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**14. ABSTRACT**

Nearly 70% of males exhibit a profound loss of fertility following spinal cord injury. While the mechanisms underlying this loss have been discussed for decades, our laboratories (Grill and Loose) discovered that spinal trauma produces a significant loss in integrity of theblood-testis-barrier; a protective multi-cellular structure that maintains immune privilege of the highly-antigenic sperm andsperm cell-containing compartments within the testis. We also demonstrated that once failed, the BTB remains permeable, essentially for the life of the subject. The goal of our proposal has been two-fold: 1) to develop a greater understanding of the molecular, biochemical and structural pathologies underlying BTB breakdown post-SCI, and 2) to determine whether a novel therapeutic, can help preserve BTB integrity when introduced during the acute phase of SCI using a clinically-relevant rat spinal contusion model. We have found that the drug, licofelone, preserves blood-spinal cord barrier integrity and enhances locomotor function in rats when given early following injury. During this third year, we have completed all planned spinal cord injuries (24 hour out to 90 day time points); collected testis tissues and have analyzed these samples by metabolomic analysis and expression

**15. SUBJECT TERMS**

BTB, blood-testis-barrier; inflammation; oxidative stress; spinal cord injury, SCI, metabolism, expression microarray

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**1: INTRODUCTION** Males who have received a spinal cord injury (SCI) face a lifetime of sensory and motor deficits. In addition to these well-described pathological outcomes, a majority of men will also experience greatly diminished fertility. This should be clearly understood to be separate from SCI-dependent erectile dysfunction that is due to a loss of neural input into the male sexual organs. SCI-dependent male infertility is characterized by a significant
reduction in numbers and quality of functional sperm. The mechanism(s) underlying this deficit has previously been unknown. We have explored the effects of spinal trauma on tissues that exhibit "barrier" properties, or properties in which specialized tissues regulate the flow of materials from the blood stream into compartments throughout the body that are "immune privileged". Our focus has been on the blood-spinal cord barrier (BSCB) and how trauma collapses this important spinal vascular specialization; producing an environment that encourages long-term inflammatory conditions and permits infiltration of normally excluded immune. We recently asked whether spinal trauma had any effect on the blood-testis-barrier (BTB), a specialized set of cellular structures located within the testis which protects sperm (immature through mature) as well as sperm precursor/stem cells from the immune system. We reported that a contusive injury to the rat spinal cord causes a profound and sustained loss of BTB integrity; resulting in the formation of both inflammatory and pro-oxidative conditions within the sperm producing compartments. In addition, we detected the loss of structural elements that comprise the BTB as well as significant cell death and immune cell infiltration. The goal of this project is to: 1) further elaborate the early and long-term biochemical, molecular and structural deficits to the BTB elicited by spinal trauma, and 2) determine whether these pathological changes can be prevented or at least minimized by pharmacological modulation. We have recently found that a novel anti-inflammatory drug, licofelone, provided significant protection to the BSCB when administered to rats orally during the acute phase of SCI. Licofelone is a first generation anti-inflammatory drug that targets BOTH cyclooxygenase AND 5-lipoxygenase pathways of arachidonic acid metabolism; the two main pathways used to generate arachidonic acid-derived proinflammatory compounds (prostaglandins and leukotrienes, respectively). In the second aim of the current project (and a focus of this year's annual report), we describe how acute licofelone treatment results in attenuation of SCI-dependent pathological events that influence inflammation and oxidative stress. More importantly, we report that the attenuation of these pathological events resulting from early, acute licofelone treatment produce a sustained beneficial effect on these SCI-induced pathologies within the testes long after cessation of licofelone treatment. Our results to date, are intriguing and suggest that SCI produces a wide range of pathologies, both acute as well as sustained over a long time period, within the testes. Our goal is to determine whether licofelone treatment can be subsequently translated as a novel therapeutic for the treatment of male infertility following SCI.

**2: Keywords:** Blood-testis-barrier (BTB), spinal cord injury (SCI), blood-spinal cord-barrier (BSCB), testes, testis,
inflammation, oxidative stress, metabolism, genomic, metabolomic

## 3: OVERALL PROJECT SUMMARY:

**What were the major goals of the project?** The overall hypothesis and aims of this proposal were asfollows:

**Hypothesis:** SCI induces biochemical, structural and functional changes within the BTB that contribute to a loss of sexual function in males. We further hypothesize that the application of a novel antiinflammatoryintervention will protect both BTB integrity and male reproductive capabilities.

**Specific Aim 1:** Explore the molecular, biochemical and structural changes that occur to the BTB as a functionof time following the delivery of a clinically-relevant spinal contusion injury.

**Specific Aim 2:** Determine whether treatment with the new generation anti-inflammatory drug, licofelone, canprotect BTB integrity and enhance germ cell and sperm viability over time following SCI.

We estimate that we are upwards of 90% complete with both aims. All testis samples, blood samples, a samples from additional tissues have been collected. We have validated several of the observed metabolomic and gene array changes using commercially-available ELISA kits, Western blot analysis.

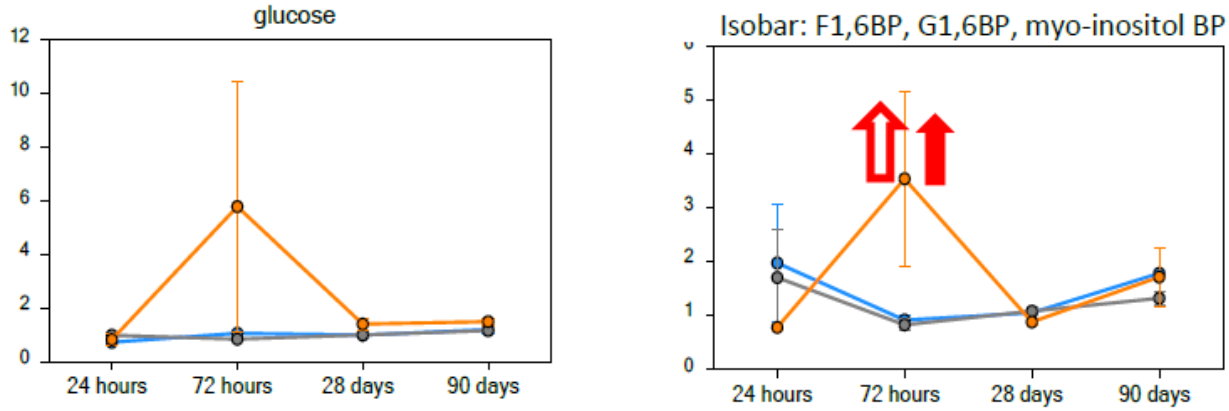
Lt. Ryan Fortune,who is an MD/PhD student in at UTHealth was a student in Dr. Grill's laboratory. With Dr. Grill's move to the Medical College of Mississippi, Lt. Fortune has continued to work on the project under the mentorship of Dr. Loose. He has performed the majority of the spinal cord injury studies in rats and has assisted in all endstage tissue collections. He has completed the metabolomics and expression microarray studies.

**What was accomplished under these goals? (during the 2014-2015 period):**

We will briefly review progress described in prior progress reports during the 2013-2014 period as well as describe our progress in the current reporting year (2014-2015).

