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TITLE: Matrix Metalloproteinases as a Therapeutic Target to Improve Neurologic Recovery After Spinal Cord Injury

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**14. ABSTRACT**

**Purpose:** We are evaluating efficacy of GM6001, a matrix metalloproteinase (MMP) inhibitor in a murine model of spinal cord injury (UCSF) and in dogs (Texas A & M, TAMU) that sustain naturally occurring spinal cord injuries resulting from spontaneous intervertebral disk herniation (IVDH).

**Scope:** These studies focus on efficacy of GM6001 in the context of an optimal therapeutic window and dependency on injury severity, using clinically relevant neurologic and urologic outcome measures.

**Major findings:**

- Spinal cord injury (SCI) in mice resulted in marked injury severity-dependent changes in locomotor and bladder function.
- GM6001 has an extended therapeutic window. When given up to at least 8 hours post injury, GM6001 resulted in injury severity dependent efficacy in a murine model of SCI. GM6001 treatment resulted in both neurologic and urologic benefit after a moderate level of SCI. This recovery was associated with a decrease in spinal cord lesion volume, greater spared white matter volume, and decreased muscle hypertrophy as assessed by bladder wall thickness. We further show that gelatinase activity is increased in the bladder after SCI. Thus, improved bladder function may be due at least in part to a more direct effect of GM6001 on the bladder wall by attenuating aberrant remodeling remodeling. In contrast to these beneficial effects seen after a moderate level of SCI, GM6001 did not rescue locomotor or bladder function in mice with severe SCIs.
- Pharmacokinetic study of GM6001 in 10 dogs supports the short-term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial (Levine et al PLOS ONE, 2014, Appendix).
- Developed cystometry protocol on 10 uninjured dogs.
- Enrolled 17 dogs with acute IVDH-associated SCI into serial cystometry study. Dogs with SCI that were non ambulatory lacked normal voiding reflex and had increased residual bladder volume compared to control dogs. Residual volume was significantly higher immediately following injury compared to 42 days post-injury.
- Have currently enrolled 77/90 SCI dogs in a therapeutic trial evaluating GM6001+DMSO versus DMSO.

**Significance:** GM6001 is efficacious when the therapeutic window is extended up to at least 8 hours after murine SCI of moderate severity. However, a similar benefit is not seen after a more severe SCI. The extended therapeutic window offers greater opportunity for translation to the theater for those soldiers who have sustained moderate SCIs. The pharmacokinetics of GM6001 in the dog has been completed. We have now enrolled 77/90 into the clinical trial, which will evaluate the efficacy of GM6001 in a cohort of dogs with naturally occurring injuries. Validation of GM6001 in two species would be a powerful argument for advancing this drug to human clinical trials.

**15. SUBJECT TERMS**

spinal cord injury, matrix metalloproteinase inhibitor, intervertebral disk herniation, mouse, dogs, urologic function, neurologic function

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## Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusion.....	14
References.....	15
Appendices.....	16
Supporting Data.....	36
Quad Chart.....	66

## INTRODUCTION

The primary objectives of this research are to evaluate the efficacy of a general inhibitor of matrix metalloproteinases, GM6001, in both a murine model of spinal cord injury (SCI) and in dogs who have sustained a naturally occurring SCI resulting from the sudden rupture of an intervertebral disk. The study builds upon our earlier work in a murine model of SCI, which showed that GM6001 significantly improved neurologic outcome when given 3 hours post injury after a moderate SCI (1). Thus, the goal here was to determine if GM6001 is likewise efficacious if the window of therapeutic intervention is extended and if the injury is more severe. An additional objective was to determine if GM6001 improves bladder function. We tested the efficacy of GM6001 dissolved in DMSO when administered subcutaneously at 8 hours after either a moderate or severe injury in mice. GM6001 improved both neurologic and urologic outcomes in the moderately injured group but not in the more severely injured group. Findings from the mouse studies have served to inform the dog preclinical trial, where the focus has been on the initial categorization of dogs according to severity of injury and assessment of GM6001 efficacy as determined by both neurologic and urologic assessments.

Please note that each task, described below, is indicated in bold. The requested and approved changes are indicated in bold italics.

## BODY

### UCSF Site:

#### *Specific Aim 1*

#### **Task 1. Refine the therapeutic window for GM6001 in mice**

##### **1a. Obtain animal use protocol approval to study 165 mice (months 1-4)**

We received approval from the UCSF IACUC and ACURO to conduct these studies.

##### **1b. Compare neurologic recovery in 30 mice when GM6001 is initiated at 8 hours post injury. (months 5-6)**

For reasons described below (Texas A & M, Specific Aim 2, Task 1b) GM6001 was not available until month 8<sup>th</sup> of this project. In the interim, we refined our murine SCI model so that we could reproducibly generate both moderate and severe SCIs (as required for Specific Aim 1, Task 2a) and defined a series of abnormal urologic parameters that are present after SCI including uninhibited bladder contractions and changes in peak bladder pressure, bladder volume, and bladder weight. These experiments have provided a foundation for Specific Aim 1, Task 3A. Finally, beginning in month 10, we began Task 1b. Below summarizes our findings.

To confirm a reproducible, graded model of SCI, male, C57Bl/6 mice were subjected to a 2 gm weight dropped 5 cm (mild injury), a 2 gm weight dropped 7.5 cm (moderate injury), or a 3 gm weight dropped 5 cm (severe injury) onto the cord exposed at the T 9 vertebral level (**Figure 1**). Severity was defined based upon the Basso Mouse Scale (BMS) where a score of 0 indicates hindlimb paralysis and a score of 9 reflects normal hindlimb locomotor function. The more mildly injured animals showed scores of about 7.5 (frequent to consistent stepping and mostly coordinated in their locomotion). The moderately injured group scored about 3.5 (occasional plantar stepping). The severely injured animals scored about a 2 (hindlimb movement limited to extensive ankle movement). Representative urodynamic tracings (**Figure 2**), resulting from awake cystometry in mice subjected to mild, moderate, or severe SCIs, revealed distinct differences between injury severities with mild injuries showing qualitatively the most prominent bladder contractions relative to the moderately and severely injured group.

We initially addressed long-term urologic status after mild and moderate SCIs, focusing on 4 measures- namely uninhibited bladder contractions (UICs), residual urine, bladder weight, and bladder volume. As might be expected mild and moderate injuries resulted in more prominent uninhibited bladder

contractions than sham controls (Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test). **(Figure 3, Upper Panel)**. Residual urine **(Figure 3, Bottom Panel)** was similar between mild SCIs relative to shams but was elevated in the moderately injured group relative to shams (Kruskal-Wallis followed by Dunn’s Multiple Comparison Test). In contrast, peak voiding pressure showed no differences between mild and moderate SCIs and sham groups **(Figure 4)**. Finally, we analyzed bladder volumes and bladder weights using the same statistical approaches. Bladder volume remained unchanged, relative to shams, after a mild injury, whereas significantly increased after a moderate injury **(Figure 5)**. Bladder weight showed incremental increase in response to injury **(Figure 6)**.

In summary, we have successfully generated reproducible graded levels of injury severity based upon the BMS. Urologic status shows injury severity-dependent changes with UICs being most pronounced after a more mild injury than a moderate injury. We believe that reduced uninhibited bladder contractions with greater severity of SCI may reflect prolonged over distension of the bladder wall, which may damage the muscle layer. We further found greater residual urine, bladder volume and bladder weight in the moderate injured group relative to the sham controls. These findings suggest aberrant remodeling of the bladder wall, which could contribute to increased weight of this structure and reduced voiding.

We next evaluated the efficacy of GM6001 when give 8 hours after a moderate SCI, using a blinded, randomized experimental design with a priori exclusion criteria. A total of 25 C57Bl/6 adult male mice were subjected to a moderate SCI at T9. Two groups were studied: drug-treated (N= 12) or vehicle (carboxymethylcellulose)-treated (n= 13). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols. GM6001, (100mg/kg, i.p.) was given at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 12 GM6001 treated mice, a total of 5 were excluded from the study due to early death or early morbidity. Of 13 mice that were treated with vehicle, 4 were removed from study due to morbidity, 2 met exclusion criteria at 8 hours post injury, and 2 others had injury device malfunction. Thus, neurologic recovery was evaluated in N= 5 for the vehicle and N= 7 for drug. Two-way repeated measures ANOVA of the BMS score revealed the following: P= 0.58 for interaction, P<0.0001 for time, and P= 0.16 for treatment **(Figure 7)**.

We then evaluated improvement between the groups by comparing initial BMS scores at day 1 relative to final BMS scores at day 35 **(Figure 8)**. Based upon a Student T-test, the drug treated group shower greater improvement than the vehicle group (P= 0.025). Finally, since weight supported stepping is considered to be a very favorable outcome, we evaluated the percentage of mice that showed frequent stepping **(Figure 9)**. Statistical comparisons (2-way ANOVA) were done on percentages that were transformed into arcsin values. Approximately 60% of mice, treated with GM6001, showed frequent stepping whereas only 40% achieved that degree of recovery in the vehicle treated group. Based upon a 2-way ANOVA there was a significant effect of both treatment (P= 0.017) and time (P= 0.015). Taken together, despite the small group sizes, the behavioral data generally support improved recovery in mice treated with GM6001.

Finally, we have analyzed a cohort of bladders from these animals by awake cystometry. While we saw no differences in bladder volume, bladder weight or residual urine, the GM6001 treated group showed a significant reduction in uninhibited bladder contractions, one of the key features of dyssynergia **(Figure 10)**.

**After approval from the UCSF IACUC and ACURO, we began a 2<sup>nd</sup> set of studies to assess efficacy of GM6001 using dimethyl sulfoxide (DMSO) as the vehicle and a subcutaneous (s.c) route of delivery. This change in experimental design was prompted by the design of the TAMU dog study, which required DMSO and s.c. drug delivery.**

#### **Task 1b. Compare neurologic recovery in 30 mice when GM6001 dissolved in DMSO and injected subcutaneously is initiated at 8 hours post injury. (Months 12-14)**

We evaluated the efficacy of GM6001 dissolved in DMSO when given s.c at 8 hours after injury, using a randomized, blinded design with *a priori* exclusion criteria.

A total of 32 C57Bl/6 adult male mice were subjected to a moderate SCI at T9- and randomized to receive drug (n= 16) or vehicle (n= 16). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury (n=6) or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=3) or an absence of locomotor recovery at 14 days post injury (n=1). GM6001 in DMSO was given s.c. at 100mg/kg at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 3 met exclusion criteria at 8 hours post-injury, 1 at 14 days post-injury and 1 had device malfunction. Of 16 mice that were treated with vehicle, 1 was removed from study due to morbidity, and 3 met exclusion criteria at 8 hours post injury. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=11 for drug group (**Figure 11**).

Neurologic recovery was measured using the BMS. Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of BMS scores revealed the following: P= 0.2453 for interaction, P<0.0001 for time, and P= 0.0397 for treatment (**Figure 12A**). That is, all animals showed significant improvement in locomotor ability over time and there was a significant improvement in the drug-treated group on locomotor recovery. The average BMS score in the drug treated group at 35 days was 4.273 (indicating occasional plantar stepping), whereas the vehicle treated group had an average BMS score at 35 days of 3.292 (mice have the ability to plantar place, with or without support, but exhibit no plantar stepping). Based on these positive findings, this year, we further compared the percentage of mice between the two groups that had the ability to step at 35 days post-injury (**Figure 12B**). Seventy-three percent of the GM6001-treated mice had the ability to step as compared to only 33% in the DMSO-treated group (chi square analysis, p=0.029). Our findings demonstrate that GM6001 improves locomotor function in a model of moderate contusion injury, even when initiation of treatment is delayed by 8 hours post-injury.

This year, we further evaluated weight changes across groups (expressed as a percentage weight change over time) in moderately injured mice treated with DMSO or GM6001 [Two-way repeated measures ANOVA of body weight revealed the following: P= 0.0317 for interaction]. Since we observed significant interaction, we conducted Sidak's multiple comparisons test to analyze within treatment group changes. In response to injury, while all mice in the vehicle treated group lost weight over the first week (p<0.05 in vehicle treated group 3 day vs. pre-injury and p<0.001 in vehicle treated group 7 day vs. pre-injury), similar weight loss was not evident in the GM6001 treated group (p>0.05 in drug treated group at 3-28 days post-injury compared to pre-injury values). Furthermore, by 14 days all drug treated mice had gained weight and by 35 days they had gained significantly more weight as compared to pre-injury values (p<0.05). In contrast, the vehicle treated group did not show weight gain until day 21 (p<0.05) and at day 35, values were similar compared to pre-injury measures (p>0.05). Between treatment group analysis (Sidak's multiple comparisons test) showed that weight change at 14 days post-injury was significantly different between drug and vehicle treated groups (p<0.05) (**Figure 13**).

Taken together, the above findings serve as the 2<sup>nd</sup> independent study to validate GM6001 as a therapeutic for SCI. We have shown that when used in combination with the vehicle methylcellulose and given i.p. at a delayed time-point (8 hours post moderate injury), GM6001 improves long-term neurological recovery. In task 1b, we confirm neurological efficacy using a different route of administration (s.c.) and a different vehicle (DMSO). Using body weight as an index of overall health, we also found that mice treated with the drug were "healthier" post injury as compared to vehicle treated group.

### **Task 1c. Compare neurologic recovery in 30 mice when GM6001 is initiated at 6 or 12 hours depending on the results 1b. (Months 7-8)**

***We received permission from the Grants Office's Representative to eliminate task 1c, so that we could repeat task 1b, testing a subcutaneous route of administration.*** Please see above for the repeat dosing at 8 h with DMSO as vehicle and subcutaneous route of injection for moderate level injury severity.

## **Task 2. Determine if GM6001 will be efficacious after a more severe SCI in mice.**

### **2a. Compare neurologic recovery in 30 mice after a severe SCI. (Months 16-18)**

We evaluated the efficacy of GM6001, dissolved in DMSO, when given subcutaneously at 8 hours after severe SCI, using a blinded, randomized design with a priori exclusion criteria. A total of 31 C57Bl/6 adult male mice were subjected to a severe contusion injury at T9- Drug (N= 16) and vehicle (N= 15). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=2). GM6001 (100 mg/kg), dissolved in 99% DMSO, was given s.c. at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 1 had device malfunction and 3 met the morbidity criteria. Of 15 mice that were treated with vehicle, 2 were removed from study due to morbidity. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=13 for drug group.

Neurologic recovery was measured using the BMS. Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of the BMS score revealed the following: P= 0.9468 for interaction, P<0.0001 for time, and P= 0.7530 for treatment (**Figure 14**). That is, all animals showed significant improvement in locomotor ability over time but there was no effect of drug on motor recovery. The average BMS score in both groups at 35 days was 2.6, which means extensive ankle movement.

Body weights were evaluated over time in severely injured mice treated with DMSO or GM6001. All animals gained weight with time, but there was no effect of drug treatment at this level of injury (Two-way repeated measures ANOVA: P= 0.2662 for interaction, P<0.0001 for time, and P= 0.5362 for treatment) (**Figure 15**).

## **Task 3. Determine if GM6001, when optimally delivered, will improve bladder function in mice.**

### **3a. Compare urologic function in spinal cord injured mice treated with either vehicle or GM6001 after moderate and severe SCIs. (Months 14-20)**

At 5 weeks after a moderate or severe SCI in mice treated with GM6001 or DMSO, a PE10 catheter was implanted into the bladder dome and 2-3 days later awake cystometry was conducted. The following parameters were measured: time to first void, uninhibited bladder contractions/cycle, residual urine and voiding efficiency. Time to first void was defined as the period between when the saline infusion was initiated to the first release of fluid from the urethral meatus. To measure uninhibited bladder contractions (UIC), animals were first exposed to an equilibration period of 30 minutes during which the bladder was filled with saline. After this equilibration period, UICs were evaluated. UIC's were defined as rhythmic intravesical pressure rises (>5 cm H<sub>2</sub>O from baseline pressure) without a release of fluid from the urethra using three representative voiding cycles. The numbers of non-voiding UICs per voiding cycle were determined. Residual urine was measured after the last void. The infusion was stopped and residual volume was determined by withdrawing the residual saline through the intravesical catheter. Voiding efficiency was calculated as (total infused volume – residual urine)/ total volume \* 100.

Cystometry results after moderate SCI. In the intact spinal cord, long descending fiber tracts from the midbrain coordinate the activity of the detrusor muscle and the urethral sphincter, i.e. the detrusor muscle is relaxed while the sphincter muscle is contracted to allow for filling of the bladder, while voiding is characterized by relaxation of the urethral sphincter and contraction of the detrusor muscle. After SCI, input from the brainstem is partially lost. As such, simultaneous contractions of detrusor and sphincter emerge. In cystometry, detrusor sphincter dyssynergia (DSD) is in part represented by detrusor contractions against a closed urethral sphincter without release of fluid (i.e. uninhibited bladder contractions). In addition to the parameters defined above, this year we also completed a detailed cystometry analysis that also included baseline pressure, threshold pressure, opening pressure, maximum pressure, intermicturition interval, duration of voiding, and amplitude of non-voiding contractions. We include here the data from uninjured mice that served as baseline controls and show the development of bladder dysfunction in injured mice. Furthermore using several parameters measured during awake cystometry, we show that GM6001 ameliorates the bladder dysfunction. Also included are cystometry tracings from representative mice from each of the groups. First, cystometry outcome values were established in a group of nine uninjured mice that served as baseline controls (**Table 1**). This

cohort demonstrated normal bladder function with short voiding duration and a small bladder capacity, as signified by the short time to first void. In addition, these animals showed a near perfect voiding efficiency with low intravesical post-void baseline values and minimal residual urine. An insignificant number of non-voiding contractions (NVCs) with small NVC amplitude were seen. All these parameters were altered in response to injury (compared to vehicle treated group, **Table 1**). GM6001 treatment ameliorates bladder dysfunction as compared to the DMSO-treated group by significantly increasing voiding efficiency (Mann-Whitney test,  $p=0.010$ ) and thus decreasing post-void baseline pressure (Mann-Whitney test,  $p=0.036$ ) and residual urine (unpaired t test,  $t(20)=4.171$ ,  $p=0.0005$ ) (**Figure 16, Table 1**). The time to first void/ leak significantly decreased (unpaired t test,  $t(20)=2.836$ ,  $p=0.010$ ), indicating that the bladder is less distended and hence the bladder capacity is lower. In addition, the number of NVCs per voiding cycle (Mann-Whitney test,  $p=0.008$ ) and the amplitude (unpaired t test,  $t(20)=2.307$ ,  $p=0.032$ ) of NVCs was significantly decreased. **Figures 16E-G** show representative cystometry recordings for each of the study groups. NOTE: All the statistical analyses were run after checking data for normal distribution, data that were not normally distributed were compared by non-parametric test.

