (p=0.0334, mean fold change=3.396) as well as at 90 days (ANOVA: p=0.0274) with doses of 10 mg/kg (p=0.0218, mean fold change=1.855) and approaches significance with 50 mg/kg (p=0.0800, mean fold change=1.684).

Ascorbate (Vitamin C) is increased by treatment with licofelone at 72 hours (ANOVA: p=0.0050) in SCI animals with doses of 10 mg/kg (p=0.0183, mean fold change=1.580) 50 mg/kg (p=0.0350, mean fold change=1.516) and 100 mg/kg (p=0.0185, mean fold change=1.556) as well as at 90 days (ANOVA: p=0.0297) with doses of 10 mg/kg (p=0.0255, mean fold change=1.268) and approaches significance with 50 mg/kg (p=0.0828, mean fold change=1.276).
Figure 11c: Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.
Figure 11c: Changes to antioxidants and markers of oxidative stress in the testes after treatment with licofelone in the setting of after spinal cord injury.

Legend is the same as Figure 9.

**Oxidized glutathione** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0054) in SCI animals with doses of 10 mg/kg (p= 0.0099, mean fold change=0.6156) 50 mg/kg (p=0.0038, mean fold change=0.5865) and 100 mg/kg (p=0.0420, mean fold change=0.7029) as well as at 90 days (ANOVA: p=0.00169) with doses of 10 mg/kg (p= 0.0197, mean fold change=0.6466) and approached significance at 50 mg/kg (p=0.0642, mean fold change=0.6730).

**Reduced glutathione** is increased by treatment with licofelone at 72 hours (ANOVA: p=0.0026) in SCI animals with doses of 10 mg/kg (p= 0.0088, mean fold change=2.371) 50 mg/kg (p=0.0035, mean fold change=2.508) and 100 mg/kg (p=0.0329, mean fold change=2.083) as well as at 90 days (ANOVA: p=0.0120) with doses of 10 mg/kg (p= 0.0112, mean fold change=1.788) and approached significance with 50 mg/kg (p=0.0710, mean fold change=1.664).
Figure 12a: Attenuation of chronic unconjugated bile acid levels in the testes after treatment with licofelone in spinal cord injury.
**Figure 12b:** Acute unconjugated bile acid levels in the testes after treatment with licofelone in spinal cord injury.
**Figure 12:** Acute and chronic changes to unconjugated bile acids in the testes after treatment with licofelone in spinal cord injury. Legend is the same as Figure 9.

**Cholate** is decreased by treatment with licofelone at 90 days (ANOVA: p=0.0020) with doses of 10 mg/kg (p= 0.0001, mean fold change=0.2679).

**a-Muricholate** is decreased by treatment with licofelone at 90 days (ANOVA: p=0.0109) with doses of 10 mg/kg (p= 0.0001, mean fold change=0.2506).

**b-Muricholate** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0001) in SCI animals with doses of 10 mg/kg (p= 0.0260, mean fold change=0.5122) and is increased with 100 mg/kg (p=0.0060, mean fold change=1.684) as well significantly decreased at 90 days (ANOVA: p=0.0014) with doses of 10 mg/kg (p=0.0009, mean fold change=0.2421).

**Chenodeoxycholate** is decreased by treatment with licofelone at 72 hours (ANOVA: p=0.0010) in SCI animals with doses of 10 mg/kg (p= 0.0498, mean fold change=0.6041) and is increased with 100 mg/kg (p=0.0115, mean fold change=1.194) as well significantly decreased at 90 days (ANOVA: p=0.0057) with doses of 10 mg/kg (p<0.0001, mean fold change=0.2874) and 50 mg/kg (p=0.0412, mean fold change=0.6208).
4.c Discussion

Other than our previous studies, “little is known regarding the cascade of pathological events initiated in the testes following SCI. In order to get a broad picture of the pathology occurring, we elected to take a broad, multifaceted approach with mRNA and metabolomics. The amount of data resulting from such methods is immense, encompassing multiple pathways and systems at multiple time points. Metabolomics is a recent tool that permits the quantitative assessment in order to get a broad snapshot of a tissue’s metabolic profile. In any experiment, a certain small percentage of metabolites will show a difference where none actually exists due to the nature of statistics. With the large numbers that metabolomics deals with, the number of these false positives is significant, but largely shows up as random metabolites across the whole data set, not concentrated in one metabolic pathway. As such, metabolomics is most useful either when used as an initial screening tool, or when broad shifts in the metabolic state are evident.” (83)

This study was done semi-concurrently with that of the previous chapter. In-between running these samples at Metabolon for metabolomics assessment, they re-optimized their machines. This allowed greater accuracy and range of metabolites to be analyzed, but made combining the 2 data sets into one homogenous set a mathematical impossibility. However, the two data sets can still be compared for relative changes while remaining skeptical of exact numbers. Ideally, all time points would have been repeated in this study, but financial limitations required us to restrict our study to two time points chosen before the data from our first study could be analyzed. Because of this, some important pathological changes caused by SCI at 24 hours that may have
been reversed by licofelone were not able to be directly measured. However, seeing the broad changes that licofelone induces provides convincing evidence that these pathological changes are attenuated by licofelone.