**SCI-induced changes in testicular metabolite profile.**

We have spent a significant amount of time in the current year in developing a rigorous statistical approach to analyzing both the metabolomics and the microarray data. With respect to the metabolomics data, Metabolon performed an initial analysis that made several assumptions that we considered invalid. For example, if a metabolite was undetectable in a given animal, the lowest value detected in the remaining animals was assigned to that missing value. This skewed both the group average and distorted the actual N (number of animals) in the group. In addition, they did not perform any outlier analysis on any of the data and they applied a two-tailed T-test instead of more appropriate statistics. We developed a consistent analytical pathway that examined every datum for every metabolite from every animal. Values that diverged by more than 2 SD from the mean were eliminated from the data set. Values for all remaining animals were analyzed by ANOVA using all three groups of animals, injured, sham, and naïve. This process eliminated some of the metabolic pathways that we reported last year. For example, the initial analysis suggested that glucose metabolism was altered at 24 and 72 hours post SCI.




In these figures, the gold lines represent the injured animals, the blue bars represent sham animals and the gray bars are naïve animals. The hollow red bar means significant at p< 0.05 for injured compared to naïve, the solid arrow is p < 0.05 for injured vs sham.

Although the changes at 24 and especially 72 look significant, our analysis suggested that this is due to a single outlier animal (that had 41-times higher glucose than the rest of the animals) that was not excluded from the data set. Using the more valid ANOVA these changes are not significant. However, many of the changes we reported last year DO pass the new statistical analysis, For example, Figure 2 illustrates the effects of SCI on eicosanoid production:

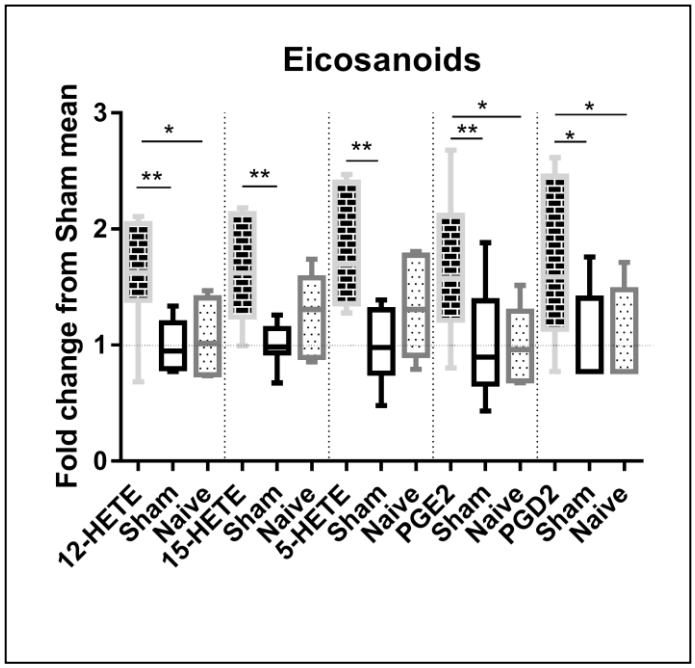



Figure 2: Metabolomic assessment of eicosanoids in the teste at 24 hours post SCI:

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).

12-HETE in injured animals is elevated (ANOVA p=.0044) compared to both sham (**: p≤.01) and naïve (*: p≤.05) with an injured mean fold change from sham mean of 1.6.

15-HETE in injured rats is elevated (ANOVA p=.0050) compared to sham (**: p≤.01) with an injured mean fold change from sham mean of 1.655.

5-HETE in injured rats is elevated (ANOVA p=.0023) compared to sham (**: p≤.01) with an injured mean fold change from sham mean of 1.828.

PGE2 in injured rats in elevated (ANOVA p=.0147) compared to sham (**: p≤.01) and naïve (*: p≤.05) with an injured mean fold change from sham mean of 1.661.

PGD2 in injured rats is elevated (ANOVA p=.0198) compared to sham and naïve (*: p≤.05) with an injured mean fold change from sham mean of 1.703.

The Metabolon panel for eicosanoids comprised these 5 metabolites. All 5 are significantly elevated at 24 hours**. This is the first observation that a whole class of pro-inflammatory molecules are elevated in the testis following SCI.**

In a comparable manner, the increase in oxidative stress that we reported last year is still valid with 2 of 5 metabolites showing changes in level that reflect such stress:

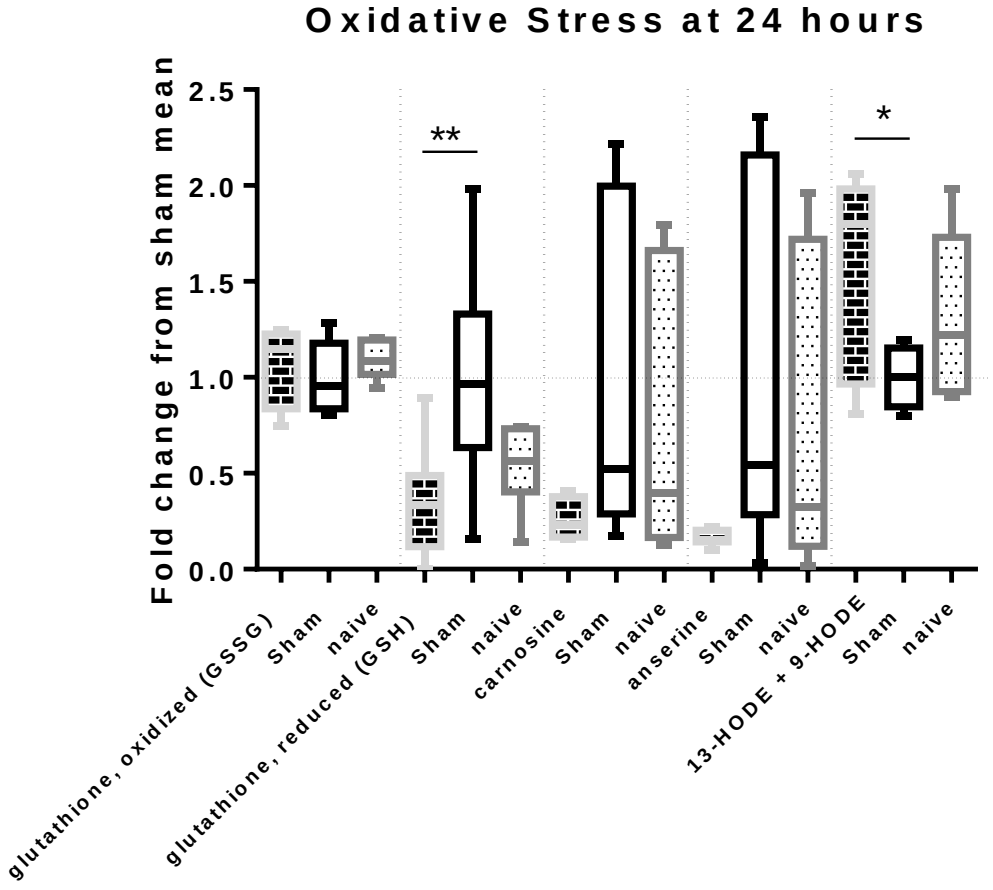



Figure 3: Metabolomic assessment of oxidative stress markers at 24 hours post-surgery:

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≤0.01, ANOVA: p=0.0064, mean fold change: 0.3376).
Carnosine and anserine were unchanged at 24 hours (ANOVA: p=0.0966 and p=0.0786, respectively).
13-HODE + 9-HODE was elevated after SCI compared to sham (p≤0.05, ANOVA p=0.0337, mean fold change: 1.550).

Reduced glutathione is arguably the most important antioxidant in the body; it is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals. Its apparent consumption following SCI reflects a reduced ability to reverse damage most likely arising from the immune system. 13-, and 9-HODE (Hydroxyoctadecadienoic acid) directly reflect oxidative stress. These metabolites derive from the lipoxygenase pathway. The may be synthesized from either damaged testicular parenchyma or from infiltrating immune cells.
Even though the reduction dipeptides carnosine and anserine did not reach statistical significance, it is worth pointing out that this was due to the marked variability in both the sham and the naïve groups. Both carnosine and anserine have antioxidant properties and both can scavenge reactive oxygen species.