SCI-induced neurogenic bladder dysfunction leads to increased bladder weight and bladder wall thickness as a result of smooth muscle hypertrophy. For this study, bladder weight was normalized to body weight. The DMSO-treated group had greater bladder weight to body weight ratios than uninjured mice (unpaired t test,  $t(19)=6.812$ ,  $p<0.0001$ ). The bladder weight to body weight ratios were not significantly different between both treatment groups; however, there was a strong trend towards lower bladder to body weight ratios in the GM6001-treated group (Mann-Whitney test,  $p=0.0509$ ) (**Figure 17A**). Since increased bladder weight may be a result of detrusor muscle hypertrophy, we next examined the bladder wall. The detrusor muscle thickness was assessed and compared separately in three regions: the bladder base, body, and dome. Comparing the DMSO-treated group to uninjured animals, there was a significant increase in detrusor muscle thickness at the base and body of the bladder (unpaired t tests; base:  $t(9)=7.523$ ,  $p<0.0001$ ; body:  $t(9)=6.402$ ,  $p=0.0001$ ; dome:  $t(9)=1.969$ ,  $p=0.0805$ ). Comparing the two treatment groups, the detrusor muscle diameter was less in each of the three regions in GM6001-treated mice as compared to DMSO-treated mice (unpaired t tests, base:  $t(10)=3.191$ ,  $p=0.010$ ; body:  $t(10)=4.729$ ,  $p=0.0008$ ; dome:  $t(10)=3.041$ ,  $p=0.013$ ) (**Figure 17B**). These findings suggest that GM6001 attenuates abnormal remodeling of the bladder wall after SCI, which mirrors the preservation of bladder function by the cystometry findings. **Figures 17C-H** show representative sections for each bladder region that is in each of the treatment groups.

Assessment of the ratio of collagen type III to collagen type I within the bladder detrusor muscle showed that there was a 40% loss in response to injury as compared to uninjured mice in the bladder base region (unpaired t-test,  $t(9)=3.203$ ,  $p=0.011$ ), a 38% loss in the body region (unpaired t-test,  $t(9)=3.38$ ,  $p=0.008$ ), and a 25% loss in the dome region (unpaired t-test,  $t(9)=2.089$ ,  $p=0.066$ ). This drop in collagen type III to collagen type I ratio was not alleviated by GM6001 treatment (base:  $t(10)=0.183$ ,  $p=0.859$ ; body:  $t(10)=0.615$ ,  $p=0.553$ ; dome:  $t(10)=0.383$ ,  $p=0.710$ ) (**Figure 18A**). Representative images from the bladder body region from uninjured, DMSO-treated, and GM6001-treated mice show the distribution of collagen type I and type III in the detrusor muscle (**Figures 18B-M**).

Given the favorable changes in bladder function, based upon awake cystometry in the GM6001-treated group, we considered the possibility that this may be attributed to a direct effect of the drug on the bladder wall. To address this hypothesis, we determined if gelatinase activity is expressed in the bladder wall. Using *in situ* zymography, we found that activity is apparent in the urothelium of the sham operated animals and that this activity appears to increase in a patchy distribution in the urothelium over time up to at least 7 days post injury (**Figure 19**). Thus, as GM6001, a nonspecific gelatinase inhibitor, is given over the first 3 days post injury there is opportunity to block this early rise in activity. Such blockade could alter the aberrant wound healing that contributes to bladder dysfunction after spinal cord injury.

Cystometry results after severe SCI. We have found that severe SCI results in a characteristic leakiness of the bladder, which likely results from a highly over distended bladder that with time disrupts the detrusor muscle.

Drug treatment did not affect urine retention (Unpaired two-tailed T- test,  $p=0.6038$ ), time to first void (Unpaired two-tailed T- test,  $p=0.9573$ ), or voiding efficacy (Mann Whitney test,  $p=0.4401$ ) (**Figure 20**). However, it did result in a decreased number of UICs per cycle (Unpaired two-tailed T- test,  $p=0.0210$ ). We believe that reduced UICs may reflect prolonged over distension of the bladder wall, which may

either damage the muscle layer or result in aberrant remodeling such that the muscle wall has reduced capability of contracting. Finally, we found that increased weight in the bladder was not attenuated by GM6001 (Unpaired two-tailed t-test,  $p=0.1390$ ) (**Figure 21**).

Collectively, these findings support the position that GM6001 shows injury severity dependent efficacy whereby moderate levels of SCI show both neurological and urological recovery.

#### **Task 4. Analysis of lesion epicenter and serotonergic fiber tracks caudal to a SCI in mice.**

##### **4a. Perfuse animals with fixative, remove the cords, and stain with Eriochrome cyanine or immunostain for serotonergic fiber tracks. (Months 5-24)**

All animals thus far studied have been perfused with fixative, cryoprotected, frozen and sectioned. We have stained and analyzed spinal cord sections from all the mice from moderate and severe injured groups with eriochrome cyanine for measurement of residual white matter and lesion volume. After fixation and cryopreservation in sucrose, 1.5 cm of the cord, encompassing the epicenter was extracted. The cord was transected caudal and rostral with the epicenter in the middle, such that length of each segment is 5 mm. All three 5 mm long pieces were placed rostral to caudal in a square cryostat mold, flush against the right side of the mold and frozen in cryopreservation medium for sectioning. Serial 20  $\mu\text{m}$  coronal sections were collected on 50 sequential Super-Frost slides, resulting in 15 sections per slide (5 per segment). An eriochrome cyanine (EC) staining protocol targeting myelin was developed, and every tenth slide was then stained. The section with the least amount of spared white matter was designated the lesion epicenter.

We have also stained half of the moderately injured mice to analyze serotonergic fiber tracks and developed stereological method of analysis for quantitation of fiber length.

##### **4b. Quantify residual white matter and serotonergic fiber tracks caudal to injury. (Months 12-32)**

The Eriochrome Cyanine stained spinal cord sections were analyzed in Stereo Investigator using the Cavalieri method. For analysis, a total of 15 sections per animal, at an interval of 200  $\mu\text{m}$  apart were measured with epicenter section in the center, analysis was performed 1.4 mm in both rostral and caudal directions. The estimated total cord, spared white matter, and lesion volumes were determined. Volumetric analysis for the axial distribution of the lesion and spared white matter across the 3 mm segment was obtained and the percentage of spared white matter and lesion size relative to the total cord were calculated.

We analyzed the axial distribution of the lesion along a 3 mm segment with epicenter in the middle. To account for spinal cord size variability, lesion volume was normalized to cord volume and expressed as percentage lesion volume. For the moderately injured group, two-way repeated measures ANOVA of percentage lesion volume revealed the following:  $P=0.0714$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment (**Figure 22A**). That is, the size of the lesion decreased at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Between groups comparisons (Sidak's multiple comparisons test) revealed significant effect of drug at 400 and 600  $\mu\text{m}$  from epicenter. This shows that drug treated mice had overall smaller lesions as compared to the vehicle treated group.

We next analyzed the axial distribution of spared white matter in the moderately injured mice treated with either GM6001 or vehicle. Two-way repeated measures ANOVA of percentage spared white matter revealed the following:  $P=0.1001$  for interaction,  $P<0.0001$  for distance, and  $P=0.0001$  for treatment (**Figure 22B**). That is, there was greater amount of spared white matter at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Between groups comparisons (Sidak's multiple comparisons test) revealed significant effect of drug at 400 and 600  $\mu\text{m}$  from epicenter. **Figures 22C and 22D** are reconstructions of the spinal cords of representative animal from the vehicle and drug treated group to demonstrate lesion (**Figures 22E and 22F**) and gray matter volumes (**Figures 22G and 22H**). This positive effect of drug on lesion volume and spared white matter was not apparent in the severely injured group (**Figures 23 and 24**).

We developed a method for unbiased stereological analysis to measure serotonergic fiber length, but due to limited number of sections that were available, we got a very high coefficient of error for our

analysis and will not be able to do this type of analysis for the moderately injured group that showed an effect of drug treatment.

**4c. Statistically analyze data. (Months 30-36).**

We have all completed statistical analyses of data that were collected at UCSF.

**We are working on a manuscript on the murine spinal cord injury model to be submitted to Experimental Neurology for publication. In addition we are collaborating with group at Texas A & M to analyze the dog bladder cystometry data. Here we show the first analysis for residual urine comparisons in dogs enrolled in Phase 1 of the study (Figure 3, Supporting data from Texas A & M).**

**REPORT FROM Texas A & M**

**Specific Aims 2-3**

**Task 4. Measure MMPs in CSF in dogs**

**4a. Collect serum from dogs, conduct fluorogenic assays, and analyze data in approximately 125 dogs. (months 12-30)**

Our group entered into a collaboration with Dr. Michael Heller at UC San Diego. Through that work, we have now been able to demonstrate that GM6001 has *in vitro* activity against MMP-2 and MMP-9 at concentrations that approximate those achieved in dog plasma (40-80 ng/mL) 72 hours following a single 100 mg/kg dose subcutaneously. These data together with the complete canine pharmacokinetics support the relevance of this strategy and also suggest that single dosing is likely adequate to achieve reasonably sustained MMP inhibition in dogs. We have already used these assays to analyze canine CSF and serum from dogs with SCI that were administered GM6001 in an NIH funded study. We have now been able to show serum elevation of MMP-2/MMP-9 following SCI and *in vitro* inhibition of serum MMP-2/MMP-9 3 days following delivery of a single 100 mg/kg dose of GM6001. Moreover, this novel assay will serve as a complementary approach to work at UCSF to address MMP activity in CSF using fluorogenic assays.

Additionally, we have begun a collaboration with Mayland Chang at University of Notre Dame to more critically examine metalloproteinase and ADAM activation following SCI. Using banked CSF from dogs with SCI not included in the DoD-funded trial, we have been able to show that the only active MMP detected is MMP-9 and the only active ADAM is ADAM-7 (see supporting data, Figure 1).

**BODY**

**Texas A & M Site:**

On 11/6/11 a sub-award agreement between UCSF and Texas A&M University (TAMU) was reached, permitting the ordering of materials to begin work at TAMU. Approvals for key purchases including urodynamics equipment (Laborie Goby), study drug (GM6001, SAI Advantium, India), and pharmacokinetic analysis (KCAS LLC, Kansas, USA) were obtained by mid-December 2011. Specific Aim 2, Task 1 was completed in July 2012, on schedule. Dog enrollment for Specific Aim 2, Task 2 and Specific Aim 3, Task 2 began in November 2012. Dog enrollment for specific Aim 3 task 1 began in July 2012.

Findings, partially supported by this funding, have been submitted for consideration for publication in *Brain*. (Appendix)

## **Specific Aim 2**

### **Task 1. Study of pharmacokinetics of GM6001 in 10 purpose bred dogs (months 1-12)**

#### **1a. Obtain animal use protocol approval at Texas A&M University (months 1-4)**

Animal use protocols were approved at TAMU on 8/12/11

#### **1b. Order GM6001 drug (months 1-4)**

We were able to obtain permission through our Office of Sponsored Research (OSR) to order GM-6001 in mid-December 2011. A contract was executed with SAI Advantium and processing of the drug began in early January 2012. On March 22 2012, production of 110 g of GM-6001 at HPLC > 98% was completed. The drug was received at TAMU on 4/7/12. Unfortunately, delays associated with obtaining a sub-contract agreement, executing a contract with SAI, and actual drug production resulted in GM 6001 being available in month 8 of the study as opposed to the planned month 4.

#### **1c. Order 10 purpose bred dogs (month 4)**

Beagle-like dogs were obtained through the TAMU comparative medicine program in late April 2012, following the availability of GM6001. Dog purchase was delayed as a result of the delays in obtaining GM6001.

#### **1d. Receive purpose bred dogs, allow for acclimatization (month 5)**

Dogs were received and acclimatized by early May 2012.

#### **1e. Perform physical examination and obtain complete blood count, chemistry, and urinalysis (month 5.5-6)**

#### **1f. Anesthetize dogs, place jugular catheters, and deliver GM6001 as a single 100 mg/kg subcutaneous dose (5 dogs) and two 100 mg/kg doses separated by 12 hours (5 dogs) (month 5.5-6)**

(Figure 2, In Supporting Data)

#### **1g. Serial serum acquisition (month 5.5-6)**

Objectives 1e-1g were accomplished in mid-May 2012.

#### **1h. Samples stored at -80C and shipped to PharmCats for gas chromatography (month 6)**

KCAS was selected as an alternative vendor for pharmacologic studies as they had a lower bid than PharmCats and more rapid turn-around. Samples were shipped to KCAS in mid-May 2012.

#### **1i. Samples processed by gas chromatography at PharmCats (months 6-10)**

By mid-June 2012, KCAS generated pharmacokinetic data from dogs. These data were available within the anticipated time frame.

#### **1j. Dr. Fajt to analyze pharmacokinetic data (months 10-12). Dr. Fajt will calculate drug elimination half life, peak drug concentration, time to peak concentration, area under the curve, and absorption half life. If serum levels remain elevated beyond the target duration of <5 in the single dose group, drug dose in the IVDH study population will be appropriately adjusted. If serum GM6001 levels are not present for at least 3 days with a single dose protocol, we will consider a 2 dose paradigm in the IVDH study population.**

Dr. Fajt received pharmacokinetic data in mid-June 2012 and completed her analysis by July 1<sup>st</sup> 2012, 2 months ahead of the SOW schedule.

**Summary Task 1:** Delivery of GM6001 was accomplished in 10 purpose bred dogs. All dogs were clinically normal prior to drug administration based on physical examination, neurological examination, complete blood count, serum biochemistry, urinalysis, and CSF analysis. There were few adverse events associated with drug delivery: 10/10 dogs exhibited mild regional hyperesthesia at the delivery site which abated within 1-3 minutes and 10/10 dogs developed transient swelling at the delivery site. Swelling at the delivery site was 2-5 cm in diameter and at the time of the conclusion of the study had

decreased in size to 1-3 cm. We have recognized similar swellings in a 4 dog safety study of GM6001 our group previously completed and in 35 dogs that have been administered the drug at 100 mg/kg S.C.

Analysis of the pharmacokinetics of GM6001 delivered S.C. in dogs suggests a rapid absorption and initial elimination followed by long-elimination half-life ("flip-flop phenomenon"). This pattern required a non-compartmental analysis. GM6001 was detected in plasma at the earliest time point following delivery (5 minutes) and had a mean time to maximal concentration (T<sub>max</sub>) of 0.7 hours (S.D. +/- 1.3 hours). The mean maximal concentration (C<sub>max</sub>) was 1370 ng/mL (S.D. +/- 361 ng/mL). The calculated elimination half-life for a single dose is 524 hours (S.D. +/- 428 hours). The mean concentration of GM6001 following single dose delivery was 80 ng/mL (S.D. +/- 20 ng/mL) at 96 hours. These data have been published in Levine et al PLoS ONE 2014.

## **Task 2. Compare motor recovery in dogs with IVDH (intervertebral disk herniation) associated SCI that receive saline placebo, DMSO vehicle, or GM 6001 (months 1-36)**

### **2a. Obtain animal use protocol approval at Texas A&M University (months 1-4)**

Animal use protocols were approved at TAMU on 8/12/11

### **2b. Obtain Clinical Research Review Committee approval (months 1-4)**

Clinical Research Review Committee approval was granted at TAMU on 8/12/11

### **2c. Advertise clinical study via electronic brochures (months 6-18)**

In February 2012 UCSF and TAMU began efforts to announce the study to media in order to develop interest in the general public. Stories were featured in the NY Times, ABC News, MS NBC, and on the Today Show website describing this unique collaboration. On June 1 2012 TAMU began efforts to advertise the study to our referring veterinarian population. These efforts included: 1) communications sent to a listserve ("Texasvets") comprised of veterinarians in July 2012, November 2012, February 2013, June 2013, August 2013, and February 2014 and 2) mailing electronic PDF brochures to referring veterinarians in November 2012, March 2013, and March 2014.

### **2d. Advertise clinical study via referring veterinarian seminars (months 6-18)**

We have held continuing education events that have featured this study at Veterinary Medical Associations in Montgomery County (September 2012 and February 2013), Washington County (January 2013), and Brazos County (December 2012 and October 2013).

### **2e. Advertise clinical study via print media (months 6-18)**

The study was featured in our College's news magazine, "CVM Today" in October 2012 and again in summer 2014.

### **2f. Development of standardized databases (months 8-10)**

Databases for the study were developed between January and February 2012. Data entry began at the start of enrollment.

### **2g. Enrollment of dogs with IVDH (months 13-30)**

Enrollment was initiated in November 2012 (month 15). Currently (month 36), 77/90 dogs have been enrolled. The actual enrollment is a few months behind the projected Sept 1 2014 completion date for this study. This has resulted in a request for a 1 year no cost extension

**Summary Task 2:** Just prior to the beginning of enrollment, the study design was altered so that the saline control was eliminated; ACURO was contacted concerning this modification. This was done to enhance our power to detect differences between the DMSO group and GM6001 group. Enrollment has been progressing smoothly, although it we are slightly behind reaching our benchmarks (77/90 dogs)

## **Specific Aim 3**

### **Task 1. Compare urodynamic measures in purpose bred dogs and dogs with IVDH (months 1-12)**

#### **1a. Obtain animal use protocol approval at Texas A&M University (months 1-12)**

Obtained 8/12/2011

#### **1b. Obtain Clinical Research Review Committee approval (months 1-4)**

Completed 8/12/2011

#### **1c. Order urodynamic equipment (month 1-4)**

Urodynamic equipment was ordered in mid-December 2011 and arrived at TAMU in February 2011

**1d. Order purpose bred dogs (month 4)**

Purpose bred dogs were ordered in April 2012. As stated previously, this order was delayed due to delays in the production of GM6001.