Our most unexpected finding from our first chapter was the chronic elevation of bile acids within the testes. Clinically, patients with spinal cord injury are well known to develop biliary sludge and gallstone disease at higher rates. (33) In regards to male infertility, increased bile acids can lead to sperm cell sloughing, apoptosis, and BTB breakdown in mice via FXR-a and TGR5. (48, 110) After treatment with 10 mg/kg licofelone, the level of chronic bile acids is decreased by approximately the same fold change that it was increased by SCI. While undoubtedly a beneficial change in the testis environment for spermatogenesis and BTB integrity, we find this result particularly interesting. Although this change could be explained by protective effects in the liver and gall bladder, an optimistic interpretation of this finding would be that licofelone attenuates the BTB break down, resulting in less sequestration of bile acids by receptors in the seminiferous tubule. Even more unexpected was the effect of licofelone on levels of bile acids at 72 hours. Two bile acids were lowered by treatment 10 mg/kg licofelone, and increased by 100 mg/kg licofelone. To our knowledge, no one has looked at the effects of licofelone on bile acids in a rat model. This finding could point to a narrow therapeutic window for a side effect of licofelone on systemic bile acids, or further indicate preservation of the blood testes barrier. Future directions would need to include analyzing licofelone for its systemic effects on bile acids, the liver, and gall bladder.
Lysolipids are an integral part of cell membrane maintenance and function, but are indicative of pathologic processes, like oxidative stress, when elevated. LysoPCs in particular have been shown to be released by apoptotic cells to act as a powerful chemotactic mediator for phagocytic cells, presumably to promote phagocytosis of the remains that are left after apoptosis. (103, 104, 106, 115) After treatment with 50 mg/kg of licofelone, 4 of the 9 lysoPCs that were elevated in chapter 1 were decreased. These findings may indicate that licofelone reduces the severity of pathologic apoptosis occurring in the testis after SCI and that the chemotactic recruitment of phagocytic cells is reduced. This could not only help preserve healthy developing sperm cells, but also reduce the infiltration of neutrophils previously shown by our lab. (61, 83) It would be worthwhile to investigate whether this also has an effect on the chronic elevation in t-cells. (83)

Previously, we had shown that there was an increase in inflammatory and oxidative stress markers in the testes at 24 hours post SCI. (83) Here, we present robust findings that indicate licofelone has an anti-oxidant and anti-inflammatory effect in the testes. This effect is present acutely at 72 hours and chronically up to at least 90 days. Although this is not direct evidence of a reduction in oxidative stress and eicosanoids at 24 hours, the consistent improvement in anti-oxidant capacity, their precursors, their metabolites, and decreased inflammatory mediators provides enough convincing evidence that we believe licofelone attenuates these pathological processes. We find it particularly interesting that the effect continues 90 days after injury, or 80 days since the last dose of licofelone. Although our previous study did not show a statistically significant chronic increase in inflammatory or oxidative markers, it has been shown
before that SCI causes long term inflammation and oxidative stress in peripheral tissues and organs. (116) As with bile acids, inflammation and oxidative stress are detrimental to spermatogenesis and these data are an encouraging result. (100)
Chapter 5: Discussion

Spinal cord injury causes a sustained breakdown of blood testis barrier integrity. In order to evaluate potential therapeutic targets, we took a broad approach to understanding the changes in local testes environment at both acute and chronic time points. At the same time, we tested a promising novel therapeutic that has had success in other areas of medicine.

At 24 hours, we showed that SCI causes a robust inflammatory and oxidative environment, consistent with earlier preliminary data by our lab. Early treatment with licofelone causes both acute and chronic anti-inflammatory changes, as well as improving the testes anti-oxidative capabilities. Although this is not direct evidence of attenuating the pathologic changes at 24 hours, its broad and sustained effect is convincing nonetheless. We also showed that steroidogenesis mRNA, and testosterone production, is suppressed by SCI. This effect is not changed by treatment with licofelone, indicating a different mechanism for the decline. While transient decreases in testosterone and LH/FSH after SCI is documented, research on exogenous testosterone’s role in treatment has been mixed and is still being studied. (97, 117, 118)