Our metabolomic analysis revealed that lysolipids are greatly induced in the testis following SCI. Quantitatively the changes seen in the lysolipid profile are highly coherent (consistent across the whole class of metabolites) and of considerable magnitude,averaging about 6-fold.

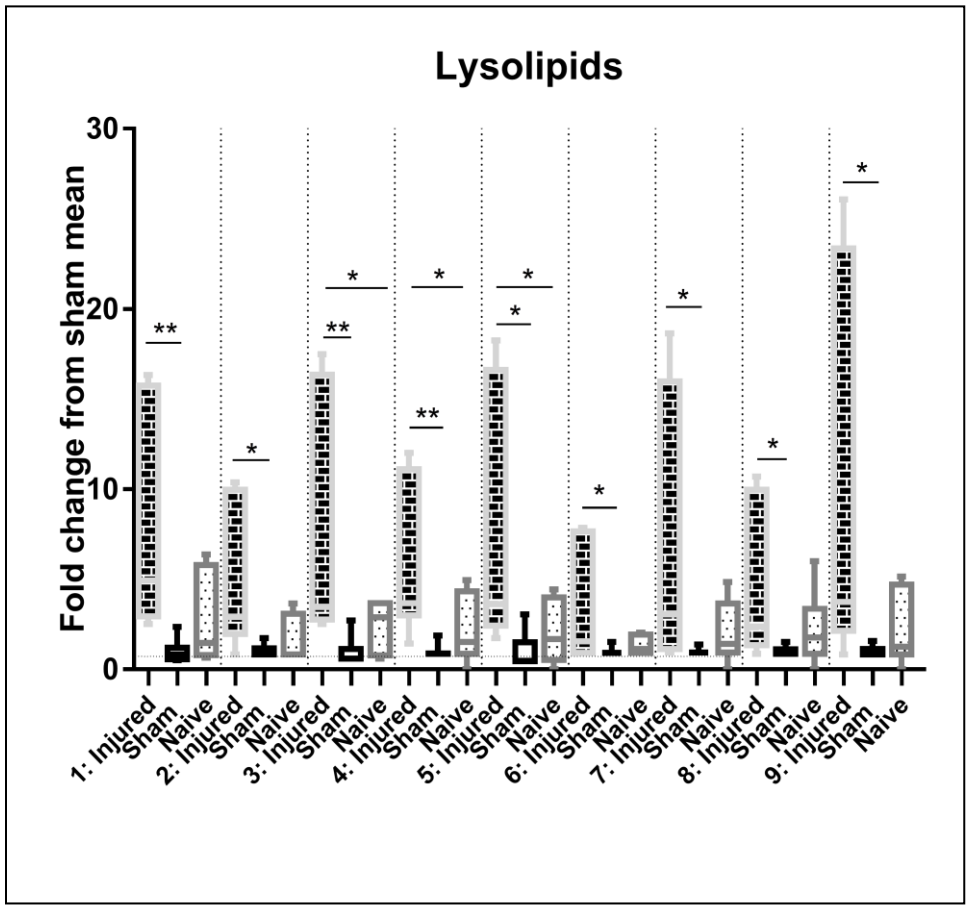
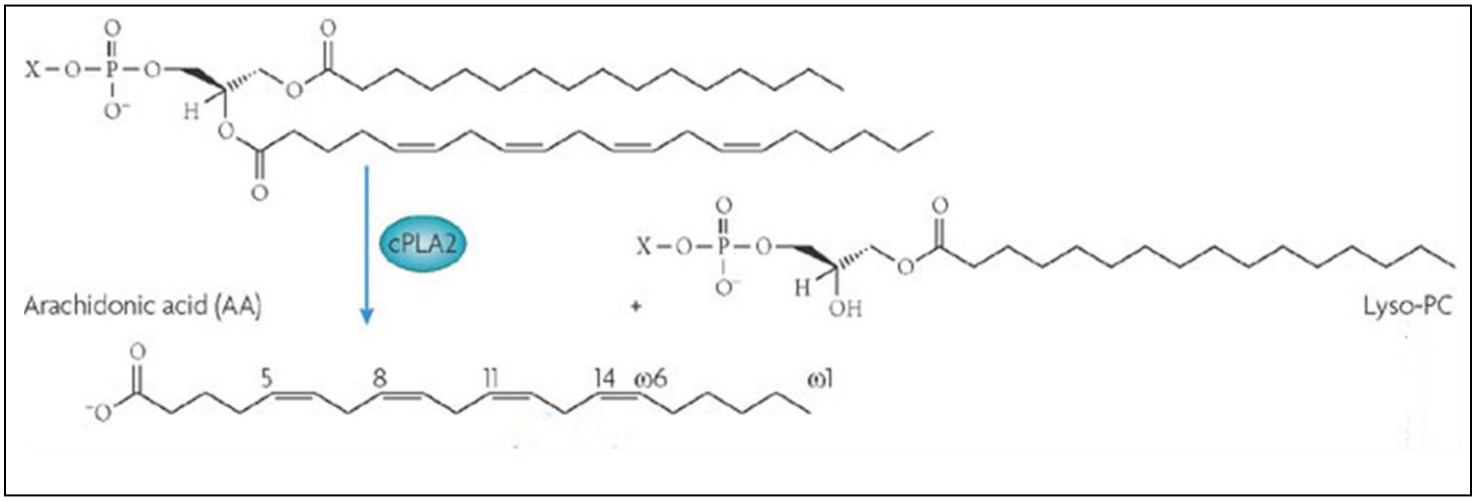


Figure 4: Metabolomic assessment of lysolipids at 72 hours post-surgery:
Legend is the same as Figure 2.
Compared to sham, lysolipids that were elevated after SCI were
1: 1-palmitoylglycerophosphocholine (p≤0.01, ANOVA: p=0.0055, mean fold change: 8.118),
2: 2-palmitoylglyerophosphocholine (p≤0.05, ANOVA: 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= .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).

Lysolipids are formed by the action of lipases which remove one of the fatty acid chains from a phospholipid. In the example below, phospholipase A2 acts on a membrane phospholipids to release arachidonate and a phosphocholine lysolipid.




Lysolipids can be formed from any class of phospholipid: phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositide etc. As illustrated in Figure 4, we identified 9 lysolipids that were increased following SCI. Importantly, **ALL 9** are phosphatidylcholine derivatives. We did not detect changes in any other class of diacylglycerol even though they all were well represented on in the Metabolon profile.
Unique to the phophatidylcholine (PC) derived lysolipids is there ability to act as chemoattractant agents – in fact they establish a positive feed back loop as illustrated in Figure 5. PC-derived lysolipids act as chemoattractants for monocytes and macrophages. These immune cells secrete numerous cytokines that can stimulate the biosynthesis and activity of phospholipase A2; phospholipase A2 acts to produce more lysolipids. Dr. Grill has demonstrated that in the rat model,SCI causes acute increases in PLA2. The rate limiting enzyme in the biosynthesis of eicosanoids (Figure 2) is PLA2.