**1e. Receive purpose bred dogs, allow for acclimatization (month 5)**

Purpose bred dogs were obtained and acclimatized. The acclimatization process was completed in early May 2012.

**1f. Perform urodynamic studies in purpose bred dogs (month 6).**

Ten purpose bred dogs will be utilized. Dogs will be sedated and will have a dual lumen urinary catheter, rectal catheter, and perineal volume following micturition will be recorded and voided volume and voiding efficiency will be calculated. Baseline pressure (vesical pressure after voiding), maximal voiding pressure (maximal vesical pressure during micturition) or leak point pressure (maximal vesical pressure in an animal without voluntary voiding, prior to urine overflow), voiding duration, and voiding interval (the frequency of voiding during filling) will be determined. The number of uninhibited bladder contractions will be recorded on each study. Finally, the timing of external anal sphincter EMG activity in relation to the voiding will be examined. Dogs with phasic contractions of the external anal sphincter during voiding that exhibit subsequent interrupted urine flow and elevated voiding pressure will be classified as having reflex dyssynergia. Voided volume and voiding efficiency will be calculated. Bladder ultrasound will be performed in all dogs immediately following voiding on the same days as urodynamic studies to determine residual urine volume. Animals will be placed in cages and provided water for 8 hours. Upon removal from the cage, dogs will be walked in a large outdoor area and allowed to voluntarily void without manual assistance. Immediately following voiding, an ultrasound machine will be used to measure transverse depth, transverse width, longitudinal length, longitudinal depth, and longitudinal width of the bladder. These measurements will be utilized to calculate residual bladder volume as has been previously described in dogs with IVDH.

Ten healthy beagle-like dogs were utilized to generate experimental data in early May 2012. At the outset of this study, it became clear that Ketamine sedation would be inadequate as it produces excessive spasticity in dogs, which may interfere with the assessment of urodynamic measures. We modified our AUP and received ACURO approval to utilize dexmedetomidine as an alternative sedative agent.

**1g. Perform urodynamic studies in dogs with IVDH (months 6-12). A total of 25 dogs not enrolled in the GM6001 delivery trial will be utilized. Measurements will be performed at admission, and 3 days, 7 days, and 42 days following IVDH surgery. The same cystometric data as outlined in 1f will be recorded.**

On June 1 2012 we opened enrollment to this clinical arm of the study. We have slightly modified inclusion criteria so that dogs lacking deep nociception are excluded due to the severity of the injury. Dogs lacking deep nociception have represented a small fraction (20%) of our IVDH associated SCI caseload and we did not believe that in a 25 dog population of dogs lacking deep nociception to make meaningful conclusions relative to typical urodynamic profile.

To date (9/1/13) we have enrolled 17 of the 25 dogs for this study, 16/17 of which have completed all time points. Data have been exported electronically to the Noble lab at UCSF for measurement of critical study variables. Analysis will occur when all animals have been enrolled.

**1h. Dr. Fosgate will analyze data (month 12). Descriptive statistics will be calculated for all urodynamic outcome measures in dogs. Evaluation of descriptive statistics and the Anderson-Darling test will be used to assess the normality assumption. The coefficient of variation will be calculated for the 3 replicates within unaffected dogs to assess the repeatability of the urodynamic measures. Urodynamic measures will be compared between normal and non-trail IVDH dogs at presentation using Student t tests for normally distributed variables and Mann-Whitney U tests otherwise. Outcome measures will be ranked based on the ability to distinguish normal versus affected dogs using scatter plots of standardized values and P values from the statistical comparisons. The outcome measure that best distinguishes affected from normal dogs**

will be used for subsequent statistical analyses. The most efficient urodynamic measure will also be compared between normal and IVDH-affected dogs at the 42 day recheck evaluation. Repeated measures ANOVA will be used to identify factors associated with improvement of urodynamic measures over time within the IVDH-affected dogs. Predictors that will be evaluated include time from injury until surgery, severity of injury at presentation, surgery duration, and age. Analyses will be performed in commercially available programs and results will be interpreted at the 5% level of significance.

Formal analysis of these data has not occurred to date as enrollment has not been completed.

**Summary Specific Aim 3, Task 1:** Cystometric measures and post-voiding bladder ultrasound was obtained in 10 Beagle-like dogs with few complications. In 2/10 dogs, hematuria was present following cystometry, but resolved within 24 hours. No dogs developed significant systemic complications as a result of cystometry.

To date, 17 dogs with IVDH-associated SCI have been enrolled in the serial cystometric study. At the time of cystometry, all dogs have been non-ambulatory. A control population of 10 healthy dogs has also been examined using cystometry techniques. We have performed a preliminary analysis comparing residual urine volume in dogs with SCI to control dogs. The analysis suggests that dogs with SCI have significantly larger residual volume at the time of injury compared with control dogs. Additionally, at day 42 post-SCI, residual urine volume is significantly lower than 0-48 hours following injury (Figure 3). Following the completion of this study and the clinical trial, a wider spectrum of cystometric variables will be assessed including baseline pressure, leak point pressure, bladder capacity, and voiding efficiency.

Figures 4 and 5 are cystometrograms obtained at the time of admission and 3 days following admission in one dog that demonstrate patterns seen during injury and recovery. At admission, there was an absence of voiding reflex, leak point pressure of 12 cm H<sub>2</sub>O, 75 mL of residual urine volume, and evidence of un-inhibited bladder contractions (Marked "U"). At 3 days following injury there was an absence of un-inhibited bladder contractions, a maximal voiding pressure of 40 cm H<sub>2</sub>O, voided volume of 78 mL with 28 mL of residual urine, and a baseline pressure following voiding of 8 cm H<sub>2</sub>O.

**Task 2: Compare urodynamic measures in dogs with IVDH enrolled in the GM 6001 delivery trial (months 13-30).**

Enrollment for this arm of the study started in month 15 and to date 77/90 dogs have participated.

## KEY RESEARCH ACCOMPLISHMENTS

### UCSF Site:

- Developed reproducible models of graded SCIs in the mouse.
- Defined key parameters to assess urologic status in mice after SCI
- Conducted the first study to assess efficacy of GM6001 when delivered 8 hours after a moderate SCI injury in mice. Though group sizes were small, these data showed promising results in terms of improving neurologic and urologic function.
- Completed all proposed studies in mice to assess efficacy of GM6001 when delivered 8 hours after two levels of injury severities. Here we mirrored the delivery route and vehicle to the dog clinical trial, so drug was dissolved in DMSO and injected subcutaneously.
- We show therapeutic efficacy of GM6001 in mice with a moderate level of spinal cord based upon both neurological and urological outcomes. However, similar efficacy as not seen after a severe spinal cord injury.
- Completed data analysis of other parameters of bladder function collected during the awake cystometry to assess bladder function in the moderately injured group treated with vehicle or GM6001 along with uninjured mice that served as baseline controls.
- Conducted 3D reconstruction of representative spinal cords from the moderately injured -vehicle and GM6001 treated mice.
- Completed histological analysis of the bladder wall thickness of the moderately injured -vehicle and GM6001 treated mice.
- Performed in situ gelatinase assay on fresh bladder sections from uninjured and 7 days post-injury.
- Developed unbiased stereology assay to assess serotonergic fiber length in the injured spinal cord.
- Stained bladders for collagen I and III and developed assay and analyzed bladders from moderately injured mice treated with vehicle or GM6001.

### TAMU Site:

- Completed pharmacokinetic study of GM6001 in 10 dogs. The study supports the rapid development of maximal plasma concentration after S.Q. delivery, the presence of plasma drug levels capable of inhibiting MMPs in vitro, and the short term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial. Data published in Levine et al PLoS ONE 2014.
- Completed normal dog cystometry in 10 dogs
- Enrolled 17 dogs with acute IVDH-associated SCI into serial cystometry study (Specific Aim 3.1). As expected, dogs with SCI that are non-ambulatory lack normal voiding reflex, have larger bladder capacity, have elevated post-cystometry baseline pressure, and have larger residual volume compared to measures taken during recovery.
- Enrolled 77 dogs with acute IVDH-associated SCI into clinical trial (Specific Aims 2.2 and 3.2)

## **REPORTABLE OUTCOMES**

### **UCSF Site:**

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale. Abstract was entitled "URINARY BLADDER DYSFUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY: RELATIONSHIP BETWEEN INJURY SEVERITY AND MEASURES OF UROLOGIC STATUS". Abstract is provided in the Appendices.

Invited speaker, International Symposium on Neuroregeneration, December 7, 2011, Asilomar, CA  
MATRIX METALLOPROTEINASES (MMPS) AND SPINAL CORD INJURY

Invited speaker, Ohio State University, January 14, 2013, Columbus, Ohio  
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, Rutgers University, W.M. Keck Center for Collaborative Neuroscience January 31, 2013, Piscataway, New Jersey  
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL  
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS  
Abstract is provided in the Appendices.

Invited speaker, University of California, September 10, 2013, San Francisco, CA  
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY. A PATHWAY OF DISCOVERY FROM MICE TO DOGS

Presenter at nanosymposium, Society for Neuroscience annual meeting, November 9-13, 2013, San Diego, CA. Abstract was entitled, "ACUTE TREATMENT WITH THE MATRIX METALLOPROTEINASE INHIBITOR GM6001 IMPROVES LONG-TERM LOCOMOTOR AND BLADDER FUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY". Abstract is provided in the Appendices.

Poster presentation at International Symposium on Neuroregeneration, December 11-15, 2013, Asilomar, CA. Abstract was entitled, "MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY". Abstract is provided in the Appendices.

Poster presentation at the American Urology Association Meeting, May 16 - 21, 2014 in Orlando, Florida. Abstract was entitled "A MURINE MODEL OF SPINAL CORD INJURY: EFFECT OF THE GENERAL MATRIX METALLOPROTEINASE INHIBITOR GM6001 ON LONG-TERM LOCOMOTOR AND BLADDER FUNCTION". Abstract is provided in the Appendices.

Poster presentation at the National Neurotrauma Society Symposium, June 29-July 2, 2014 in San Francisco, CA. Abstract was entitled "MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SUPPORTING UROLOGIC RECOVERY IN A MURINE MODEL OF SPINAL CORD INJURY". Abstract is provided in the Appendices.

### **TAMU Site:**

Levine JM et al EFFICACY OF A MATRIX METALLOPROTEINASE INHIBITOR IN SPINAL CORD INJURED DOGS. PLOS ONE 2014; 9:e96408. Paper in pdf format is provided in the Appendices

## CONCLUSIONS

- In a preliminary study, GM6001 (dissolved in 4% carboxy methyl cellulose and delivered via intraperitoneal route) when given 8 hours after a moderate SCI in the mouse, results in improvement in long-term neurologic recovery and a significant reduction in abnormal bladder contractility.
- Studied efficacy of GM6001 when delivered subcutaneously at 8 hours after moderate and severe SCI in the mouse. GM6001 treatment resulted in a long-term improvement in locomotor function and increased percentage of mice with the ability to step. Histological analysis of the injured spinal cord showed greater sparing of white matter with a corresponding reduction in lesion volume in the moderate injury group treated with GM6001. Awake cystometry revealed bladder dysfunction in the moderately injured mice treated with vehicle, which was ameliorated by drug treatment, as measured by a reduction in residual urine, uninhibited bladder contractions, reduction in time to first void and increased bladder efficiency. This was confirmed by histological analysis of the bladder, where injury led to increased bladder wall thickness and a significant reduction was observed in the GM6001 treated group relative to the vehicle control. In addition, to sparing white matter tracts, GM6001 may also act directly on the bladder urothelium, as seen by increased gelatinase activity at 7 days post-injury in untreated bladder.
- In contrast to these beneficial effects of GM6001 seen in the moderate injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs.
- GM6001 dosed subcutaneously at 100 mg/kg in dogs is safe and results in a pharmacokinetic profile that lends itself to the duration of MMP inhibition demonstrated to be effective in rodent neurotrauma work.
- In dogs with IVDH associated SCI, urinary voiding impairment can be assessed by cystometry and bears similarity to what is seen in humans with per-acute injury. Voiding recovery happens rapidly in dogs with mild or moderate SCI (non-ambulatory with or without limb movement but with intact deep nociception).

## REFERENCES

1. Noble LJ et al *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. Journal of Neuroscience* 22, 7526 (2002)

## APPENDICES:

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale.

TITLE: Urinary bladder dysfunction in a murine model of spinal cord injury: Relationship between injury severity and measures of urologic status

Thomas M. Fandel, Jonathan Levine, Kayleen Gimlin, Haoqian Zhang,  
Alpa Mahuvakar, and Linda J. Noble-Haeusslein

**Presenter's Name:** Linda J. Noble-Haeusslein, Ph.D.

**PURPOSE/AIMS:** The purpose of this study was to determine the extent to which severity of an incomplete spinal cord injury (SCI) influences bladder function in a murine model of SCI.

**DESIGN:** Mice were randomized to sham, (n=8), mild (n=5) or moderate (n=7) SCI and treated with Enrofloxacin for 10 days subcutaneously followed by food supplemented with Enrofloxacin until euthanasia. Neurological status was evaluated at 1 and 3 days post injury and weekly thereafter for 3 weeks. At 4 weeks post-injury, awake cystometry was performed (n= 3-7/group). At the completion of cystometry and after residual urine was determined (n=3-6/group) the bladders were removed and weighed (n= 5-7/group). All observers were blinded to the experimental condition.

**POPULATION/SAMPLE STUDIED:** Adult, male, C57Bl6 mice subjected to laminectomy only or mild or moderate SCI.

**METHODS:** SCI was produced by dropping either a 2 g (mild injury) or 3 g (moderate injury) weight onto the exposed spinal cord at the T9 vertebral level. Neurological status was based upon the BMS scale. At 3 weeks post-injury, a PE50 polyethylene catheter was implanted into the bladder dome and tunneled subcutaneously to emerge in the interscapular area. One week later, cystometry was performed in the awake restrained animal using saline at an infusion speed of 16-20 / minute (Catamount Research, St. Albans, VT). Residual urine was determined at the end of cystometry. The urinary bladders were removed, blotted dry, and weighed.

**DATA ANALYSIS:** Two-way repeated measures (RM) analysis of variance (ANOVA) was used to evaluate neurological recovery. Residual urine and bladder weight were analyzed using 1-way ANOVA followed by Bonferroni's Multiple Comparison Test. Unpaired Student's T-test was used when two groups were specified. Significance was defined at  $P \leq 0.05$ . All data are expressed as means +/- SEM.

**FINDINGS:** BMS scores revealed an effect of both time ( $p=0.0001$ ) and injury severity ( $p=0.0182$ ). While both injury groups showed improved performance over time, BMS scores were lower in the 3 g ( $1.786 \pm 0.3595$ ) relative to the 2 g ( $6.000 \pm 1.508$ ) group ( $p=0.0097$ ) at 21 days post injury. Moreover, a 3 g injury led to qualitatively more uninhibited bladder contractions and greater residual urine ( $0.9293 \pm 0.1346$ ) and bladder weight ( $0.1475 \pm 0.2238$  g) relative to residual urine ( $0.3980 \pm 0.0080$ ) and bladder weight in the 2 g injury ( $0.07160 \pm 0.0072$  g) ( $p < 0.01$ ).

**CONCLUSIONS/RECOMMENDATIONS:** There are injury severity dependent abnormal changes in both weight and function of the urinary bladder after SCI.

**IMPLICATIONS:** While bladder dysfunction is a common problem in human SCI, analyses of bladder function are typically neglected in murine models of SCI. Characterization of bladder function, relative to injury severity, provides a clinically relevant benchmark for establishing efficacy of candidate therapeutics.

**FROM/TO TIME PERIOD OF STUDY:** From September 30, 2011 to April 25, 2012

**FUNDING:** DOD Spinal Cord Injury Program SC100140

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL

TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS

**Presenter's Name:** Linda J. Noble-Haeusslein, Ph.D.

Dogs sustain naturally occurring spinal cord injuries (SCIs) represent a clinically relevant population to confirm therapeutic targets that have been identified in rodent models of spinal cord injury (SCI). Spinal cord injured mice, genetically deficient in the gelatinase MMP-9 or treated with GM 6001, a broad-spectrum inhibitor of matrix metalloproteinases (MMPs), beginning 3 hours post injury, show improved long-term neurological outcomes that correspond to the early reduction of leukocytes in the injured cord and stabilization of the blood-spinal cord barrier. Here we find that serum levels of gelatinases are acutely elevated in spinal cord injured dogs, suggesting that these proteases may likewise be a determinant of recovery. After confirming safety and defining the pharmacokinetics of GM 6001 in normal dogs, a large scale, randomized, placebo controlled study was performed in dogs with acute SCIs. Duration of SCI was required to be  $\leq 48$  hours and dogs were stratified according to injury severity. Three groups were studied: GM 6001 + DMSO, DMSO, or saline. As GM 6001 is not soluble in an aqueous solution, DMSO was selected as the vehicle, recognizing that this agent has broad anti-inflammatory actions in models of CNS injury. Only the GM 6001-treated group, given shortly after admission to the clinic, resulted in a reduction in serum gelatinase activity. Utilizing post-hoc statistical techniques, there was a therapeutic benefit of GM 6001+DMSO over DMSO or saline in dogs with mild-moderate spinal cord injuries. These encouraging findings provide the first evidence that MMPs are a determinant of recovery after SCI in dogs. Such validation in a 2nd species reinforces the adverse interactions of these proteases in SCI, and suggests that GM 6001 may likely hold promise for human SCI.

Presenter at Nanosymposium, Society for Neuroscience annual meeting, November 9-13, 2013, San Diego, CA.

ACUTE TREATMENT WITH THE MATRIX METALLOPROTEINASE INHIBITOR GM6001 IMPROVES LONG-TERM LOCOMOTOR AND BLADDER FUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY.