At 72 hours, we showed that there was a constellation of findings that pointed toward a wave of cell death. Neutrophils, recruited into the testes by the prevalent amount of lysolipids, showed an increased presence in the testes, a tissue that is particularly susceptible to a mounted local immune reaction. However, a lack of an increase in caspase activity points to localized areas of apoptosis, much like that in heat induced testicular injury, coupled with a decrease in spermatogenesis’ normal
apoptosis, a finding supported by the decrease in mRNA transcripts involved in the cell cycle. Alternatively, it has been shown in vitro that testosterone withdrawal causes apoptosis in sperm cells via a mechanism that does not elevate caspase but would still result in the extensive positive TUNNEL staining shown before. (119, 120) Licofelone shows promising effects by lowering chemotactic lysolipids. This could point to both a decreased amount of cell death that would lead to lysolipid release, and lowered neutrophilic recruitment. Future investigation would necessitate histological assessment of the licofelone treated animals, especially TUNNEL staining. We had intended to do just that, but a freezer malfunction resulted in losing all of our samples earmarked for that task. An alternative method of assessing cell death/apoptosis in the testes would be to isolate the DNA and use gel electrophoreses to quantify the amount of 3’ end labeling. This has been used by other labs to successfully compare changes in cell death levels for heat induced testicular injury and testosterone withdrawal. (43, 50) As discussed above, testosterone withdrawal and heat induced testicular injury result in stage specific apoptosis of germ cells. Determining which stages of spermatogenesis and which segments of the seminiferous tubule are negatively affected after spinal cord injury would help us determine the particular mechanism or mechanisms that lead to testicular injury after spinal cord injury. Frankly, this study would require collaboration with a lab with expertise in identifying sperm cell stages in chaotic tissue.

Finally, at 28 days and 90 days post SCI, we showed an elevation in bile acids, which was our most unexpected finding. Even more surprising was that it was reversed by treatment with licofelone. If chronically elevated bile acids were responsible for maintaining the permeability of the BTB, then this reversal by licofelone would be very a
very encouraging finding. We also showed increased numbers of T-cells in the testes 1.5 years after SCI, indicating a sustained immune response in the testes.

The reason for the different responses to the licofelone doses presents an interesting question. 10 doses of licofelone early after injury causes lower bile acids in the testes 90 days later, with a clear negative dose response curve. Similar responses, although less pronounced, can be seen among many of the metabolites presented. While SCI could alter licofelone metabolism and lead to toxicity, we think it is much more likely that the level of eicosanoids in untreated animals is pathologically high due to the overwhelming response to SCI, while in those with higher doses of licofelone some of the protective and beneficial effects of eicosanoids are blocked. (62) Similar to treating spasticity after SCI, finding the right balance for blocking inflammatory reactions would be optimal for treatment. Regardless, some metabolites respond better to 10 mg/kg and others to 50 mg/kg of licofelone, indicating multiple optimal doses depending on the desired result or target. This has been demonstrated before when targeting different pathologies in both mice and humans. (121)
5.a Other Future Directions

As with every scientific study, there are both questions left unanswered and new questions that arise based on the answers we found. In addition to the future targets discussed above, these studies naturally lead into other areas.

A natural progression of these studies would be to analyze functional changes in sperm motility after treatment with licofelone. This would require the capability to harvest samples, as well as the ability to analyze massive numbers of sperm cells for their motility parameters. With the right equipment, such as computer assisted semen analysis, this study would be rather straightforward.(122) If successful, transition to clinical trials for a drug that has already passed staged III clinical trials would be easier than an untested new pharmaceutical.

Originally, the testes from these experiments were to be analyzed histologically. Unfortunately, the testes that were marked for these experiments were located in a -80°C freezer that completely thawed due to a faulty electrical part. We wanted to explore TUNNEL staining as discussed above, but we also wanted to evaluate the status of various proteins that make up the blood testes barrier and their disorganization over time. Were all components of the BTB as disrupted as occludin? Additionally, are the recruited neutrophils in the interstitium or within the seminiferous tubule? We also wished to identify which stages of spermatogenesis were affected. These basic questions are still valid and should be eventually addressed.
Not enough is known about licofelone’s effects on the liver, gall bladder, and bile acids. The unexpected acute effects of licofelone on bile acid levels in the testes urge further investigation.

To my knowledge, no one has studied the effects of SCI on the blood retinal barrier. Similar to the BBB, it is a vascularly associated tight junctional network that creates an immune privileged environment for antigenic proteins like the BTB. With how many seemingly unrelated tight-junctions being disrupted by SCI, it would almost be more surprising to see a null result. However, one difference between the BRB and other tight junctions is that it is in an area completely innervated by cranial nerves, which do not route anywhere near the spinal cord. We harvested the eyes of enough of the animals from these experiments to adequately assess the possibility of spinal cord injury also affecting this barrier integrity. We fully intend to answer this question in the future.


Vita

Ryan Dean Fortune was born in Dallas, Texas and lived in the surrounding areas until dropping out of Coppell high school to attend the Texas Academy of Mathematics and Science at the University of North Texas. After graduation in 2006, he entered Texas A&M University in College Station, Texas as a member of the Corps of Cadets. He received the degree of Bachelor of Science with a major in genetics and a certificate in leadership from Texas A&M in May, 2008. In June of 2008, he entered his first year of the University of Texas MD/PhD Program at Houston, a dual degree program of The University of Texas McGovern Medical School and MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. He has served as a 1st Lieutenant in the Texas Army National Guard’s Texas Medical Command since before his 2nd year of medical school. He will be starting UTHealth’s Family Medicine Residency Program in the summer of 2018.