Although we have not proven that this feedback loop is established following SCI, all experimental evidence supports that hypothesis. **The increase in 7 of the 9 lysolipids was blocked by administration of licofelone (10 mg/kg) prior to SCI.**

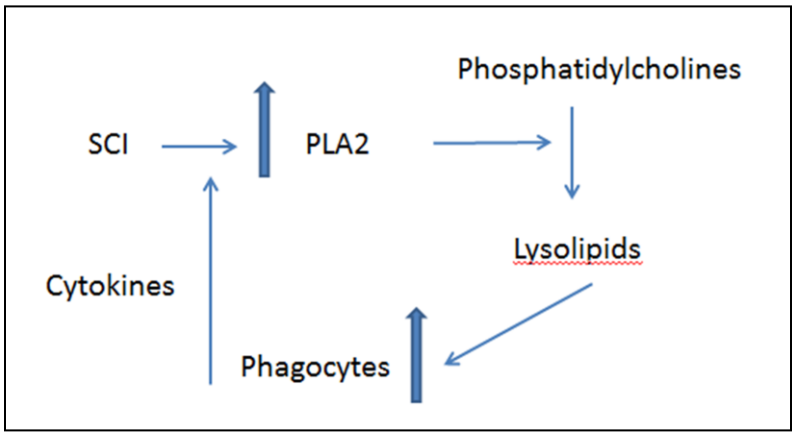
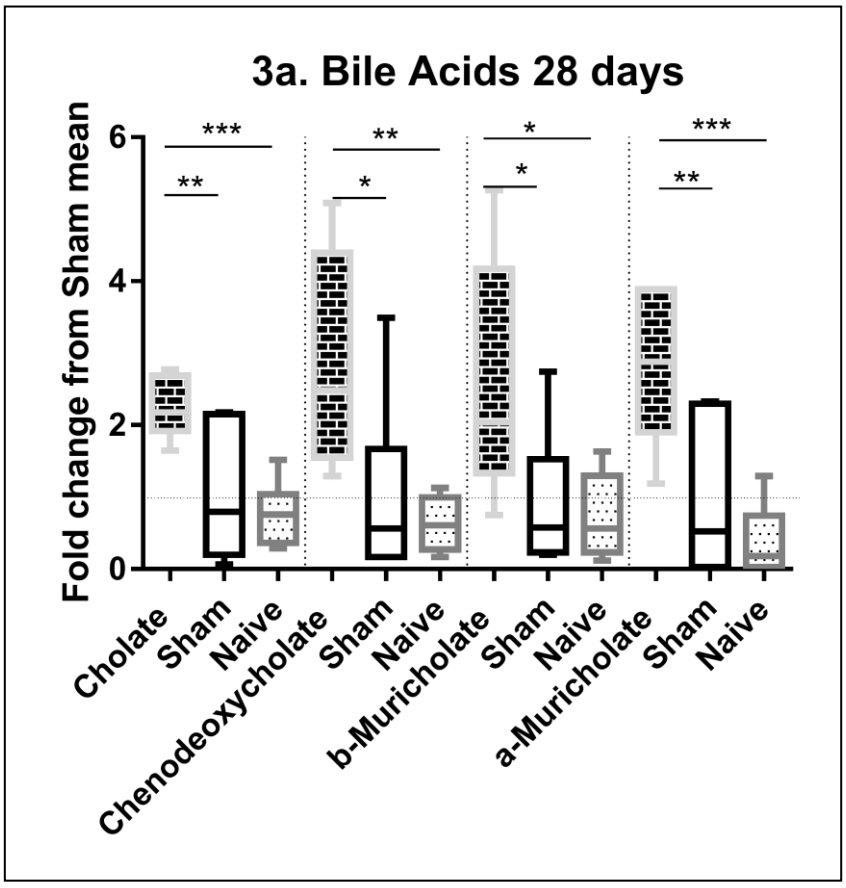



Figure 5. Potential positive-feedback loop established by PLA2 mediated production of lysolipids and the recruitment of phagocytes.

We reported in the previous annual report that some bile acids were increased within the testis following SCI. We have validated these results both by additional analysis of the Metabolon data and analysis of plasma bile acids.



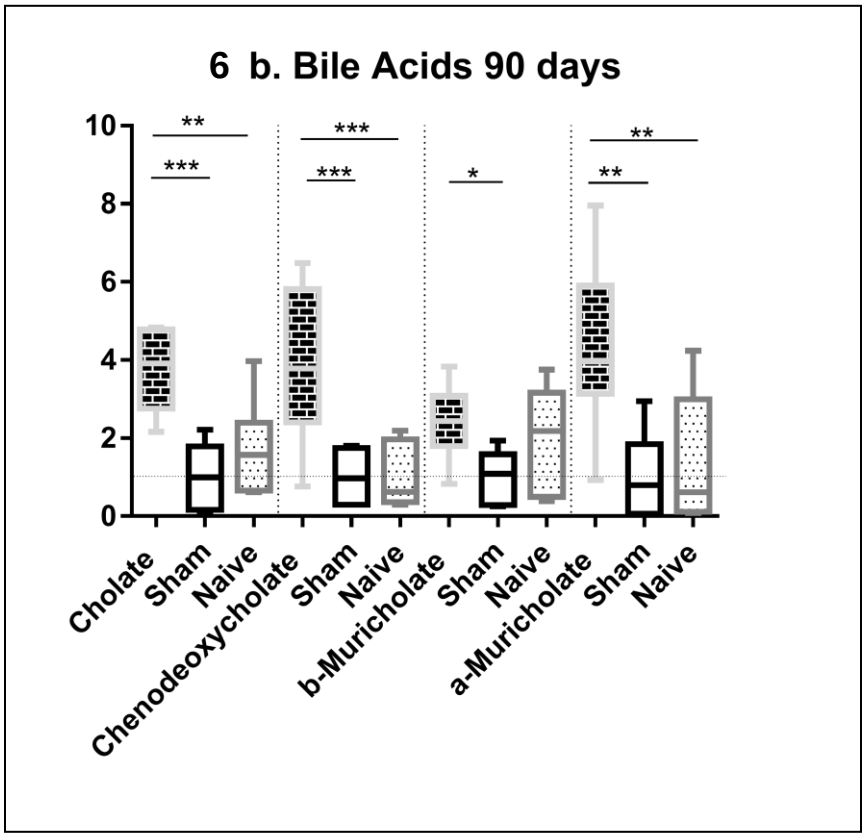



Figure 6a: Legend is the same as Figure 2.
5a: Metabolomic assessment of unconjugated bile acids in the teste 28 days post SCI:
Cholate in injured rats was elevated (ANOVA: p=.0003) compared to sham (**: p≤.01, mean fold change: 2.238) and naïve (***: p≤.001).
Chenodeoxycholate in injured rats was elevated (ANOVA: p=.0016) compared to sham (*: p≤.05, mean fold change: 2.844) and naïve (**: p≤.01).
B-Muricholate in injured rats was elevated (ANOVA: p=.0096) compared to sham (*: p≤.05, mean fold change: 2.534) and naïve (*: p≤.05).
a-Muricholate in injured rats was elevated (ANOVA: p=.0001) compared to sham(**: p≤.01, mean fold change: 2.815) and naïve (***: p≤.001)

6b: Metabolomic assessment of unconjugated bile acids in the teste 90 days post SCI:
Cholate in injured rats was elevated (ANOVA: p=.0001) compared to sham (***: p≤.001, mean fold change: 3.760) and naïve (**: p≤.01).
Chenodeoxycholate in injured rats was elevated (ANOVA: p=.0002) compared to sham (***: p≤.001, mean fold change: 3.836) and naïve (***: p≤.001).
B-Muricholate in injured rats was elevated (ANOVA: p=.0303) compared to sham (*: p≤.05, mean fold change: 2.420).
a-Muricholate in injured rats was elevated (ANOVA: p=.0010) compared to sham (**: p≤.01, mean fold change: 4.352) and naïve (**: p≤.01).