T. M. FANDEL, A. TRIVEDI-MAHUVAKAR, K. GIMLIN, H. ZHANG, J. M. LEVINE, A. F. MARTINEZ, L. J. NOBLE-HAEUSSLEIN

**Presenter's name:** Thomas M. Fandel

We have previously shown that the general matrix-metalloproteinase inhibitor GM6001, when given beginning 3 hours after moderate spinal cord injury (SCI), attenuates secondary injury to the murine spinal cord and improves locomotor recovery. Here we determine efficacy of GM6001 on locomotor and bladder function after more severe levels of SCI and when the initial treatment is delayed to 8 hours post injury. Adult, male, C57Bl/6 mice were subjected to moderate-severe (n=23) or severe (n=25) SCI, produced by dropping either a 2g or 3g weight respectively onto the exposed spinal cord at the T9 vertebral level. Injured mice were randomized to receive drug (GM6001 at 100mg/kg, n=11/12) or vehicle (99% DMSO, s.c. n=12/13) starting 8 hours after injury and then twice daily for 3 consecutive days. Neurological status was evaluated at 1 and 3 days post-injury and weekly thereafter for 5 weeks using the Basso Mouse Scale (BMS). At 5-6 weeks, a PE10 catheter was implanted into the bladder dome and conscious cystometry was performed 2-3 days later. Two-way repeated measures analysis of variance and unpaired T-Tests were used to evaluate locomotor recovery and bladder function, respectively. Significance was defined as  $p < 0.05$ . All observers were blinded to the experimental conditions. There was an effect of both time ( $p < 0.0001$ ) and treatment ( $p = 0.04$ ) on BMS score in the moderate-severe injury group. In contrast, in the severe injury group, there was an effect of time ( $p < 0.0001$ ) but not of treatment on locomotor recovery ( $p = 0.08$ ). Cystometry after moderate-severe injury revealed a therapeutic effect of GM6001 on post-void baseline pressure ( $p = 0.01$ ), residual urine ( $p = 0.0004$ ), the duration of the intermicturition interval ( $p = 0.004$ ), the number of uninhibited bladder contractions (UBC)/voiding cycle ( $p = 0.01$ ), and the pressure increase of UBC ( $p = 0.01$ ). GM6001 treatment in the severe injury group had a positive effect on certain metrics; namely, the duration of the intermicturition interval ( $p < 0.05$ ), the number of UBC/voiding cycle ( $p = 0.004$ ), and the pressure increase of UBC ( $p = 0.02$ ). Here we provide the first evidence that GM6001 improves both locomotor and bladder function when treatment is delayed to a more clinically relevant time point of 8 hours and after a moderate severe spinal cord injury. Such findings support the candidacy of this drug for clinical trials. While GM6001 did not improve locomotor function after a severe SCI, we are encouraged by the benefit seen in bladder function, which we speculate may result from the direct action of this drug on the bladder wall.

Poster presentation at International Symposium on Neuroregeneration, December 7, 2013, Asilomar, CA  
CA

MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL  
AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY.

A Trivedi, T. M. Fandel, A. F. Martinez, H. Zhang, J. M. Levine, and L. J. Noble-Haeusslein

**Presenter's name:** Alpa Trivedi (Mahuvakar), Ph.D.

We have shown that the matrix-metalloproteinase (MMP) inhibitor, GM6001 improves locomotor function in a murine model of moderate spinal cord contusion injury (SCI), when treatment is initiated at 3 hours post-injury. However, this timing of administration of GM6001 is not easily achievable in the clinical scenario. As infiltrating neutrophils peak at 12 hours post-injury and are a major source of MMPs, we determined if efficacy could be achieved when the timing of administration of GM6001 was extended beyond 3 hours post-injury. In this randomized and blinded study, adult male C57Bl/6 mice were subjected to a moderate-severe or a severe SCI. Animals were randomized to receive drug (GM6001 at 100 mg/kg) or vehicle (99% DMSO) starting 8 hours after injury and then every 12 hours for 3 consecutive days. Using the Basso Mouse Scale (BMS), neurological recovery was assessed at 1 and 3 days post-injury and once per week for 5 weeks. After 5 weeks, awake cystometry was conducted to assess bladder function. GM6001 treatment resulted in a long-term improvement in locomotor function and greater sparing of white matter with a corresponding reduction in lesion volume in the moderate-severe injury group. Awake cystometry revealed reduced residual urine, uninhibited bladder contractions, and bladder wall thickness relative to the vehicle control. In contrast to these beneficial effects of GM6001 seen in the moderate-severe injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs. These findings demonstrate that GM6001 shows injury-severity dependent efficacy in terms of both neurological and urological recovery. Importantly, this broad-based efficacy is achieved when the drug is administered as late as 8 hours post-injury, a feature which offers promise for the spinal cord injured patient.

Poster presentation at the American Urology Association meeting, May 16 - 21, 2014 in Orlando, Florida.

A MURINE MODEL OF SPINAL CORD INJURY: EFFECT OF THE GENERAL MATRIX METALLOPROTEINASE INHIBITOR GM6001 ON LONG-TERM LOCOMOTOR AND BLADDER FUNCTION.

Thomas M. Fandel\*, Alpa Trivedi, Aida F. Martinez, Haoqian Zhang, Jonathan M. Levine, and Linda J. Noble-Haeusslein

**Presenter's name:** Thomas M. Fandel

**Introduction and Objectives**

We have previously shown that GM6001 improves locomotor function in a murine model of moderate spinal cord contusion injury (SCI), when treatment is initiated at 3 hours post-injury. Here we determine efficacy of GM6001 on locomotor and bladder function after two levels of SCI and when the initial treatment is delayed to 8 hours post-injury.

**Methods**

Adult, male, C57Bl/6 mice were subjected to moderate-severe (n=23) or severe (n=24) SCI, produced by dropping either a 2g or 3g weight respectively onto the exposed spinal cord at the T9 vertebral level. Injured mice were randomized to receive drug (GM6001 at 100mg/kg, n=11/12) or vehicle (99% DMSO, s.c. n=12/12) starting 8 hours after injury and then twice daily for 3 consecutive days. Nine animals served as uninjured controls. Neurological status was evaluated at 1 and 3 days post-injury and weekly thereafter for 5 weeks using the Basso Mouse Scale (BMS). At 5-6 weeks, a PE10 catheter was implanted into the bladder dome and conscious cystometry was performed 2-3 days later. Two-way repeated measures analysis of variance and unpaired T-Tests were used to evaluate locomotor recovery and bladder function, respectively. Significance was defined as p<0.05.

**Results**

On BMS score in the moderate-severe injury group, there was an effect of both time (p<0.0001) and treatment (p=0.04). In contrast, in the severe injury group, there was an effect of time (p<0.0001) but not of treatment on locomotor recovery (p=0.8). Cystometry after moderate-severe injury revealed a therapeutic effect of GM6001 on the number of uninhibited bladder contractions (UBC)/ voiding cycle (p=0.007), time to first void (p=0.0004), residual urine (p=0.004), and voiding efficacy (p=0.008). GM6001 treatment in the severe injury group reduced the number of UBC/ voiding cycle compared to its injury control (p=0.03).

**Conclusions**

Here we provide the first evidence that GM6001 improves both locomotor and bladder function when treatment is delayed to a more clinically relevant time point of 8 hours post-injury and after a moderate severe spinal cord injury. Such findings support the candidacy of this drug for clinical trials.

Changes in bladder function following spinal cord injury

	Uninjured Controls	Moderate-severe SCI Controls	Moderate-severe SCI GM6001	Severe SCI Controls	Severe SCI GM6001
Uninhibited bladder contractions/ voiding cycle	1.7 ± 0.6	17.2 ± 2.7	8.4 ± 1.4**	10.6 ± 2.0	4.9 ± 1.2*
Time to first void (min)	12.4 ± 2.0	45.9 ± 4.5	19.7 ± 4.0***	30.1 ± 6.9	29.5 ± 4.4
Residual urine (ml)	0.007 ± 0.004	0.648 ± 0.047	0.249 ± 0.087**	0.463 ± 0.088	0.403 ± 0.073
Voiding efficacy (%)	93.1 ± 3.7	0.2 ± 0.1	35.5 ± 10.7**	18.6 ± 8.6	20.9 ± 11.4

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 vs. respective injury controls

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Poster presentation at the National Neurotrauma Society symposium, June 29-July 2, 2014 in San Francisco, CA.

**MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SUPPORTING UROLOGIC RECOVERY IN A MURINE MODEL OF SPINAL CORD INJURY**

Fandel, T.M., Trivedi, A., Martinez, A.F., Zhang, H., Levine, J.M., Noble-Haeusslein, L.J.

**Presenter's name:** Thomas M. Fandel

Matrix-metalloproteinases (MMPs), and in particular MMP-9, are upregulated in the acutely injured spinal cord and their transient, short-term blockade with a general MMP-inhibitor (MMPI), beginning 3 hours post-injury and for the next 3 days, results in long-term locomotor recovery and greater sparing of white matter. As sparing of white matter may at least in part reflect preservation of long descending fiber tracts including those involved in the control of bladder function, we hypothesized that acute blockade of MMPs would lead to improved urological function. Testing this hypothesis, we conducted a randomized, blinded pre-clinical study, in which adult male C57Bl/6 mice were subjected to a moderate contusion injury (n=23) at the level T9 and were treated with either an MMPI or vehicle. As neutrophils are a major source of MMP-9, treatments were initiated 8 hours after injury, a time corresponding to prominent neutrophilia in the humoral compartment. Neurological and urological recovery was assessed using the Basso Mouse Scale and conscious cystometry, over a period of 5 weeks and at 6 weeks post-injury, respectively. Stereology was used to determine lesion volume and white matter sparing. As bladder dysfunction is associated with aberrant wound healing resulting in increased bladder wall thickness, this parameter was measured at the time of euthanasia. In the MMPI-treated group there were significant long-term improvements in locomotor function, sparing of white matter and voiding function, as evidenced by decreased post-void residual urine and enhanced voiding efficacy. Moreover, there were fewer uninhibited bladder contractions per voiding cycle, an indicator of decreased bladder over-activity, and detrusor wall thickness was significantly less compared to vehicle controls. In summary, delayed treatment with an MMPI improved both locomotor and bladder function. These findings, together with an extended therapeutic window, offer promise for translation to the clinical setting.

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Keywords: Spinal cord injury, Neurogenic bladder dysfunction

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# Efficacy of a Metalloproteinase Inhibitor in Spinal Cord Injured Dogs

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## Abstract

Matrix metalloproteinase-9 is elevated within the acutely injured murine spinal cord and blockade of this early proteolytic activity with GM6001, a broad-spectrum matrix metalloproteinase inhibitor, results in improved recovery after spinal cord injury. As matrix metalloproteinase-9 is likewise acutely elevated in dogs with naturally occurring spinal cord injuries, we evaluated efficacy of GM6001 solubilized in dimethyl sulfoxide in this second species. Safety and pharmacokinetic studies were conducted in naïve dogs. After confirming safety, subsequent pharmacokinetic analyses demonstrated that a 100 mg/kg subcutaneous dose of GM6001 resulted in plasma concentrations that peaked shortly after administration and were sustained for at least 4 days at levels that produced robust *in vitro* inhibition of matrix metalloproteinase-9. A randomized, blinded, placebo-controlled study was then conducted to assess efficacy of GM6001 given within 48 hours of spinal cord injury. Dogs were enrolled in 3 groups: GM6001 dissolved in dimethyl sulfoxide (n = 35), dimethyl sulfoxide (n = 37), or saline (n = 41). Matrix metalloproteinase activity was increased in the serum of injured dogs and GM6001 reduced this serum protease activity compared to the other two groups. To assess recovery, dogs were *a priori* stratified into a severely injured group and a mild-to-moderate injured group, using a Modified Frankel Scale. The Texas Spinal Cord Injury Score was then used to assess long-term motor/sensory function. In dogs with severe spinal cord injuries, those treated with saline had a mean motor score of 2 (95% CI 0–4.0) that was significantly ( $P < 0.05$ ; generalized linear model) less than the estimated mean motor score for dogs receiving dimethyl sulfoxide (mean, 5; 95% CI 2.0–8.0) or GM6001 (mean, 5; 95% CI 2.0–8.0). As there was no independent effect of GM6001, we attribute improved neurological outcomes to dimethyl sulfoxide, a pleotropic agent that may target diverse secondary pathogenic events that emerge in the acutely injured cord.

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## Introduction

Matrix metalloproteinases (MMPs) are endopeptidases that degrade the extracellular matrix [1]. Several members of the MMP family, including MMP-9 (gelatinase B) and MMP-12, have been implicated in early secondary pathogenesis after spinal cord injury (SCI) [2–4]. These MMPs are released by local cells as well as by infiltrating leukocytes and result in reduced cell-cell adhesion, disruption of the blood-spinal cord barrier, up-regulation of pro-inflammatory cytokines, and demyelination [1,4,5].

Early blockade of MMPs confers neuroprotection after SCI [2,6,7]. Short-term administration of the broad-spectrum MMP inhibitor, GM6001, results in sparing of white matter and improves locomotor function when the drug is given over the

first 3 days post-injury [2]. Several lines of evidence suggest that one likely target of GM6001 is MMP-9. This protease is not actively expressed in the uninjured spinal cord and is up-regulated over the first 3 days post-injury, corresponding to the time-course for infiltration of neutrophils [8]. While there are local sources of MMP-9, including glia and endothelial cells, neutrophil depletion studies confirm that these leukocytes are the major source of MMP-9 in the acutely injured cord [7]. As this protease is not complexed with tissue inhibitor of MMP-1, degranulation of neutrophils results in release of activated MMP-9 [9], which then may disrupt the barrier and facilitate transmigration of leukocytes into the injured spinal cord. It thus is not surprising that early administration of GM6001 attenuates the trafficking of neutrophils

into the injured spinal cord and stabilizes the blood-spinal cord barrier [2]. There are other members of the MMP family that are also determinants of recovery after SCI including MMP-12 and ADAM-8 (a disintegrin and metalloprotease domain) [3]. Thus, broad inhibitors of MMPs may offer greater benefit than specific inhibitors of these proteases.

In this study, we have used dimethyl sulfoxide (DMSO) in combination with GM6001 [10,11]. While DMSO is commonly used as a vehicle to increase solubility of a drug, it has been reported to have neuroprotective properties in traumatic brain injury and SCI [12,13]. The putative neuroprotective activity of DMSO is thought to arise from its ability to block voltage-sensitive sodium channels and calcium influx into cells, and mitigate opening of ionotropic channels that are activated by glutamate [14].

Few studies have considered a pre-clinical platform involving dogs with naturally occurring SCIs resulting from intervertebral disk herniation (IVDH) [15–17]. This approach mimics pathologic aspects of human SCI including compressive/contusive injuries and a pro-inflammatory response that includes the infiltration of neutrophils and up-regulation of MMP-9 [18–20]. Moreover, these naturally-occurring injuries provide a means for studying therapeutics in the challenging context of varying degrees of injury severity, common in human SCI, but without confounding factors such as anesthetics that are necessary during creation of injury in experimental models.

Here we evaluate the efficacy of GM6001 in dogs with IVDH. Based on a double-blind, randomized, placebo-controlled trial, consisting of 3 groups (GM6001 in DMSO, DMSO alone, or saline) we show enhanced neurological recovery in dogs sustaining severe SCIs when treated acutely with GM6001 solubilized in DMSO or DMSO alone, relative to the saline group. Such findings implicate DMSO in improving neurological recovery, which is consistent with its reported ability to attenuate secondary pathogenic events in various models of neurotrauma [14].

## Materials and Methods

### Study Design and Inclusion Criteria

A preliminary drug tolerance study was constructed based on Food and Drug Administration guidelines (<http://www.fda.gov/AnimalVeterinary/default.htm>) and performed in 4 healthy, purpose-bred Beagles. Ten healthy, purpose-bred Beagles were obtained to evaluate pharmacokinetics (PK); this sample size was based on similar animal studies and general recommendations for canine PK investigations [21].

Guidelines for the conduct of SCI trials developed by the International Campaign for Cures of Spinal Cord Injury Paralysis were utilized to assist with the design of a randomized, double-blinded (clinicians and clients were unaware of treatment group), placebo-controlled canine trial including inclusion/exclusion criteria, randomization protocol, data handling, and the *a priori* definition of outcome metrics and statistical approaches.[22]. Consolidated Standards of Reporting Trials (CONSORT) Statement Guidelines were used to assist with trial performance and data reporting [23,24]. Client-owned dogs with IVDH-associated SCI, admitted to the Texas A&M University Veterinary Medical Teaching Hospital between September 2008 and February 2012, were recruited. The study interval was selected to generate a sample size of >100 dogs, which was considered robust based on previous human phase II and III SCI studies [25,26], animal model studies of SCI using MMP blockers [2], and completed canine SCI studies [27,28]. A formal power calculation was not

performed due to the absence of a phase I canine study examining the effects of GM6001.

Dogs had to meet the following criteria to be included in the clinical trial population: 1) duration of SCI was required to be  $\leq 48$  hours; 2) IVDH-associated SCI had to result in non-ambulatory paraparesis or paraplegia at enrollment; 3) IVDH-associated SCI had to be identified between the T8-L6 vertebral articulations and treated via surgical decompression. The exclusion criteria were: 1) concurrent disseminated neoplasia or systemic inflammation; 2) a history of recent breeding/pregnancy; and, 3) glucocorticoid treatment within 7 days of SCI.

The primary outcome of the clinical trial was a validated ordinal SCI score (the Texas Spinal Cord Injury Score [TSCIS]) conducted at 42 days post-injury [29]. The secondary outcome was TSCIS at 3 days after SCI. Dogs were stratified into those with behaviorally severe SCI (absent pelvic limb movement and deep nociception) and those with mild-to-moderate SCI (intact pelvic limb deep nociception with or without movement) at study entry to examine primary and secondary outcomes. This *a priori* stratification was utilized because a substantially lower proportion of dogs with severe SCI recover independent ambulation at long-term follow-up time-points (approximately 50–60%) in comparison to dogs with mild-to-moderate SCI (approximately 85 to 95%); thus, injury severity might influence the ability to detect treatment-related effects [30–33].