Four unconjugated bile acids were detected in the metabolomics profiling (Figure 5) with all 4 elevated in the SCI animals compared to shams (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. Interestingly, we detected no changes in conjugated (tauro- or glycol-) bile acids. There is strong evidence of bile acid metabolism changes in SCI; **but no one has reported changes in bile acid concentration in the testis following 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.[1-4] This, together with our data, suggests a more fundamental change in the bile acid metabolism than just decreased mechanical function. We found that all 4 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 hepaticsystemic change in bile acid metabolism and provides a strong case to further investigate into bile acid metabolism changes after SCI. In addition to their cytotoxic effects activity due to detergent action and, elevated bile acids have been shown to reduce male fertility via FXR-$\alpha$ (farnesoid X receptor alpha) 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.[5-6]

**SCI-induced changes in testicular gene expression profile.**
As reported in the last annual progress report we detected numerous changes in transcript levels in the testis following SCI. I will not reiterate those data, but will highlight two pathways that we have validated by further analysis.

We detect a rapid decline in transcripts involved in steroidogenesis with the testis (Figure 7).

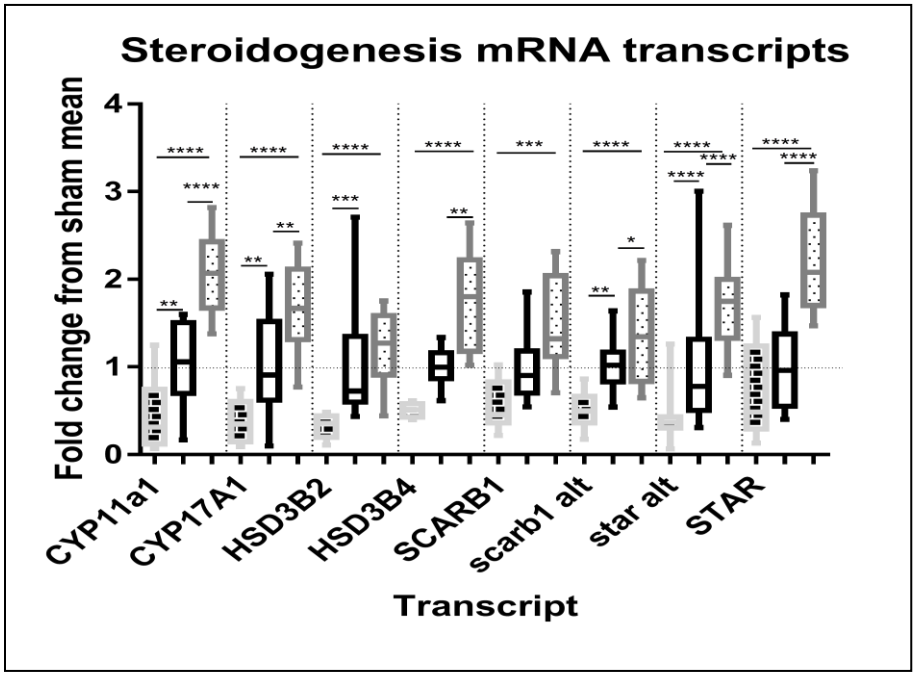


Figure 7: Array assessment of the expression of steroidogenesis genes' mRNA transcripts at 24 hours post SCI:
Legend is the same as Figure 2.
CYP11a1 mRNA expression is suppressed in sham animals (sham vs naïve****: p≤.0001), and even more suppressed in injured animals (injured vs sham**: p≤.01, Injured vs naïve****: p≤.0001) with a mean fold change vs sham of .4486 (ANOVA: p<.0001).
CYP17a1 mRNA expression is suppressed in sham animals (sham vs naïve**: p≤.01), and even more suppressed in injured animals (injured vs sham**: p≤.01, Injured vs naïve****: p≤.0001) with a mean fold change vs sham of .3878 (ANOVA: p<.0001).
HSD3B2 mRNA expression is not suppressed in sham animals but is suppressed in injured animals (injured vs sham***: p≤.001, Injured vs naïve****: p≤.0001) with a mean fold change vs sham of .3214 (ANOVA: p<.0001).
HSD3B4 mRNA expression is suppressed in both sham animals (sham vs naïve**: p≤.01) and injured animals (Injured vs naïve****: p≤.0001) with a mean fold change vs naive of .2882 (ANOVA: p<.0001).
SCARB1 mRNA expression is not suppressed in sham animals but is suppressed in injured animals (Injured vs naïve***: p≤.001) with a mean fold change vs naive of .3653 (ANOVA: p=.0012).
SCARB1 alt (alternative transcript) mRNA expression is suppressed in sham animals (sham vs naïve*: p≤.05), and even more suppressed in injured animals (injured vs sham**: p≤.01, Injured vs naïve****: p≤.0001) with a mean fold change vs sham of .5190 (ANOVA: p<.0001).
STAR alt mRNA expression is suppressed in sham animals (sham vs naïve****: p≤.0001), and even more suppressed in injured animals (injured vs sham****: p≤.0001, Injured vs naïve****: p≤.0001) with a mean fold change vs sham of .4382 (ANOVA: p<.0001).
STAR mRNA expression is suppressed in both sham animals (sham vs naïve****: p≤.0001) and injured animals (Injured vs naïve****: p≤.0001) with a mean fold change vs naive of .3082 (ANOVA: p<.0001).

We detected changes in 8 transcripts involved in steroid biosynthesis in the testis. Testicular function, including maintenance of Sertoli cell function and spermatogenesis, is dependent on testosterone. Of the 8 steroidogenic transcripts, StAR (steroidogenic acute regulatory protein)is of critical importance and is rate limiting for testosterone production on the testis. StAR protein has been shown to be instrumental in the acute regulation of steroid hormone biosynthesis through its action in mediating cholesterol transfer to the inner mitochondrial membrane and the cholesterol side chain cleavage enzyme system.[7-9] Maintenance of steroidogenesis in the testis is dependent of LH released from the anterior pituitary. We hypothesize that SCI causes a general shock to the CNS and disrupts normal neuroendocrine pathways including the release of LH. This is testable by ELISA assay of plasma for LH, which we would predict would fall prior to 24 h. Unfortunately we did not collect blood samples at this time point so pursuing this line of investigation would require additional animals and is beyond the SOW of this proposal.

**SCI-induced changes in testicular immune cell profile profile.**

Many of the changes found in our studies suggested a major alteration in immune cell dynamics or function. We have formed a collaboration with Dr. Christine Beeton of Baylor College of Medicine to begin to look a immune cells within the rat testis following SCI (Figure 8). Although these studies were not part of the original SOW, they were accomplished at no cost to the DOD and were done as a true collaboration; we provided only small aliquots of whole testes tissue from the animals we had already used in the metabolomic and genomic studies or some chronic animals due for sacrifice because of Dr. Grill's move to Mississippi.

Immune cells in the testis were quantitated by flow cytometry in Dr. Beeton's laboratory. A single-cell suspension was prepared from rat testes using a 70 um cell strainer (BD) as described.[10] Isolated cells were washed with ice-cold flow cytometry wash solution (PBS + 2% goat serum + 2% BSA), stained with fluorophore-conjugated antibodies or with ShK-F6CA, a fluorophore-conjugated peptide selective for Kv1.3, a marker of activated effector-memory 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 for acquiring 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.[11]
Neutrophil counts were significantly increased at 72 hours in the acute SCI animals versus both sham and naïve animals (26.4% and 30.8% increase respectively, $p<0.05$), but were not elevated in the chronic 1.5 year post-surgery SCI animals. In the 1.5 year chronic SCI animal we observed an elevation in T cells in the testes (41.6% increase, $p<0.001$) that was not seen at 72 hours. There was no significant change in subsets of T-cell phenotypes at either time point. The 72 hours is significant since it is the time point where we found maximal increases in lysolipids which can recruit neutrophils.