### Ethics Statement

All animal procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2007–115; AUP 2011–057; AUP-2011–145) and in the case of client-owned dogs were performed with signed consent. All studies adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Drug Preparation, Drug Tolerance, and Pharmacokinetic Procedures

For all canine studies, GM6001 (SAI Advantium, Hyderabad, India) was dissolved in 90% DMSO (Domoso, Fort Dodge Corp, Fort Dodge, IA) at a concentration of 250 mg/mL. The solution was sterilized using a 25-mm syringe filter with 0.22- $\mu$ m HT Tuffryn membrane (Pall Corporation, East Hills, NY).

Dogs, participating in the drug tolerance study, were acclimatized for 14 days and then randomized as follows: DMSO (at a volume equivalent to that present in a 100 mg/kg GM6001 treatment), 100 mg/kg GM6001, 150 mg/kg GM6001, or 300 mg/kg GM6001 subcutaneously (SC) every 12 hours for 3 days. The doses of GM6001 were selected to exceed those reported previously in a murine model of SCI [2]. A SC route of administration was selected as 1) GM6001 does not remain solubilized in DMSO when exposed to hydrophilic solutions such as blood, prohibiting intravenous delivery and 2) intraperitoneal drug administration is not generally permitted in client-owned dogs at our institution, due to challenges in managing any local drug reactions. Adverse event monitoring was performed for 7 days following the completion of drug administration. All dogs had physical examinations, injection site evaluations, and assessment of food and water intake twice daily. A complete blood count, serum biochemistry profile, urinalysis, and coagulation profile were performed 3 and 7 days following the completion of drug administration. Following the completion of this study, the vehicle and 300 mg/kg GM6001 dogs were euthanized via intravenous administration of 120 mg/kg pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). The brain, heart, liver, kidney, lung, intestine, and injection sites were evaluated.

For PK assessments, a single 100 mg/kg SC administration of GM6001 was delivered in 5 dogs with 5 additional dogs receiving a second 100 mg/kg SC of GM6001, 12 hours following the first dose. In dogs with single dosing, serial plasma samples were obtained at 5, 15, and 30 minutes and 1, 2, 3, 6, 12, 24, 36, 48, and 96 hours after GM6001 delivery. In dogs with multiple dosing, blood samples were collected shortly after the second dose, and then at 24, 48, 72, and 96 hours. All samples were stored in a  $-80^{\circ}\text{C}$  freezer until analyzed by high performance chromatography (Thermo Electron Co., Waltham, MA) and tandem mass spectroscopy (MDS-Sciex/Applied Biosystems API3000, Concord, ONT) (LC-MS/MS). Concentrations of GM6001 ( $m/z$  389.0 $\rightarrow$ 356.0) were determined, using MMP Inhibitor III ( $m/z$  364.0 $\rightarrow$ 356.0, Calbiochem, Billerica, MA) as the internal standard. A standard curve was created with blank dog plasma at concentrations 10.0 to 10,204.0 ng/mL, with linear regression and weighting of concentrations ( $1/x^2$ ). After thawing and addition of internal standard (300  $\mu\text{L}$  of 100 ng/mL in 0.5% acetic acid in methanol), plasma samples or standards (100  $\mu\text{L}$ ) were centrifuged and reconstituted with 30/70 methanol/10 mM ammonium formate buffer, pH 3.0, for protein precipitation. Supernatant (100  $\mu\text{L}$ ) was collected, vortexed, and refrigerated until injection on LC-MS/MS. The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile/methanol/formic acid (40:60:0.1, v/v/v) (B) with a flow rate of 0.30 mL/minute using a linear gradient starting at 40% B from 0 to 0.01 minutes, to 80% B at 1.5 minutes, to 90% B at 3.5 minutes, to 40% B at 3.6 minutes, with a total run time of 5 minutes.

#### Randomized, Placebo Controlled Study in Dogs with IVDH-associated SCI

Dogs, enrolled in the clinical trial, had physical and neurological examinations, complete blood count and serum biochemistry profile. Anesthesia was induced with propofol (Rapinovel, Schering-Plough Animal Health Corp, Union, NJ) and maintained with inhalant sevoflurane (SevoFlo, Abbott Laboratories, North Chicago, IL). Diagnostic imaging consisting of myelography, computed tomography (CT), or MRI was performed to identify IVDH. Cerebrospinal fluid (CSF) was collected from the cisterna magna for routine analysis and a 200- $\mu\text{L}$  aliquot was stored at  $-80^{\circ}\text{C}$  for determination of MMP-2/MMP-9 activity. Six mL of whole blood were obtained at the time of CSF collection and 3 days following treatment delivery; serum was isolated and frozen at  $-80^{\circ}\text{C}$ .

Immediately after collection of CSF and blood, dogs were randomized to receive 100 mg/kg GM6001+ DMSO, DMSO, or saline placebo. The dose of both DMSO and saline was 0.4 mL/kg, a volume equivalent to that of 100 mg/kg GM6001+DMSO; this approach was taken to maintain blinding. A randomization sequence was developed prior to the initiation of this trial and randomization was accomplished by blocking the dogs by gender status in a 1:1:1 ratio to each of the treatment groups. Sealed envelopes contained treatment allocations and were delivered to a central location where treatments were formulated by individuals not involved in the assessment of animals. Treatments were covered and marked only with animal identifiers to ensure blinding.

Following surgical decompression, all dogs were recovered in an intensive care unit for 24 hours and during that time were provided post-operative opioid analgesia and bladder evacuation. Physical rehabilitation protocols were standardized for dogs participating in this study. Dogs received thoracic limb and pelvic limb passive range of motion exercises beginning 24 hours post-operatively and until dogs could independently ambulate. Each

limb was gently flexed and extended at the carpal, elbow, and hip joints in 3 sets of 10 repetitions, 2 times daily. Supported standing exercises were performed twice daily for 5 minutes by placing a sling immediately cranial to the pelvic limbs and continued until dogs could independently ambulate. Dogs that were non-ambulatory were walked using a sling placed immediately cranial to the pelvic limbs for 5 minutes twice daily. Independently ambulatory dogs were permitted to walk on a leash for 5 minutes 3–4 times per day during hospitalization and were allowed to continue this activity until 42-day re-check. Participating dogs were housed in cages that permitted limited additional activity until 42-day re-check evaluation.

#### Neurological Assessments

Clinicians responsible for neurologic scoring were blinded to treatment assignments. Two ordinal SCI scores were used to address injury severity at study entry, day 3 post-treatment, and day 42 post-treatment. In both scoring systems, dogs were considered ambulatory if they could spontaneously rise, bear weight, and take at least 10 steps without falling. Dogs that were non-ambulatory had pelvic limb movement evaluated using tail support. Postural responses were evaluated by placing the dorsum of the pes on a non-slick surface while manually supporting the animal and waiting for limb correction. Pelvic limb deep and superficial nociception were evaluated by applying hemostats to a nail-bed or interdigital webbing, respectively and evaluating for the presence of a behavioral or physiological response.

A modified Frankel scale (MFS) was developed to broadly parallel the American Spinal Cord Injury Association Impairment Scale (AIS) [15,29]. Dogs were scored as paraplegic with absent deep nociception (0; equivalent to AIS A), paraplegic with absent superficial nociception (1; equivalent to AIS B), paraplegic with intact nociception (2; equivalent to AIS B), or non-ambulatory with identifiable pelvic limb movement (3; equivalent to AIS C). The MFS was not a primary trial outcome, but instead was used to describe the baseline population (overall and by treatment group) and to stratify the study population for analysis.

The Texas Spinal Cord Injury Score (TSCIS) was used to assess pelvic limb gait, posture and nociception. This is a more refined scale than the MFS [15] with a larger array of sub-categories, including gait assessment that parallels the Basso, Beattie, Bresnahan Scale [34]. The TSCIS gait score ranges from 0 to 6 in each pelvic limb and correlates to the degree of limb protraction and weight bearing. The gait classifications include: no voluntary movement seen when the dog is supported (score = 0); intact limb protraction with no ground clearance (1); intact limb protraction with inconsistent ground clearance (2); intact protraction with ground clearance  $>75\%$  of steps (3); ambulatory with consistent ground clearance and significant paresis-ataxia that results in occasional falling (4); ambulatory with consistent ground clearance and mild paresis-ataxia that does not result in falling (5); and normal gait (6). Pelvic limb postural responses using the TSCIS were scored in each limb as absent (0), delayed (1, correction occurred  $>1$  second after positioning), and present (2). Nociception was scored in each limb as absent (0), deep nociception only present (1), or both deep and superficial nociception present (2).

#### Magnetic Resonance Imaging (MRI)

Vertebral column MRI was performed on enrolled dogs, except in cases where animals were evaluated outside of normal operating hours or the scanner was unavailable due to mechanical failure. Between September 2008 and July 2011, a 1.0 T system (Siemens Magnetom, Malvern, PA) was utilized to acquire images; for the remainder of the trial images were generated using a 3.0 T MRI

(Siemens Verio, Malvern, PA). Dogs that received MRI had sagittal T2-weighted (T2W) images reviewed by 1 investigator (JML) using commercially available software (eFILM, Merge Healthcare, Chicago, IL) prior to un-blinding. The acquisition parameters for sagittal T2W images generated at 1T and 3T included a repetition time of 3500 ms, echo time of 90 ms, and slice thickness of 2.0 mm. The presence of spinal cord T2W hyperintensity was determined by visually comparing injured parenchyma to surrounding spinal cord. This technique has been used extensively in human and canine MRI studies and produces repeatable results that correlate with behavioral measures of SCI severity and recovery [35,36].

### MMP-2/MMP-9 Activity in CSF and Serum

CSF and serum samples ( $n = 16$ /treatment group) were randomly selected at the end of the trial by computerized sorting on random numbers. Purpose-bred Beagle dogs ( $n = 5$ ) were sampled as controls. Serum samples with overt hemolysis were excluded from analysis.

Activity of MMP-2 and MMP-9 in serum and CSF samples was assessed in a blinded manner using a previously developed electrophoretic method [37–39] that included a synthetic peptide (AAPPtec, Louisville, KY) (sequence: Ac-NGDPVGLTAGAGK-NH<sub>2</sub>), tagged with a fluorophore BODIPY-FL-SE (Invitrogen, Carlsbad, CA). The substrate was mixed with either serum or CSF, with phosphate buffered saline as the negative control. After reacting for 1 hour, aliquots were loaded onto 20% polyacrylamide gels and the samples were electrophoresed. Gels were imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA). The image was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) with ImageQuant v5.2 software and fluorescent signal was quantified using ImageJ (1.440, National Institutes of Health, Bethesda, MD).

To assess ability of GM6001 to inhibit MMP-9, activity (described above) was determined using human recombinant MMP-9 (Sigma, St. Louis, MO) that was serially diluted in DMSO to final concentrations of 0.01  $\mu$ M to 200  $\mu$ M. Controls consisted of enzyme and substrate only and substrate and GM6001 only.

### Statistical Analyses

Noncompartmental pharmacokinetic analysis was performed (Phoenix WinNonLin 6.3, Pharsight, St. Louis, MO), and estimates of the parameters of  $T_{max}$ ,  $C_{max}$ ,  $T_{1/2}$ , and area  $AUC_{0-obs}$  and  $AUC_{0-\infty}$  were calculated for the single dose.

Activities of MMP-2/MMP-9 in CSF and serum were compared between healthy control dogs and the dogs with SCI using the Wilcoxon rank-sum test. The Wilcoxon rank-sum test was also used to compare CSF and serum MMP-2/MMP-9 activities between dogs with and without selected characteristics that were potential modifiers of MMP-2/MMP-9 (i.e., age, breed, sex, and markers of disease duration or severity). To compare serum MMP-2/MMP-9 values among treatment groups, the serum MMP-2/MMP-9 activities were converted to ranks, and the ranks were compared using a generalized linear model; multiple pair-wise comparisons between treatments were made using the method of Sidak. Model fit was assessed graphically using diagnostic plots of residuals.

For the clinical trial data, a strategy for analysis of data was developed *a priori*, including our decision to stratify the population based on SCI severity at admission. Baseline characteristics were compared among the 3 treatment groups to determine whether there was any evidence of differences among groups. Categorical variables were compared using chi-squared analysis and continuous or ordinal variables were compared using Kruskal-Wallis

tests. The primary outcome for the trial was the TSCIS score on day 42. The TSCIS on day 3 was considered a secondary outcome. The association of TSCIS with treatment group and other individual variables was assessed using generalized linear modeling. Individual variables significantly associated with TSCIS were analyzed using multivariable generalized linear modeling using maximum likelihood estimating methods. Multiple comparisons among groups were adjusted using the method of Sidak. Model fit was assessed graphically using diagnostic plots of residuals. Comparisons of proportions among treatments groups (e.g., frequency of adverse events) were made using chi-squared or, when appropriate, Fisher's exact tests. Significance was set at  $P < 0.05$  for all analyses. Analyses were performed using S-PLUS statistical software (Version 8.2, TIBCO, Inc., Seattle, WA).

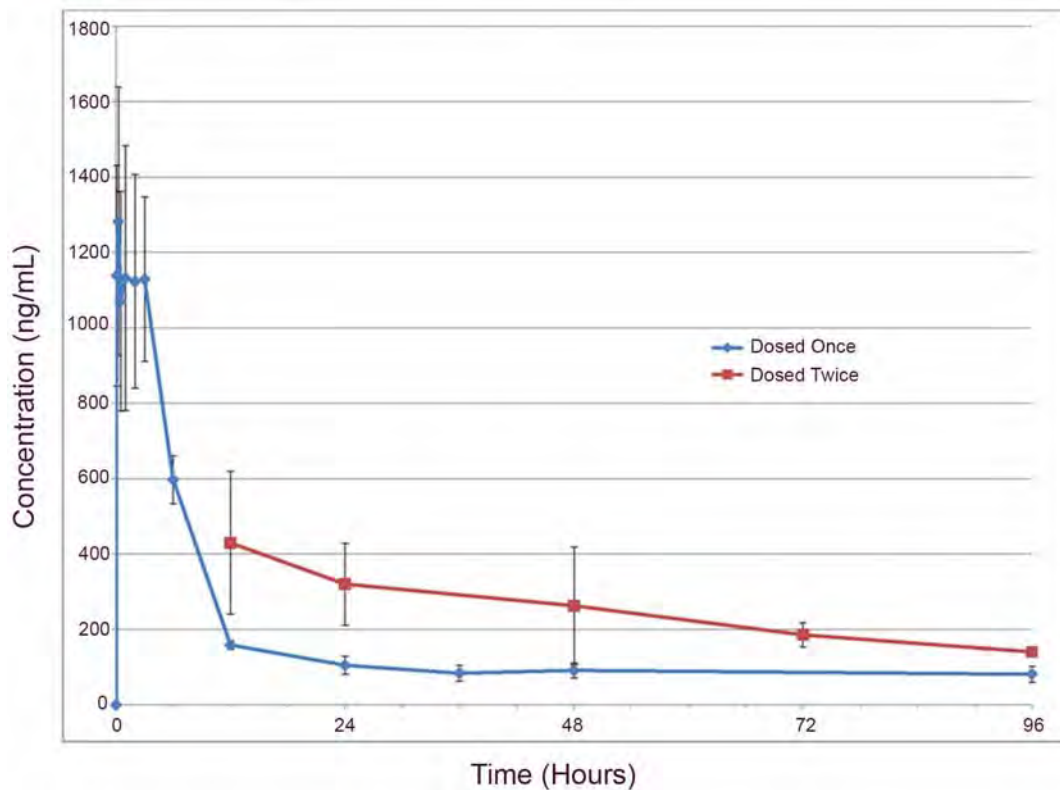
## Results

### GM6001 is Well Tolerated in Naive Dogs

We first addressed the safety of GM6001 using a dose tolerance study. Four healthy dogs were randomized to receive DMSO vehicle, 100 mg/kg GM6001, 150 mg/kg GM6001, or 300 mg/kg GM6001 SC every 12 hours for 3 days. Following drug delivery, all studied parameters were within normal limits, with the following exceptions: 1) increase in body temperature in all GM6001-treated dogs which peaked 6 days after treatment was completed (Fig. S1); 2) transient decrease in food consumption during the 3 days of drug delivery (mean percentage of food consumed, 64.5%  $\pm$  12.9%) in comparison to the 3 days following delivery (mean percentage of food consumed, 91.7%  $\pm$  16.3%); and 3) the presence of subcutaneous nodules at the drug delivery sites that regressed in size following delivery in all animals (Fig. S2). No lesions were detected via necropsy or histopathology in the dog that received vehicle. In the dog receiving 300 mg/kg GM6001 twice daily for 3 days, sites of subcutaneous drug deposition were surrounded by a connective tissue capsule with minimal inflammation; additionally, there was mild bile duct hyperplasia. The absence of substantial adverse events in this tolerance study suggested that GM6001 would have an acceptable safety profile in injured dogs.

### GM6001 is Rapidly Detected in Plasma After Subcutaneous Administration

As the PK of GM6001 might differ from that in rodent [40], we determined the PK in normal dogs. GM6001, administered once at 100 mg/kg SC, was detected in plasma at 5 minutes in all dogs, with a mean time to peak concentration ( $T_{max}$ ) of 0.7 hours (S.D.  $\pm$  1.3 hours) (Fig. 1). The mean peak concentration ( $C_{max}$ ) was 1370 ng/mL (S.D.  $\pm$  361 ng/mL), mean apparent elimination half-life ( $T_{1/2}$ ) was 524 hours (S.D.  $\pm$  428 hours), and the mean plasma concentration of GM6001 at 96 hours was 80 ng/mL (S.D.  $\pm$  20 ng/mL). Mean area under the curve (AUC) from time 0 to last observed concentration ( $AUC_{0-obs}$ ) (16,100 hr\*ng/mL  $\pm$  2981) and mean AUC from time 0 to infinity ( $AUC_{0-\infty}$ ) (58,225 hr\*ng/mL  $\pm$  37,054) resulted in an extrapolated percentage of AUC of 65%. The only notable adverse event was the presence of focal subcutaneous nodules that regressed with time. The GM6001 utilized in the clinical trial had marked *in vitro* MMP-9 inhibition at concentrations approximating those achieved in dog plasma 96 hours post-drug delivery (Fig. 2). As the objective was to target the acutely injured cord, we selected a single 100 mg/kg SC dose of GM6001 in dogs to achieve plasma drug concentrations which would peak almost immediately after delivery and be sustained at levels sufficient to inhibit MMPs *in vitro* for at least 96 hours following delivery.