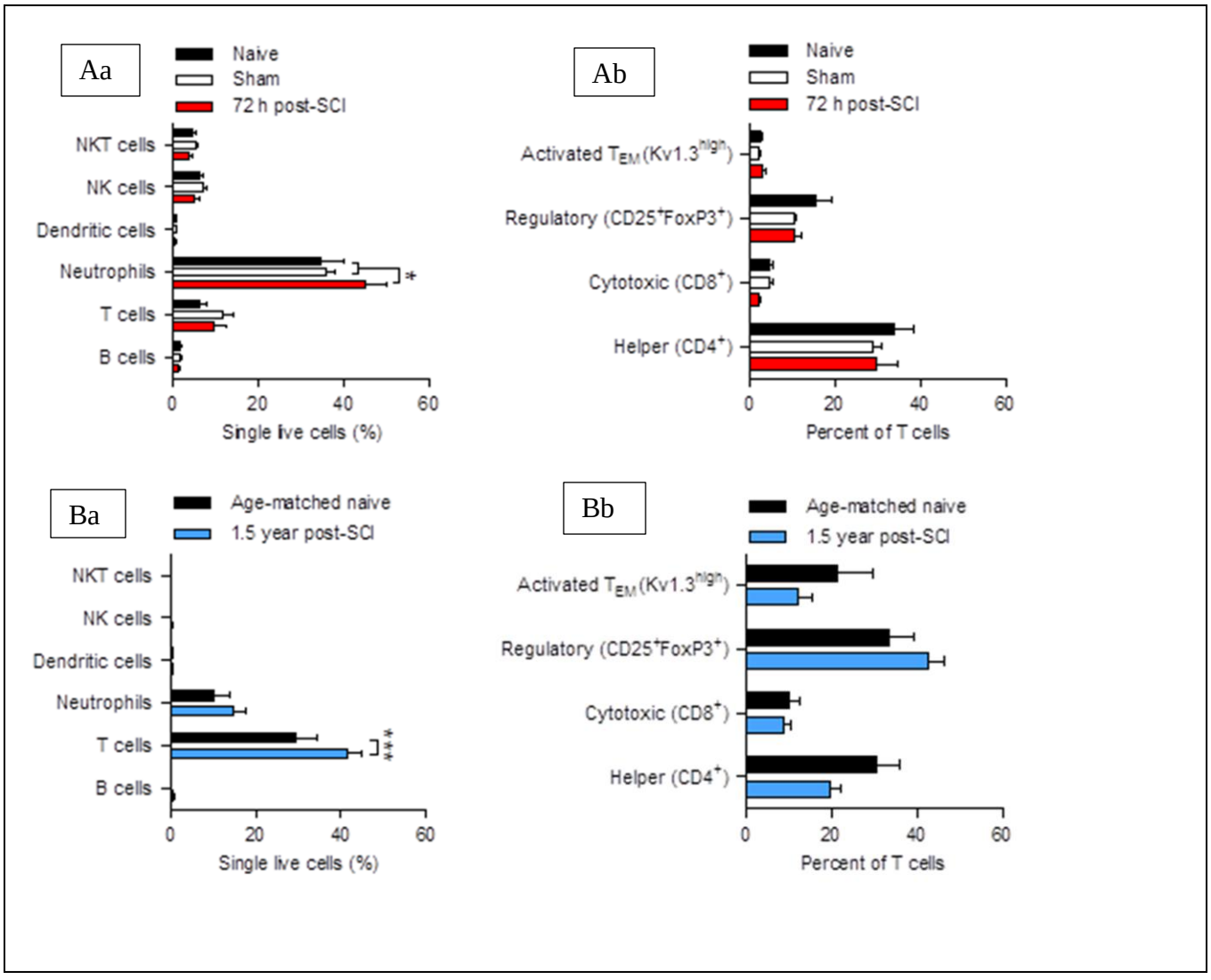


Figure8: Flow assisted cell sorting (FACS) of testes tissue for immune cell phenotypes:
Aa: Neutrophils were elevated 72 hours post SCI compared to sham and naïve(26.4% and 30.8% respectively, *$p<0.05$).
Ab: T-cell phenotypes were unchanged after injury.
Ba: T-cells were elevated 1.5 years post SCI (41.6%, ***$p<0.001$).
Bb. T-cell phenotypes were unchanged after injury.

## 4. KEY RESEARCH ACCOMPLISHMENTS:

- We have clearly demonstrated that SCI causes marked systemic and testicular changes in numerous metabolomic pathways. These include eicosanoid, lysolipid, bile acid and in markers of oxidative stress.
- The lysolipid pathway is of key importance since it can establish a positive-feedback loop between metabolic dysfunction and immune activation
- We are the first to identify changes in bile acid concentration within the testis following SCI.
- Some of the metabolic changes are reversed by 10 mg/kk licofelone administered prior to SCI.
- Although not as coherent as the changes in metabolites, we have quantitated pathway-based changes in gene expression.
- We have established an acute change in T-cell numbers within the testis in both an acute and a chronic model of SCI.

## 5. CONCLUSION:

Our studies, to date,confirm our original hypothesis that spinal cord injury results in pathological changes that negatively impact theblood testes barrier. We believe that the impact of our findings are nothing short of profound as, while it was previously known that SCI negatively impacted male fertility, potential reasons underlying such a pathologicalresponse have been relatively unexplored. We now know that SCI creates conditions that results in inflammation, oxidative stress and altered energy metabolism in the testes, and that, in many instances, these pathological conditions are maintained long after the initial trauma. Further, treatment with licofelone, a drug undergoing several phase III clinical trials for has been shown in our lab to effectively suppress inflammation in the damage spinal cord, attenuates many of the SCI-induced pathological events observed in the testes during the treatment period. Of greater significance, the attenuation of several SCI-dependent pathological changes to the metabolome appear to be maintained long after cessation of the treatment; suggesting that acute intervention with licofelone may preserve testicular function over chronic periods. If we are able to demonstrate that this involves the promotion of enhanced sperm production/survival, this would represent a step in the enhancement of SCI patient's overall quality of life.

**7. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

a. We are nearing completion of the first manuscript (Aim 1 of this proposal) and have begun on the second (Aim 2)

b. Abstracts and presentations:

University of Kentucky Neurotrauma Meeting
Title: “Spinal Cord Injury Causes Distinct Acute and Chronic Phases in the Testes and Blood Testes Barrier of a Sprague-Dawley Rat Model”
Ryan D. Fortune, David Loose, Christine Beeton, Raymond J. Grill
7/14/2015

University of Texas Research Retreat
Title “Blood Testis Barrier Disruption in Spinal Cord Injury”
Ryan Fortune, Raymond Grill, David Loose
Oral Presentation
4/21/15

8. **INVENTIONS, PATENTS AND LICENSES.** Although licofelone is a patented compound developed by Merckle GmbH with partners Alfa Wassermann and Lacer., we are working with the UTHealth Office on Technology transfer on a use patent.

9. **REPORTABLE OUTCOMES:** Nothing to report

10. **OTHER ACHIEVEMENTS:** Lt. Ryan Fortune passed his Ph.D. candidacy exam on 12/11/2014. His proposal and exam were based entirely on the studies outlined in this DOD proposal.

11. **REFERENCES:**

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12. **APPENDICES** I have attached an poster/abstract presented by Lt. Ryan in Kentucky. I have updated and attached the Quad chart.

**NOTE:**

**TRAINING OR FELLOWSHIP AWARDS:** Nothing to report

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

**QUAD CHARTS:** If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with **attachments [See Attached]**

**MARKING OF PROPRIETARY INFORMATION:** Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before

including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." DO NOT USE THE WORD " WHEN MARKING DOCUMENTS. See term entitled "Intangible Property – Data and Software Requirements" and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.