**Figure 1. Pharmacokinetics of GM6001 in dogs.** Administration of a single 100 mg/kg subcutaneous dose of GM6001 to dogs resulted in the rapid development of peak plasma drug concentrations with drug still detectable 96 hours post-delivery. Administration of a second dose of GM6001 to a sub-group of dogs 12 hours following initial drug delivery resulted in increased plasma drug concentrations at all assessed time points. doi:10.1371/journal.pone.0096408.g001

### Clinical Trial Enrollment

Enrolled dogs were randomized to a saline placebo group ( $n = 38$  dogs), a DMSO group ( $n = 37$ ), and a GM6001 group ( $n = 33$ ) (Fig. 3). Three dogs were euthanized prior to discharge from the hospital due to neurologic deterioration and 17 dogs did not return for 42-day follow-up examination. Of critical importance, there were no differences in baseline population characteristics such as breed, gender, or injury level among treatment groups, indicating that confounding based on these parameters was unlikely (Table 1).

### Adverse Events in Spinal Cord Injured Dogs

Adverse events were recorded during hospitalization and were classified as fever, gastrointestinal, injection site, urinary, or other (Table S1). A significantly greater number of dogs in the GM6001 group had injection site reactions (45%; 15/33) relative to either the saline control dogs (5%; 2/38) or DMSO dogs (0%; 0/36) ( $P < 0.0001$ ; Kruskal-Wallis test). These reactions were transient and consisted of focal dermal and subcutaneous swelling.

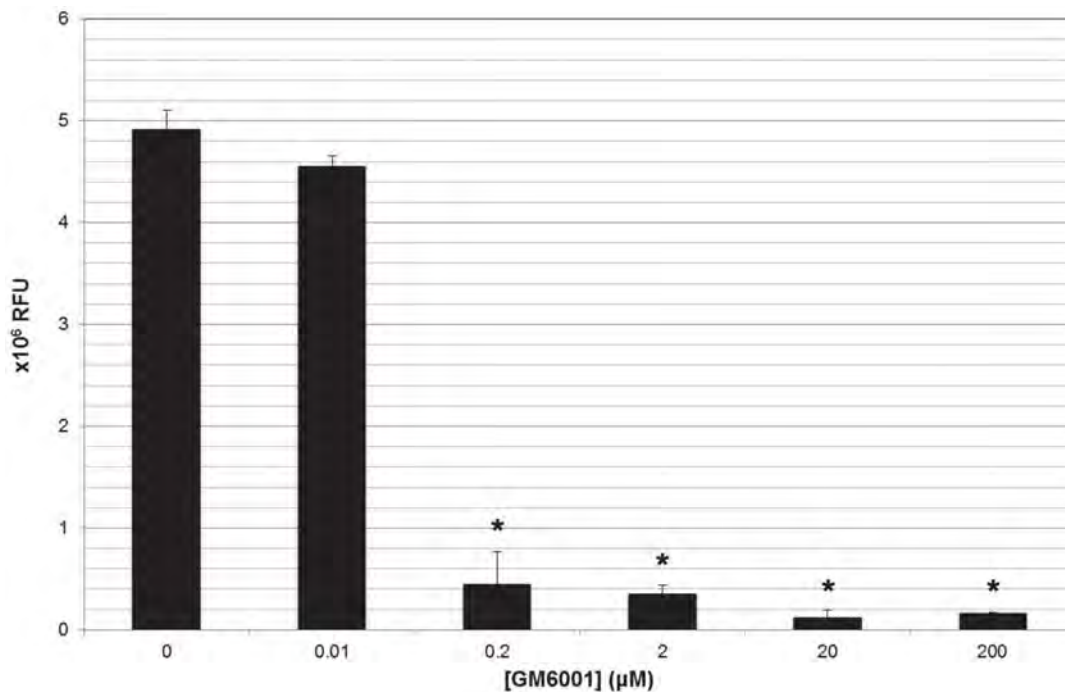
### Increased MRI T2W Signal within the Spinal Cord is Associated with Poor Recovery

Vertebral column MRI was performed on 76/107 dogs enrolled in the clinical trial. In all cases, spinal cord compression associated with IVDH was identified with variable presence of increased T2W signal (27/76 dogs) within the spinal cord (Fig. 4). High spinal cord T2 signal, suggestive of contusion, was significantly more common in dogs with severe SCIs (MFS = 0; 11/13) compared with those with mild-to-moderate SCIs (MFS > 0; 16/

63) ( $P = 0.0001$ ; Fisher's exact test). Dogs with increased T2W spinal cord also had significantly ( $P < 0.0001$ ; generalized linear model) poorer recovery of function 42 days following SCI (estimated TSCIS 9, 95% CI 7–12) compared to dogs with normal spinal cord T2W signal (estimated TSCIS 15, 95% CI 14–17). The presence of compressive SCI with variable presence of T2W hyperintensity in the spinal cord parallels what is found on MRI in humans with traumatic myelopathy, including relationships between function and spinal cord T2 signal [36].

### Characterization of Cells in CSF Following Spinal Cord Injury

CSF was acquired immediately prior to drug or placebo delivery in 102/107 (95%) clinical trial dogs; all 5 un-injured control animals also had CSF collected. Total nucleated cell count was significantly ( $P = 0.0034$ , Wilcoxon rank-sum test) higher in SCI dogs (median = 3 cells/ $\mu$ L, range 0–71) compared with control dogs (median = 0 cells/ $\mu$ L, range 0–1). Amongst dogs with CSF pleocytosis (total nucleated cell count > 5 cells/ $\mu$ L), neutrophils were most frequently identified (median 43%, range 2–89%), followed by mononuclear cells (median 25%, range 6–95%) and lymphocytes (median 18%, range 4–70%). CSF red blood cell count was likewise significantly ( $P = 0.0022$ , Wilcoxon rank-sum test) increased in dogs with SCI (median 48 cells/ $\mu$ L, range 0–15,040). Together, these findings support a pro-inflammatory state in the acutely injured canine spinal cord.



**Figure 2. *In vitro* inhibition of MMP-9 by GM6001.** Calibrated MMP-9 activity (Log 10<sup>6</sup>), as measured by a fluorescent electrophoretic technique, was dramatically attenuated by GM6001 *in vitro* at various concentrations. Plasma concentrations of GM6001, measured 96 hours following a single 100 mg/kg subcutaneous dose (range 0.16–.26 μM or 60–100 ng/mL), approximated those needed *in vitro* to robustly inhibit MMP-9 (0.2 μM or 77 ng/mL). Groups marked with an asterisk (\*) had significantly ( $P < 0.05$ ) different calibrated MMP-9 activity from reference (no GM6001) using a one tailed Student's t-test.

doi:10.1371/journal.pone.0096408.g002

### GM6001 Reduces Gelatinase Activity in Serum of Spinal Cord Injured Dogs

We utilized a fluorescent electrophoretic technique to determine if MMP-2/MMP-9 activity increases in CSF and serum in dogs with SCI and whether activity is reduced after treatment [37–39]. MMP-2/MMP-9 activity in the CSF did not differ between dogs with SCI and control dogs ( $P = 0.5011$ ; Wilcoxon rank-sum test) (Table S2, Fig. S3). Dogs with SCI had significantly ( $P = 0.0128$ ; Wilcoxon rank-sum test) higher serum MMP-2/MMP-9 activity prior to treatment compared with control dogs, but activity did not vary based on clinical factors or MRI features of SCI (Table 2, Fig. 5). Serum MMP-2/MMP-9 activity was significantly ( $P < 0.05$ ; generalized linear model) lower in dogs receiving GM6001 3 days following treatment compared to dogs receiving either DMSO or saline (Fig. 6). Thus, these findings confirm the effectiveness of GM6001 in reducing the abnormal elevation of MMP-2/MMP-9 in serum of spinal cord injured dogs.

### DMSO Enhances Recovery in Dogs with Severe Spinal Cord Injuries

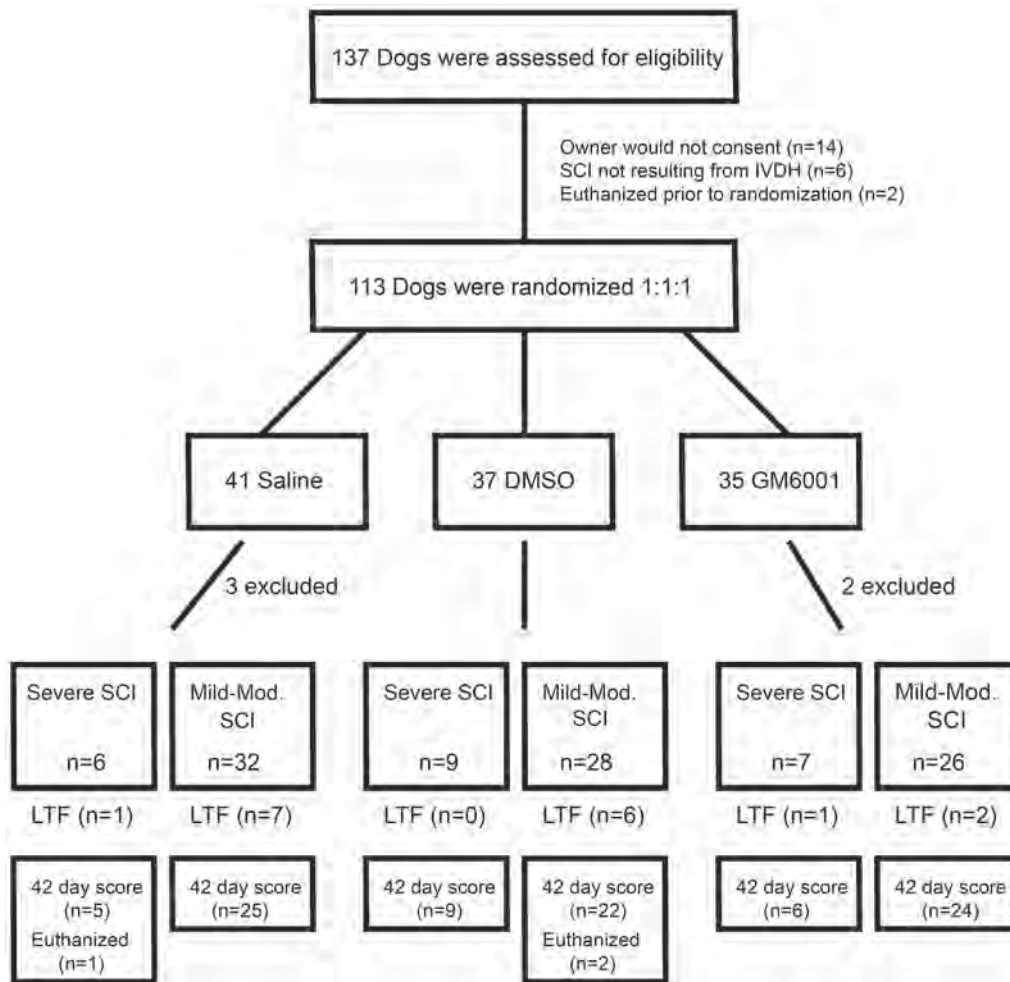
In dogs with mild-to-moderate SCI (i.e., MFS >0), there was robust recovery of function by 42 days with 64 of 71 (90%) dogs independently walking and 69 of 71 (97%) having intact pelvic limb nociception. Treatment group did not influence 3 or 42 day TSCIS (Fig. 7, Fig. S4). Dogs with mild-to-moderate SCI had significantly higher 42-day TSCIS (mean 15; 95% CI 12–18) compared to those with severe SCI (mean 7; 95% CI 4–9) ( $P < 0.0010$ ; generalized linear model).

In dogs with severe SCI (i.e., MFS = 0), those receiving either DMSO or GM6001 had significantly ( $P < 0.05$ ; generalized linear model) more robust functional recovery compared with those

receiving saline placebo (Fig. 7) at 42 days. Sub-components of the 42-day TSCIS were examined in dogs with severe SCI to better capture the influence of treatment on motor, sensory, and postural recovery. Sensory and postural scores did not differ significantly between treatment groups. Dogs receiving saline had an estimated mean motor score of 2 (95% CI 0–4.0) (suggesting absent to minimal pelvic limb movement with tail support) that was significantly ( $P < 0.05$ ; generalized linear model) less than the estimated mean motor score for dogs receiving DMSO (mean, 5; 95% CI 2.0–8.0) or GM6001 (mean, 5; 95% CI 2.0–8.0) (Fig. 8). The distribution of motor scores for both the DMSO and GM6001 treated dogs indicated that the majority of animals in these groups developed coordinated stepping movements with tail support and of those regaining movement many (6 of 12; 50%) walked without any support. Dogs that were treated with DMSO or GM6001 that regained pelvic limb movement typically (10 of 12; 83%) also recovered limb nociception. The extent of neurological recovery at 42 days post-injury was not significantly different in DMSO- and GM6001 (dissolved in DMSO)-treated groups. Such findings suggest that DMSO, rather than GM6001 contributed to enhanced recovery in these treatment groups.

### Discussion

This study was designed as a large-scale clinical trial to evaluate MMP inhibition in a clinically relevant, naturally occurring canine SCI model. Using advanced technology to measure activity of MMP-2/MMP-9, we show that these proteases are elevated in serum of dogs across all levels of injury severity and that GM001, given as a single bolus subcutaneously, significantly reduced this activity. Despite the effectiveness of GM6001 in targeting early MMP activity, both GM6001, solubilized in DMSO, and DMSO



\* Dogs were excluded from data analysis due to the fact that they were ambulatory when randomized, which was not permitted according to inclusion criteria (n= 4) or due to herniation of an adjacent disk during the course of the trial (n=1). Abbreviations: IVDH = intervertebral disk herniation; LTF = lost to follow-up; SCI = spinal cord injury.

**Figure 3. Consolidated Standards of Reporting Trials (CONSORT) Diagram.** Flow diagram depicting progress through different phases of the clinical trial including enrollment, group allocation, and follow-up. doi:10.1371/journal.pone.0096408.g003

alone produced similar levels of neurological improvement in dogs with severe SCIs, relative to saline controls. At 42 days post-injury, these dogs showed robust stepping movements that were visible with tail support and many independently ambulated; saline-treated dogs either showed no movement or had minimal limb advancement without stepping. Together, these findings demonstrate that early blockade of MMPs did not improve long-term neurological recovery. Rather, DMSO alone was responsible for the beneficial outcomes in dogs with severe SCIs.

The clinical trial described here was designed to include dogs with both severe (paraplegia and absent nociception) and mild-to-moderate SCIs (non-ambulatory with intact nociception) for several reasons. First, there is an abnormal elevation of MMP-9 in serum [20], CSF [20,41] and spinal cords of dogs [42] with IVDH across a spectrum of injury severities. Second, while long-term recovery of ambulation is common (64 of 71 dogs in this trial walked independently) in the mild-to-moderate injury group, few animals normalize with reference to motor or postural scores [43].

Thus, there is an opportunity, even within animals that are likely to show marked recovery, to examine the effect of therapeutics. We chose to stratify our population based on SCI severity to examine the effect of treatment on neurologic recovery. This approach was necessary given the well-known difference in outcome between these populations (approximately 85–95% of mildly-to-moderately and 50–60% of severely injured dogs recover independent ambulation) and the potential for differential activation of secondary injury pathways based on SCI severity [30–33]. Stratification based on SCI severity is common and accepted in human clinical trials because of expected differences in recovery between injury groups and the potential impact of this difference on evaluation of effectiveness of therapies [44,45].

GM6001 is a broad-spectrum MMP inhibitor that has been shown to exert neuroprotection in rodent models of brain and SCIs, primarily via antagonism of MMP-9 associated with neutrophils [1]. Evidence supporting this position includes a temporal association between neutrophil trafficking and MMP-9

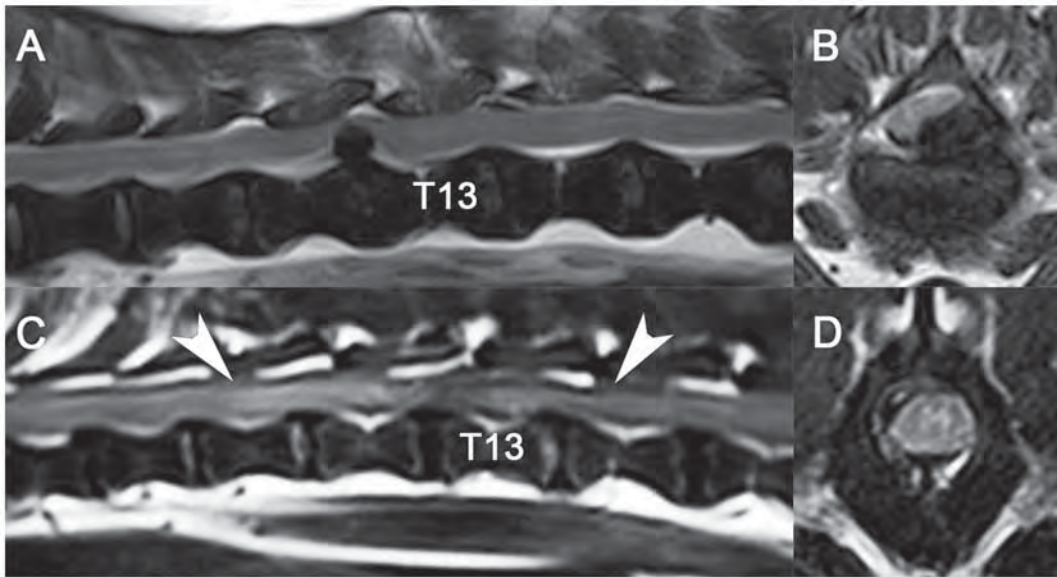
**Table 1.** Baseline characteristics did not differ significantly among treatment groups.