# The blood-testis barrier and male sexual dysfunction following spinal cord injury
# W81XWH-12-1-0481/SC1101873

PI: David S. Loose  Org: UTHealth  Award Amount $257,812

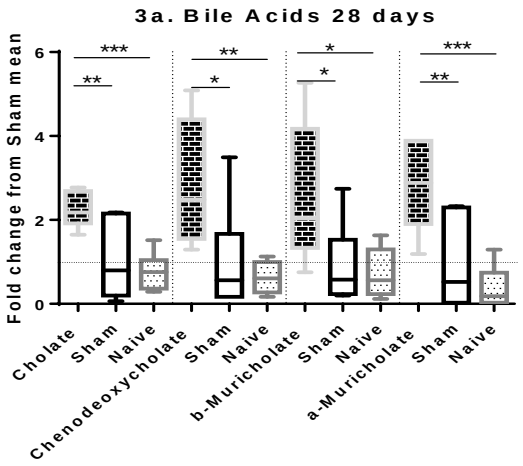
**Study/Product Aims**

**Specific Aim 1:** Explore the molecular and biochemical changes that occur to the BTB as a function of time following delivery of a clinically relevant spinal contusion injury

**Specific Aim 2:** Determine whether treatment with the new generation anti-inflammatory drug licofelone, can protect BTB integrity, biochemical changes, and enhance testicular function following SCI

**Approach:**

Utilize a clinically-relevant rat spinal contusion model of injury to to assess the early and chronic effects of the BTB as a mechanism of testicular dysfunction following spinal cord injury,




We have discovered that SCI causes a sustained (up to 90 days) accumulation of bile acids within the testis. By several different mechanisms, this accumulation could contribute to BTB damage after SCI

## Timeline and Cost

| Activities | CY 12/13 | 13/14 | 14/15 |
|---|---|---|---|
| Complete exps. Aims 1 and 2 | | | |
| Report on licofelone and bile acids, lysolipids | | | |
| Estimated Budget | $257,812 | $279,628 | NCE Carry-forward $132,264 |

## Goals/ Milestones

CY 12/13 – Initiate Aim 1 of project
CY 13/14 –Complete Aim 2
CY 14/15 –Develop appropriate statistical pipeline for data analysis. Complete data analysis.
of both aims. Determine the mechanism for bile acid accumulation in testis. Examine increased lysolipid accumulation following SCI in testis.
Assemble manuscripts on Aim1, draft of Aim2
Project Budget Expenditure to Date:$207, 669

# Spinal Cord Injury Causes Distinct Acute and Chronic Phases in the Testes and Blood Testes Barrier of a Sprague-Dawley Rat Model

Ryan D. Fortune[1], David Loose[1], Christine Beeton[2], Raymond J. Grill[1]

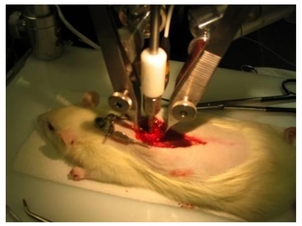
[1]Department of Integrative Biology & Pharmacology, University of Texas Health Science Center at Houston. [2]Department of Molecular Physiology and Biophysics, Baylor College of Medicine.

THE UNIVERSITY of TEXAS HEALTH SCIENCE CENTER AT HOUSTON

Mission Connect a program of TIRR Foundation

Spinal cord injury (SCI) results in devastating changes to almost all aspects of patient's lives. One symptom that remains largely uncharacterized is the development of male infertility. Erectile dysfunction and sensory loss can be significant, but are easily treated. SCI causes measureable pathology in the testis both acutely and chronically, leading to as of yet untreatable loss in sperm motility and viability. SCI has been shown previously to induce leukocytospermia, inflammatory cytokines, anti-sperm antibodies, and reactive oxygen species within the ejaculate. The Grill lab has shown that SCI causes a sustained disruption in the blood testis barrier, leading us to investigate further into the testes environment in SCI (Dulin et al, 2011). Using mRNA and metabolomics assessments, we have described the changes that occur within the testis over the course of injury. From 24 hours, 72 hours, 28 days, to 90 days post injury, the testis show an initial drop in normal sexual organ processes and initiation of an innate immune response that transitions to chronic low level immune activity. These data provide further evidence of both short- and long-term pathophysiology within the testes following SCI. In addition, these studies identify multiple, novel, avenues of exploration for treatment of male infertility in SCI.

## Methods

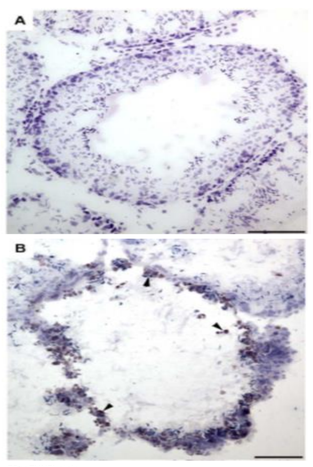


Adult, male, Sprague-Dawley rats received a spinal contusion injury at thoracic level 10 (T10) using 150 kdynes of force over a 1 second dwell time using the Infinite Horizons spinal impactor(Precision Systems and Instrumentation). Sham-injured subjects were anesthetized and received a spinal laminectomy at T10, but did not receive a spinal contusion injury. All injured subjects received appropriate post-operative care, including antibiotics, analgesic, hydration, and bladder care. To control for weaker injuries, subjects were examined using the non-invasive, Basso, Beattie, Bresnahan (BBB) open field locomotor test beginning on day 1 post-SCI. Briefly, this test scores rat hind limb function using a 21-point scale. 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.

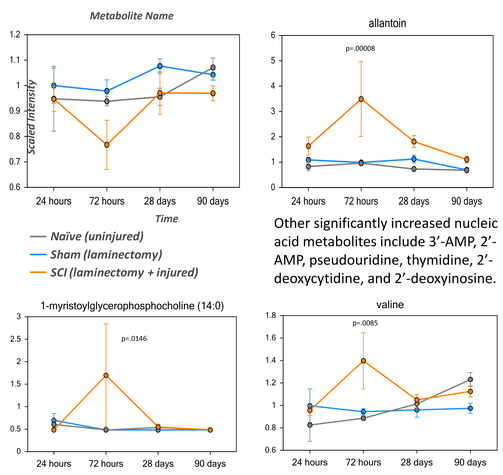
Cohorts of eight injured, sham, and naïve animals at the 24 hours, 72 hours, 28 days, and 90 days post-SCI were sacrificed and the testes immediately frozen for later analysis. To get a representative sample, testes were homogenized and separated into aliquots. We used a random sample from each animal's teste for RNA array analysis with Dr. David Loose and metabolomics assessment via Metabolon, who uses ultrahigh performance liquid chromatography and gas chromatography with mass spectrometry. Subsequent data was analyzed using ANOVA.

## Cell Death in the Testes at 72 Hours

Our data shows significant increases in multiple amino acids, lysolipids, and nucleic acid degradation products at 72 hours. These data support the findings by Dulin et al, 2011 via TUNNEL staining that shows large amounts of cell death within the seminiferous tubules 72 hours after SCI.




72hr TUNEL stain.
A) Naïve B) Injured.
Dulin JN, Grill RJ, et al.



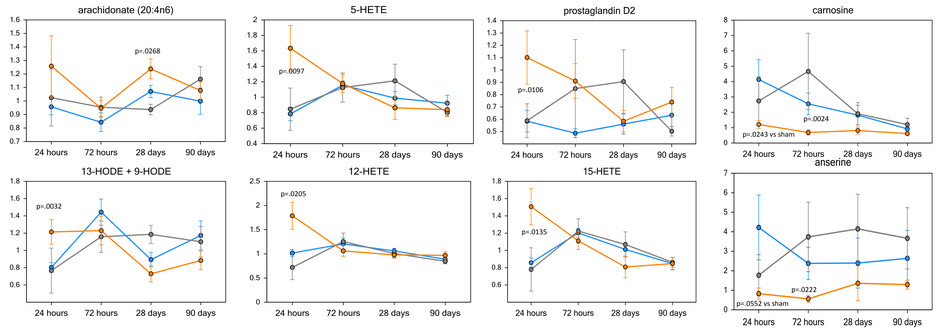

19 other lysolipids are elevated at 72 hours over sham, and 11 over naïve. P-values vary widely.

Other significantly increased nucleic acid metabolites include 3'-AMP, 2'-AMP, pseudouridine, thymidine, 2'-deoxycytidine, and 2'-deoxyinosine.

Representative graph of leucine, isoleucine, glutamine, lysine, methionine, histidine, alanine, and pseudouridine.

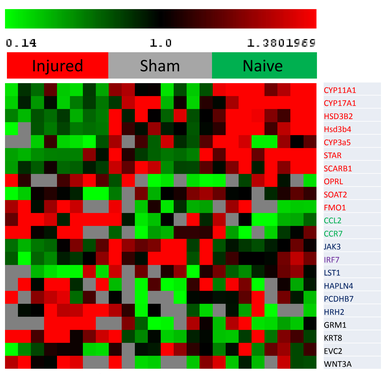
## Metabolomic Analysis: Inflammation and Oxidative Stress in the Testes following SCI

SCI elicited pro-inflammatory and pro-oxidative events in the testes. However, many of these changes were trends and not statistically significant. Changes detected via gross metabolomic analysis will require individual, more sensitive, validation

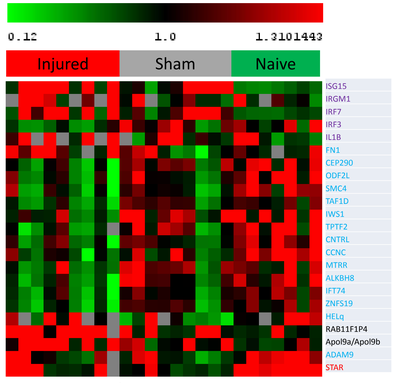



## Heatmaps of Significant RNA Changes

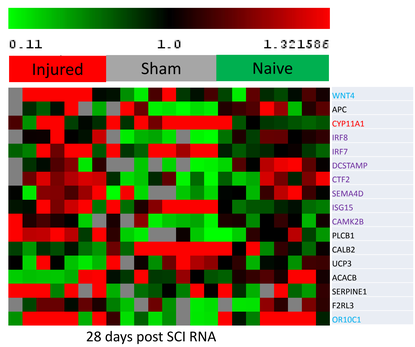
RNA array data succinctly shows the progression of SCI's effects in the testes. At 24 hours we have a decrease in 10 steroidogenesis genes and the induction of chemotactic and inflammatory genes. At 72 hours we see a greater induction of immune system genes and a decrease in a massive amount of cell cycle, DNA function, and sperm development genes. In the chronic time points we continue to see elevated expression of immune system and chemotactic genes.



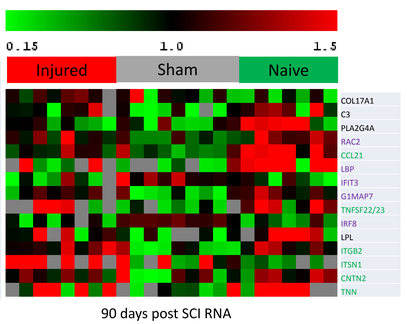

24 hours post SCI RNA




72 hours post SCI RNA



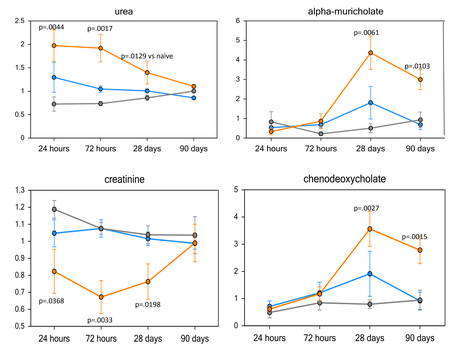

28 days post SCI RNA




90 days post SCI RNA

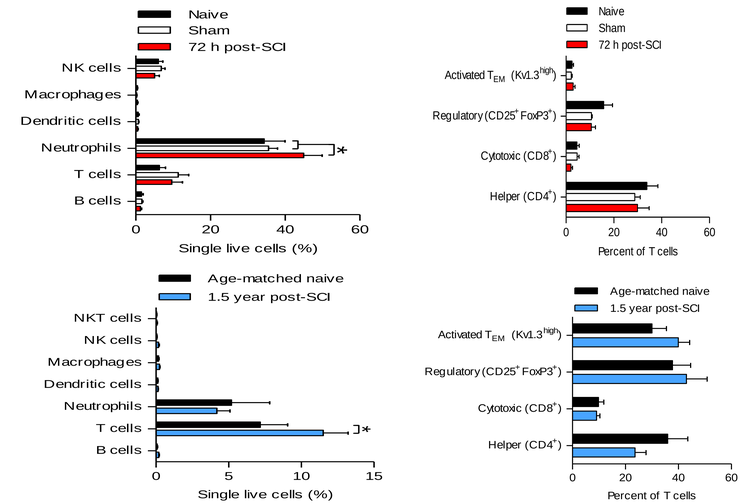
## Potential Systemic Effects

If localized to the testes, an alteration in the urea cycle at 72 hours could be further support for the hypothesis of SCI-dependent apoptosis and massive protein metabolism. However, the possibility of a systemic nitrogenous waste overload from either SCI induced mild liver toxicity or muscle tissue loss can not be ruled out. Similar changes in bile acid metabolism could indicate stronger evidence of altered liver metabolism in SCI.




## Cellular Response

Freshly harvested testes tissue was processed for immunophenotyping via florescence-activated cell sorting (FACS) for both acute (72 hour) and chronic (1.5 years) injured animals. Acutely, we see an infiltration of neutrophils, further indicating an acute inflammatory environment. Chronically, we see a continued immune response in the form of T cells, suggestive of an autoimmune response and further supporting the theory of SCI causing an immunological infertility.




It has been suggested that spinal trauma elicits both temporal and spatial changes occurring over a prolonged time frame. These data suggest that such SCI-induced changes are not restricted to the injured spinal cord, but can occur systemically; in this instance, within the testes. Since most of the inflammatory changes occurred at 24 hours, future experiments looking at the inflammatory environment will focus there, although oxidative stress continues chronically. It would be of considerable clinical interest/relevance to determine whether delayed or immediate treatment could reverse chronic pro-oxidative pathologies in the testes of chronically-injured subjects and return lost sexual function. Further evaluating the pathological changes in the testes through histological assessment, quantifying protein changes, and demonstrating any functional improvement in fertility parameters from targeted interventions are all planned subsequent next steps.

Acknowledgements: Jennifer Dulin, Ph.D.; Alissa Poteete, M.S.; UTHealth MD/PhD Program; UTHealth Graduate School of Biomedical Sciences: Cell and Regulatory Biology program; Department of Defense (SC110083); CCTS TL1 Program; Mission Connect.

Dulin JN, Moore ML, Gates KW, Queen JH, Grill RJ. Spinal cord injury causes sustained disruption of the blood-testis barrier in the rat. PloS one. 2011;6(1):e16456. PubMed PMID: 21298060.