<i>A. Continuous variables: Medians (range); P values from Kruskal-Wallis testing</i>				
Variable	Saline Controls (N = 38)	DMSO (N = 33)	Drug+DMSO (N = 33)	P value
Age (years)	5 (2 to 13)	5 (3 to 13)	5 (2 to 14)	0.9833
Duration of signs prior to admission (hours)	24 (1 to 48)	18 (4 to 36)	12 (2 to 48)	0.2246
MFS*	2 (0 to 3)	2 (0 to 3)	2 (0 to 3)	0.7409
TSCIS#	4 (0 to 10)	4 (0 to 11)	4 (0 to 10)	0.5907
<i>B. Categorical variables: P values from chi-squared testing</i>				
Variable	DMSO (N = 38)	DMSO (N = 37)	Drug+DMSO (N = 33)	P value
Sex				
Female	61% (23/38)	53% (19/36)	39% (13/33)	0.3039
Male	39% (15/38)	47% (17/36)	61% (20/33)	
Neutered				
No	16% (6/38)	22% (8/36)	18% (6/33)	0.9078
Yes	84% (32/38)	78% (28/36)	82% (27/33)	
Breed				
Dachshund	71% (27/35)	61% (22/36)	85% (28/33)	0.1560
Other	29% (8/35)	39% (14/36)	15% (5/33)	
Chondrodystrophoid				
Yes	89% (34/38)	86% (31/36)	88% (29/33)	0.9628
Other	11% (4/38)	14% (5/36)	12% (4/33)	
Level of Spinal Cord Injury				
T12-T13	34% (13/38)	36% (13/36)	24% (8/33)	0.6986
T13-L1	29% (11/38)	25% (9/36)	33% (11/33)	0.8705
L1-L2, L2-L3, or L3-L4	29% (11/38)	33% (12/36)	24% (8/33)	0.8390
T2-Weighted Hyperintensity (only available for 76 dogs)				
Absent	62% (18/29)	76% (22/29)	50% (9/18)	0.3225
Present	38% (11/29)	24% (7/29)	50% (9/18)	

Panel A summarizes continuous variables using medians (ranges) by group, with P values from Kruskal-Wallis tests; panel B describes categorical variables using proportions by group with P values from chi-squared testing.

\* MFS = Modified Frankel Score; # TSCIS = Texas Spinal Cord Injury Score.

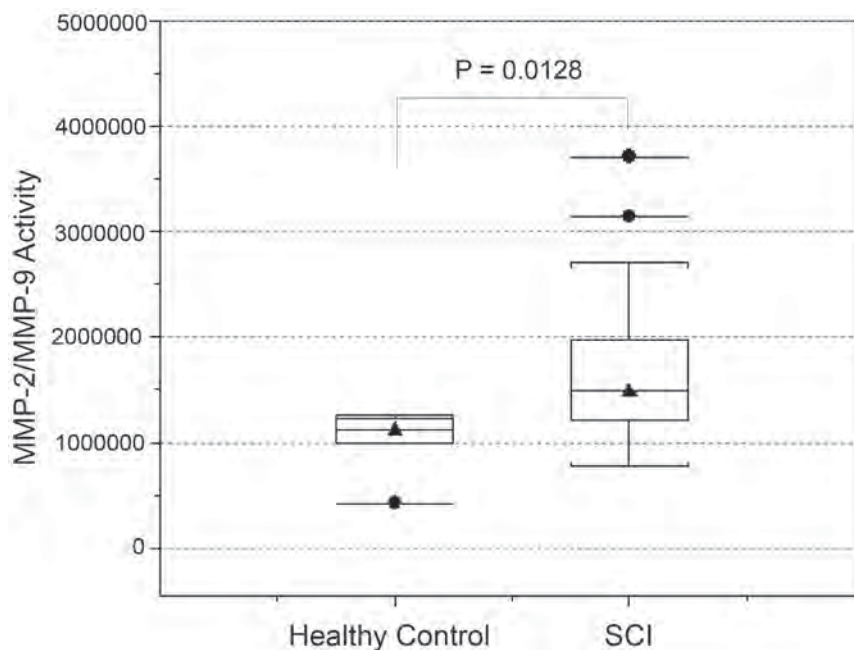
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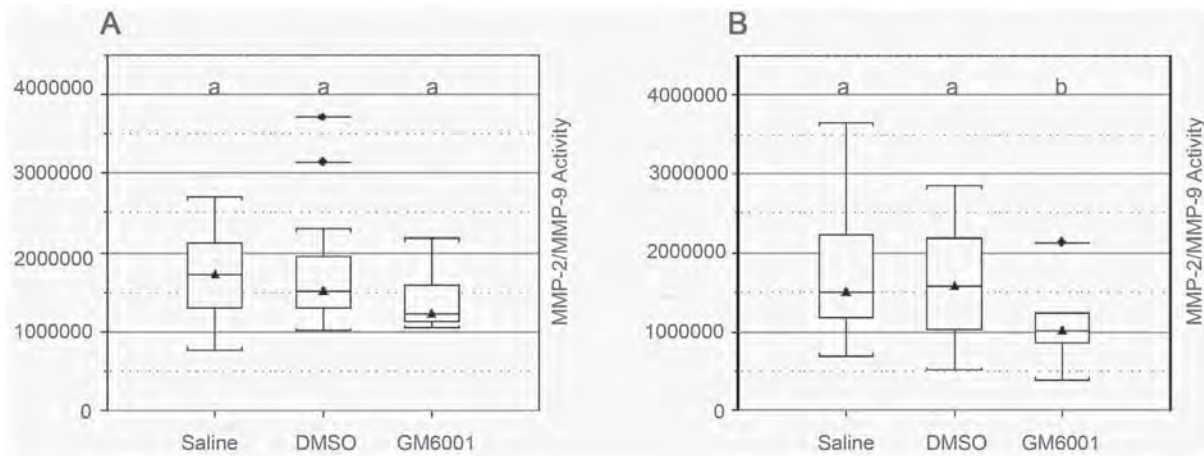
**Figure 4. T2-weighted magnetic resonance images in dogs with spinal cord injuries from intervertebral disk herniation.** In 1 dog (A, B) that was non-ambulatory with intact pelvic limb movement and sensation, there was focal ventrolateral spinal cord compression at the T12-T13 vertebral articulation without spinal cord signal change. A second dog (C, D) with paraplegia and absent pelvic limb deep nociception had compression at the T12-T13 vertebral articulation. There was extensive spinal cord T2-weighted hyperintensity (white arrows) visible on the sagittal image (C), suggestive of processes seen in contusion injuries such as edema, necrosis, hemorrhage, or cellular infiltrates. The transverse image (D, level of T13 vertebral body) indicated that T2-weighted hyperintensity was predominantly localized to the gray matter.  
doi:10.1371/journal.pone.0096408.g004

expression, reduced expression of MMP-9 in spinal cord injured mice that are neutrophil-depleted, and reduced neutrophil content within injured spinal cords of MMP-9 null mice [2,8,46]. In this

study, GM6001 was delivered SC using DMSO as a vehicle. While the high prevalence of injection site reactions and route of administration may have altered drug absorption in comparison to



**Figure 5. Serum MMP-2/MMP-9 activity in healthy and injured dogs.** Box-and-whisker plots summarizing the distribution of MMP 2/9 activity for healthy control dogs (N=5) and dogs with spinal cord injury (SCI; N=42) that had serum collected. Values of serum MMP 2/9 activities were significantly ( $P=0.0128$ ) greater for dogs with SCI included in the trial than control dogs. The horizontal lines with triangles represent the median value; the horizontal lines at the bottom and top of the boxes represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles of the data, respectively. The thin vertical lines extending up or down from the boxes to horizontal lines (so-called whiskers) extend to a multiple of  $1.75 \times$  the distance of the upper and lower quartile, respectively. Horizontal lines with circles represent values outside the limits of the whiskers.  
doi:10.1371/journal.pone.0096408.g005



**Figure 6. Serum MMP-2/MMP-9 activity pre- and post-drug delivery.** Dogs were first randomized into 3 treatment groups, and serum was obtained prior to administration of saline, DMSO, or GM6001 (panel A). There were no differences in serum MMP-2/MMP-9 activity among treatment groups at the time of admission (panel A); however, at day 3, there was a significant ( $P = 0.0482$ ) difference in serum activity between GM6001-treated dogs and the other treatment groups (panel B). See Figure 5 for a description of box-and-whisker plots. Groups marked with different letters differ significantly ( $P < 0.05$ ).

doi:10.1371/journal.pone.0096408.g006

studies in other species, our data support favorable PK via SC administration of GM6001. Furthermore, relatively small plasma concentrations of GM6001 present 3 days post-delivery appear capable of modulating MMP-2/MMP-9 activity in study dogs.

Here we studied the effects of GM6001 on MMP-2/MMP-9 activity in serum. While there was no relationship between injury severity and level of MMP-2/9 activity in serum, spinal cord injured dogs showed an increase in these proteases relative to healthy controls. Moreover, the early elevation of serum MMP-2/MMP-9 activity was significantly reduced following treatment with

GM6001, a finding which serves to confirm the effectiveness of the drug in reducing proteolytic activity.

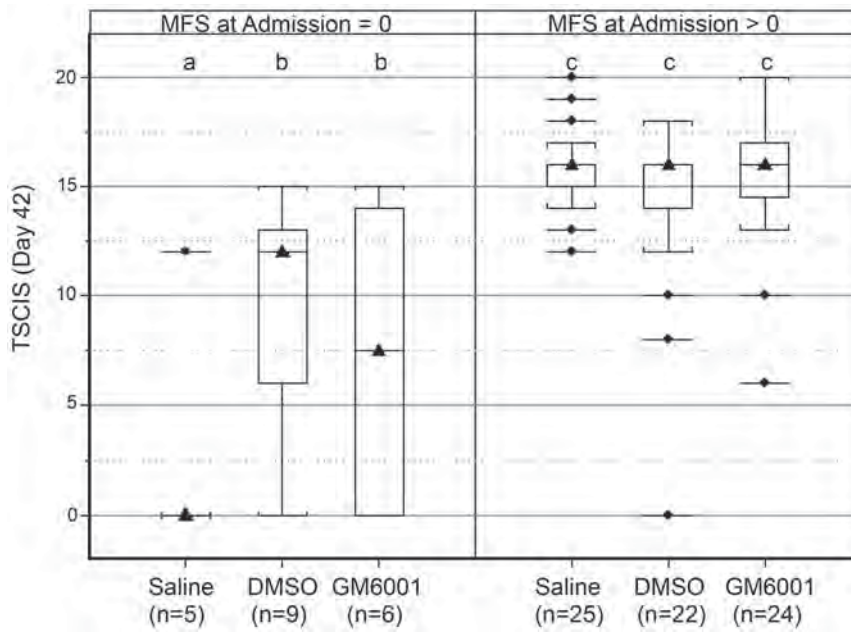
While MMP-2/MMP-9 activity was detected in the CSF of injured dogs, activity did not differ between healthy control dogs and those with SCI. The lack of a demonstrable difference in CSF MMP-2/MMP-9 between SCI and control groups may reflect the inability of this assay to distinguish between the 2 proteases. Based upon an earlier study using gelatin zymography [20], MMP-2 was found to be expressed in the CSF of normal dogs and remained unchanged after SCI. In contrast, MMP-9 was only detected in

**Table 2. Values of serum MMP-2/MMP-9 activity were not significantly associated with various clinical variables.**

Variable	Median (Range) of MMP-2/MMP-9 Activity in Serum		P value
Age	$\leq 5$ Years (N = 28)	$> 5$ Years (N = 12)	0.1458
	1,519,998 (1,009,205–3,708,449)	1,315,180 (769,191–2,700,981)	
Sex	Male (N = 22)	Female (N = 18)	0.2625
	1,504,968 (1,009,205–3,708,449)	1,455,587 (769,191–2,700,981)	
Neutered	Not neutered (N = 9)	Neutered (N = 31)	0.5881
	1,492,947 (1,009,205–2,266,638)	1,479,017 (769,191–3,708,449)	
Breed	Dachshund (N = 28)	Other (N = 12)	0.4932
	1,411,546 (769,191 - 3,144,395)	1,528,755 (941,766 - 3,708,449)	
Chondrodysplastic	Yes (N = 38)	Other (N = 2)	0.2564
	1,474,618 (769,191–3,708,449)	1,983,362 (1,975,379–1,991,345)	
Duration of clinical signs prior to admission	$\leq 12$ hours (N = 12)	$> 12$ hours (N = 28)	0.9189
	1,453,961 (1,009,205 - 3,708,449)	1,485,982 (769,191–3,144,395)	
	$\leq 24$ hours (N = 35)	$> 24$ hours (N = 5)	0.5243
	1,492,947 (941,766–3,708,449)	1,310,144 (769,191–2,306,259)	
T2-weighted hyperintensity	Absent (N = 20)	Present (N = 11)	0.6995
	1,507,977 (769,191–3,708,449)	1,523,790 (1,062,500–3,144,395)	
MFS at admission	$\leq 2$ (N = 22)	$> 2$ (N = 18)	0.4924
	1,455,587 (1,009,205 - 2,292,539)	1,504,968 (769,191–3,708,449)	

Medians (and ranges) and P values derived from Wilcoxon-rank sum tests are reported for the categorical variables listed above. MMP = matrix metalloproteinase; MFS = Modified Frankel Score.

doi:10.1371/journal.pone.0096408.t002

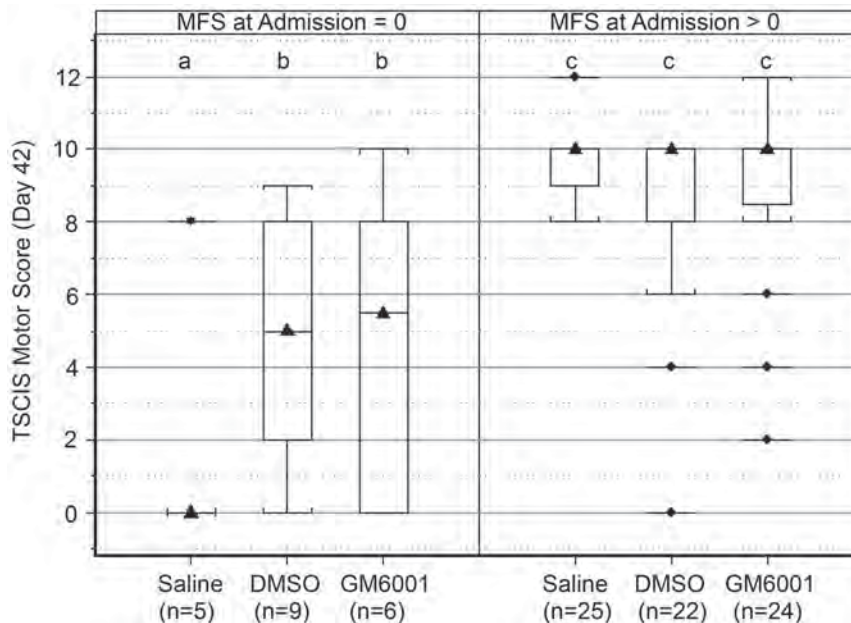


**Figure 7. Evaluation of primary outcome in dogs with SCI.** Box-and-whisker plots of TSCIS on day 42 by treatment group, stratified by MFS at admission (MFS=0, left panel; or MFS >0, right panel). There were no significant differences in TSCIS among dogs with MFS score >0 (right panel), but TSCIS was significantly ( $P<0.05$ ) greater for the GM6001 and the DMSO group than saline treated dogs with MFS=0 (left panel). See Figure 5 for a description of box-and-whisker plots. Groups marked with different letters differ significantly ( $P<0.05$ ). doi:10.1371/journal.pone.0096408.g007

spinal cord injured dogs [20,41]. Thus, in the current study, the absence of any differences between injured and control dogs may have been confounded by the constitutive activity of MMP-2 in CSF that may have masked any increase in MMP-9.

There are likely a number of possible explanations for why GM6001 failed to improve neurological recovery in spinal cord

injured dogs. First, while GM6001 has been shown to improve neurological outcomes in various rodent models of brain and spinal cord injury [2,47,48], no studies to date have evaluated efficacy in dogs. Thus, there may be species differences in responsiveness to GM6001 and/or MMP-directed pathogenesis. Additionally, effects of GM6001 demonstrated in rodents may not



**Figure 8. Evaluation of TSCIS motor score at day 42 following SCI.** Box-and-whisker plots of TSCIS motor score on day 42 by treatment group, stratified by MFS at admission (MFS=0, left panel; or MFS >0, right panel). There were no significant differences in motor score among dogs with MFS >0 (right panel), but motor score was significantly ( $P<0.05$ ) greater for the GM6001 and the DMSO group than saline treated dogs with MFS=0 (left panel). See Figure 5 for a description of box-and-whisker plots. Groups marked with different letters differ significantly ( $P<0.05$ ). doi:10.1371/journal.pone.0096408.g008

be sufficiently robust to positively influence outcome under the clinical conditions of this study [49,50]. Second, the drug was active beyond the first several days post-injury and as such could have interfered with mechanisms underlying recovery in SCI. Pharmacokinetics in healthy dogs demonstrated that plasma concentration of GM6001, present at even the 96-hour time-point, approximated or exceeded that necessary to block MMP-9 *in vitro*. As some MMPs modulate the formation of a glial scar and axonal plasticity [4], their subacute/chronic blockade may result in adverse neurological outcomes. Third, the timing between SCI and administration of GM6001 may not have been optimal. The strong association between MMP-9 expression and neutrophils suggests that an optimal therapeutic window for GM6001 is defined by the early trafficking of neutrophils into the injured cord. Such a position is supported by evidence of pronounced neurological recovery when the drug was given beginning 3 hours post-injury in a murine model of SCI [2]. In dogs treated with GM6001, median delay between injury and enrollment was 12 hours, which may have exceeded the window of efficacy for GM6001. Finally, while the use of dogs with thoracic and lumbar spinal cord lesions could have influenced our ability to detect drug-related effects [51], the proportion of dogs with lumbar lesions was similar amongst treatment groups (Table 1). Additionally, the inclusion of lesion location (lumbar versus thoracic) in multivariable generalized linear modeling did not alter the significance or magnitude of observed treatment effects (data not shown).

We found that DMSO improved motor recovery in dogs with severe SCIs. This finding is perhaps not too surprising as DMSO, under defined dosing conditions, has the ability to function as a neuroprotectant [14] and in some cases when used as a vehicle, may be synergistic. In the setting of neurotrauma, neuroprotection is exemplified in a study by Di Giorgio et al [52] which compared the antioxidant curcumin,  $\alpha$ -tocopherol, DMSO and saline in a model of traumatic brain injury. These authors reported similar levels of early neuroprotection across all agents relative to the saline control group. Beneficial effects of DMSO might also be indicated in studies where DMSO is used as a vehicle without any additional negative control group. For example, in a recent study the efficacy of an epidermal growth factor receptor inhibitor was assessed in a rodent model of SCI [53]. This inhibitor was compared against its vehicle, DMSO. Recovery of motor and bladder function was significantly greater in rats that received DMSO relative to the inhibitor. The authors concluded that the receptor inhibitor showed no efficacy relative to the “baseline” values as defined by DMSO. Based upon our study, an alternative explanation is that DMSO did not serve as the “baseline” but rather may have exerted a beneficial effect. Finally, DMSO, when co-administered with a candidate therapeutic, offers potential for synergism, by acting through separate and/or overlapping pathways. While we found no evidence of this in the current study, others have reported synergism in a model of brain ischemia where DMSO was either combined with fructose 1,6-disphosphate, an intermediate of anaerobic metabolism, or prostacyclin (PG<sub>12</sub>) which blocks aggregation of platelets and functions as a vasodilator [12,54].

The mechanisms underlying DMSO-mediated neuroprotection have been attributed to its ability to function as a free radical scavenger and suppress a variety of pathobiologic events including inflammation, calcium influx, and glutamate excitotoxicity [14]. Such broad-based, temporally-defined targets may account for the extended window of efficacy (within 48 hours of injury) in spinal cord-injured dogs.

Dogs with severe SCIs and treated with either DMSO or GM6001 in DMSO showed a consistent (>80%), improvement in

pelvic limb stepping. A critical question is whether this stepping was voluntary or mediated through the central pattern generator (i.e., spinal stepping). The vast majority of these dogs with motor recovery also regained pelvic limb nociception (10 of 12; 83%) and 50% walked independently (without tail support) when evaluated at 42 days post injury. These data would argue that pelvic limb movement was indeed voluntary in the majority of dogs with severe SCIs treated with either DMSO or GM6001.

In summary, while this study and others [12,13,52,54] underscore the potential utility of DMSO for the treatment of brain and SCI, there remain conflicting reports about the efficacy of DMSO. This is illustrated in recent studies reporting either no effects or reduced performance on behavioral tests after traumatic brain injury [55] and others suggesting improved learning ability in cerebellar mutant Lurcher mice [56]. As it is shrouded in controversy as a therapeutic yet commonly used as a vehicle, there is a need to rigorously evaluate DMSO from the standpoint of safety, dosing, and efficacy. Given that it is a common vehicle for drug delivery, there is opportunity to evaluate its synergistic properties. Such logic has been successfully applied to the treatment of human interstitial cystitis, where DMSO is given as part of multimodal regimen [57]. With FDA approval for the treatment of interstitial cystitis, there is potential for the repurposing of DMSO, capitalizing on its favorable properties as a solvent, in developing combinatorial therapies for SCI. The current study suggests that DMSO has an extended therapeutic window (up to 48 hours). As time to treatment for human SCI may be delayed for up to 1 to 3 days post-injury [58], a broader therapeutic window could potentially expand the population of spinal cord injured patients that would otherwise not qualify for treatments with more restricted windows of intervention.

## Supporting Information

**Figure S1** Rectal body temperature in healthy dogs delivered DMSO or GM6001. All dogs delivered GM6001 at 6–18 times the cumulative clinical trial dose (100–300 mg/kg six times) experienced body temperature elevations beyond normal. The elevation in body temperature qualitatively appeared greatest in dogs receiving higher doses of GM6001.

(TIF)

**Figure S2** Drug delivery site diameters in healthy dogs receiving GM6001. Delivery site diameter appeared greatest one day after administration (panel A) and diminished by day 8 post-administration (panel B) in dogs receiving 6–18 times the cumulative clinical trial dose of GM6001.

(TIF)

**Figure S3** Cerebrospinal fluid MMP-2/MMP-9 activity in healthy and spinal cord injured dogs. Although MMP-2/MMP-9 activity tended to be higher in dogs with spinal cord injuries (n = 40) than healthy controls (n = 5), the difference was not significant (P = 0.5011; Wilcoxon rank-sum test).

(TIF)

**Figure S4** Texas Spinal Cord Injury Score (TSCIS) on day 3 following spinal cord injury. There were no significant differences in TSCIS based on treatment group for dogs with severe (MFS = 0) and mild-to-moderate (MFS >0) spinal cord injuries. Box-and-Whiskers with different letters differ significantly (P < 0.05).

(TIF)

**Table S1** Frequency of adverse events and survival (died or euthanized) by treatment group. Injection site reactions were

significantly associated with GM6001 delivery, but no other adverse events were significantly associated with treatments. (DOCX)

**Table S2** Cerebrospinal MMP-2/MMP-9 activity in dogs with spinal cord injury. Cerebrospinal fluid MMP-2/MMP-9 activity in dogs with spinal cord injury was not significantly associated with signalment, duration of clinical signs, or MFS at the time of admission. Medians (and ranges) and P values derived from Wilcoxon-rank sum tests were reported for the categorical variables. (DOCX)

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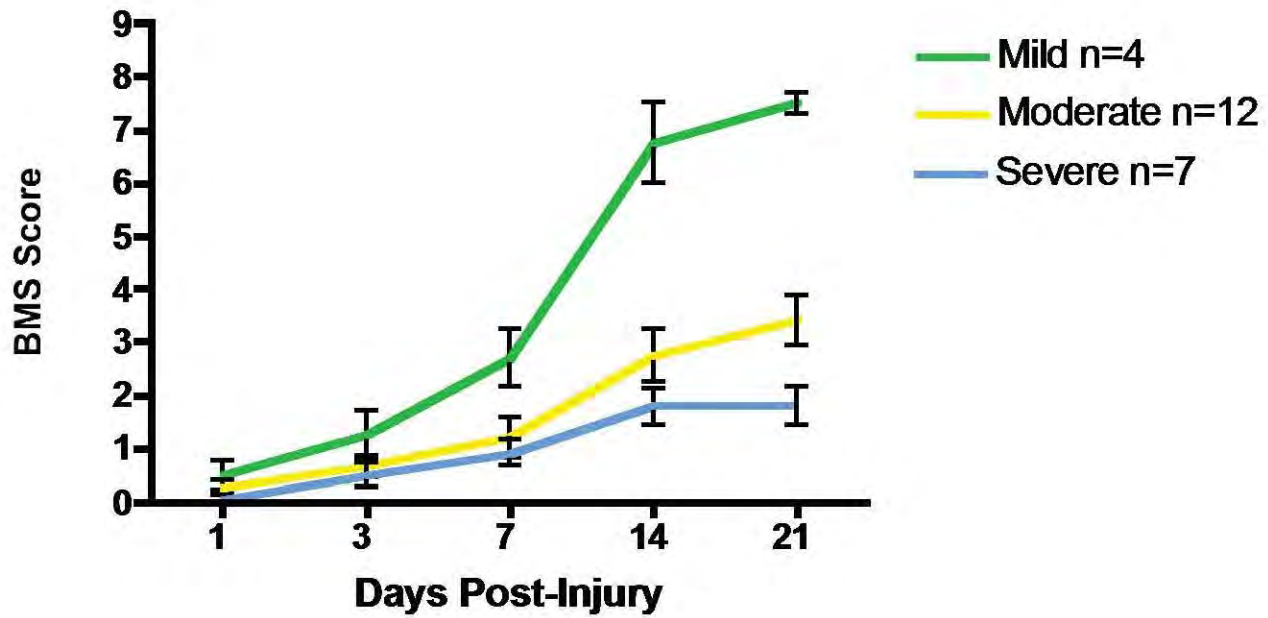
## Author Contributions

Conceived and designed the experiments: JML NDC MH VRF GJL AAT AM ZW LJN. Performed the experiments: JML GJL SCK AM LJN. Analyzed the data: NDC VRF. Contributed reagents/materials/analysis tools: JML MH AM. Wrote the paper: JML NDC MH VRF GJL SCK AAT TMF ZW AM LJN.

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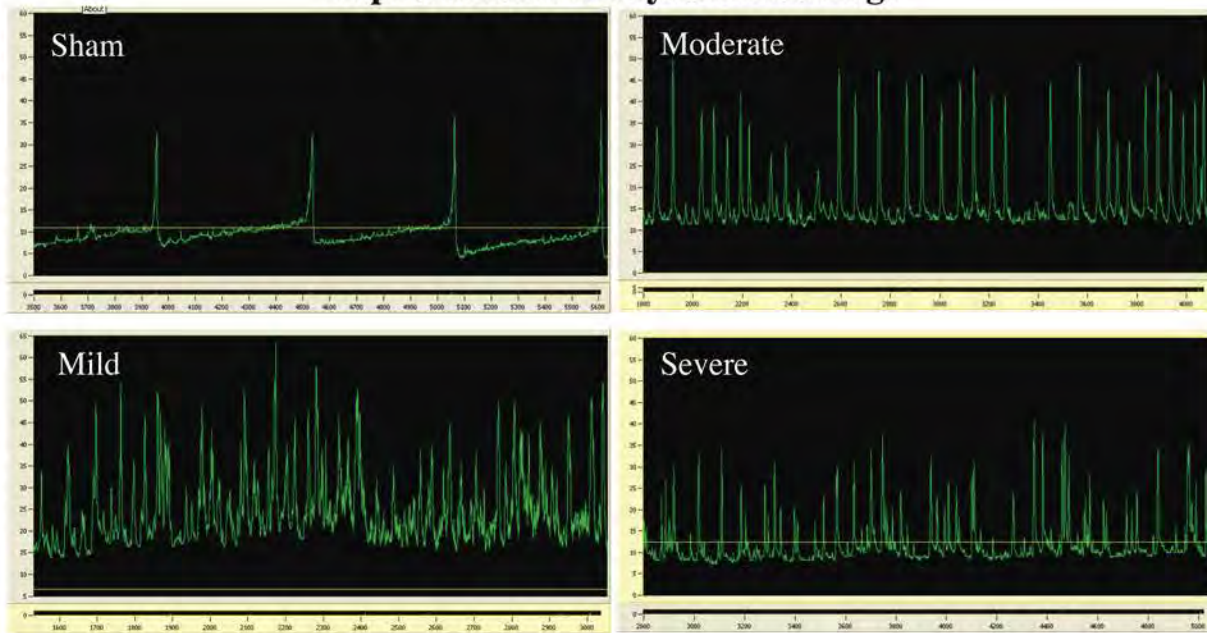
## SUPPORTING DATA: UCSF

**Figure 1:** Production of a graded reproducible model of spinal cord injury in the mouse. Two-way repeated measures ANOVA showed significant interaction ( $p < 0.0001$ ), significant effect of time ( $p < 0.0001$ ) and significant effect of injury severity ( $p = 0.0002$ ).



**Figure 2:** Uninhibited bladder contractions after mild, moderate, or severe spinal cord injury. Note the relative abundance of these contractions after mild injury relative to the more severely injured mice.

### Representative urodynamic tracings



Cystometries were performed using restrained mice and a PE10 catheter with an infusion speed of 10 microliters/ minute.

**Figure 3: Top Panel:** Quantification of uninhibited bladder contractions after a mild and moderate spinal cord injury. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, there were significantly increased numbers of bladder contractions after mild or moderate injury relative to the sham control group. **Bottom Panel:** Quantification of residual urine. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, moderate injury severity had more residual urine as compared to shams.

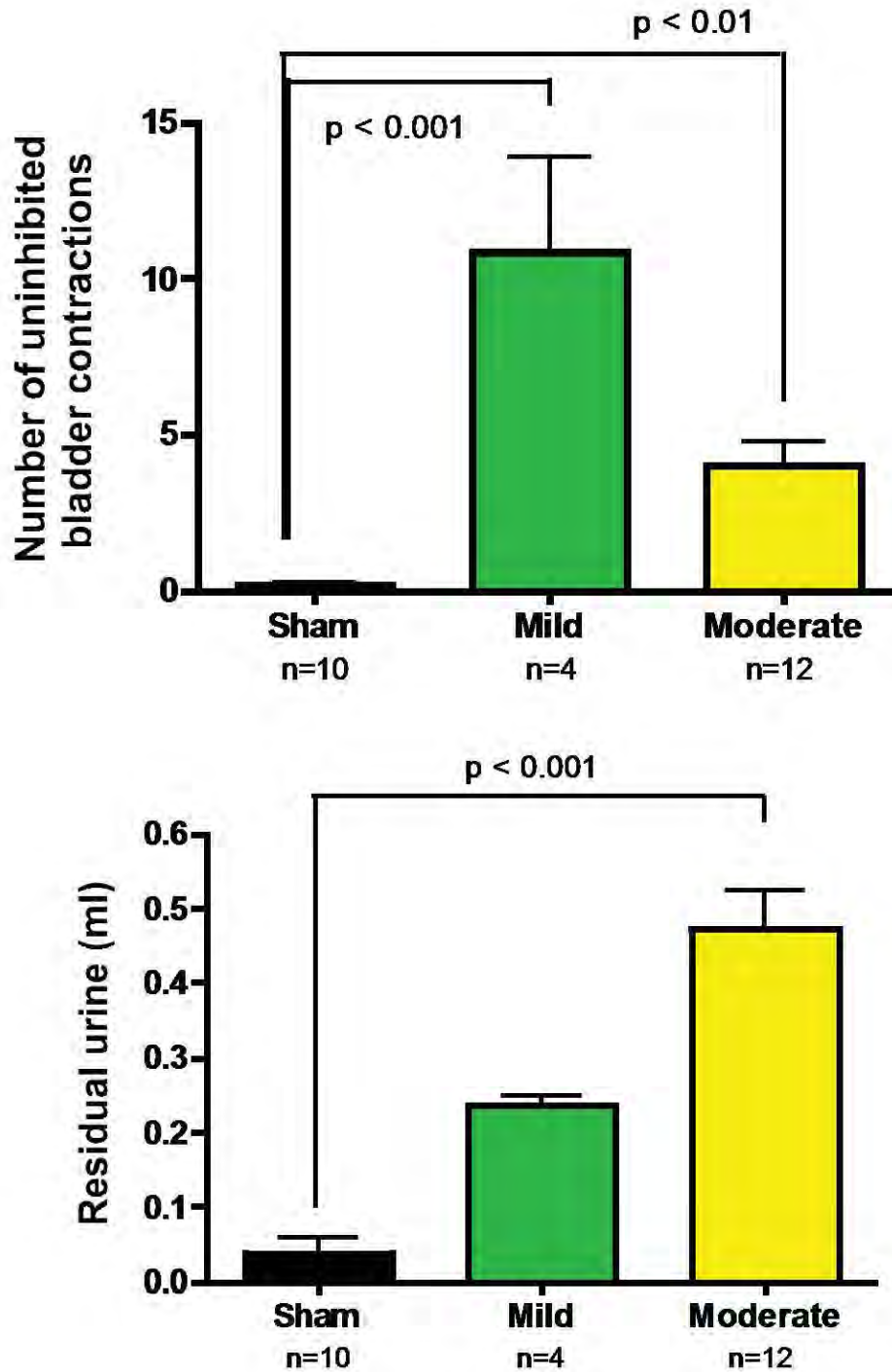
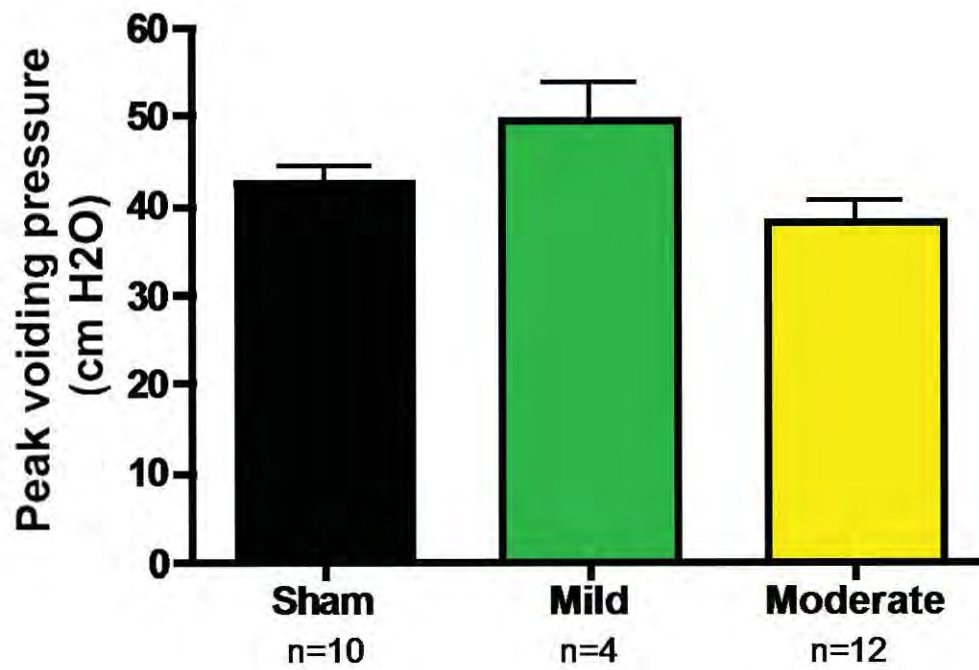
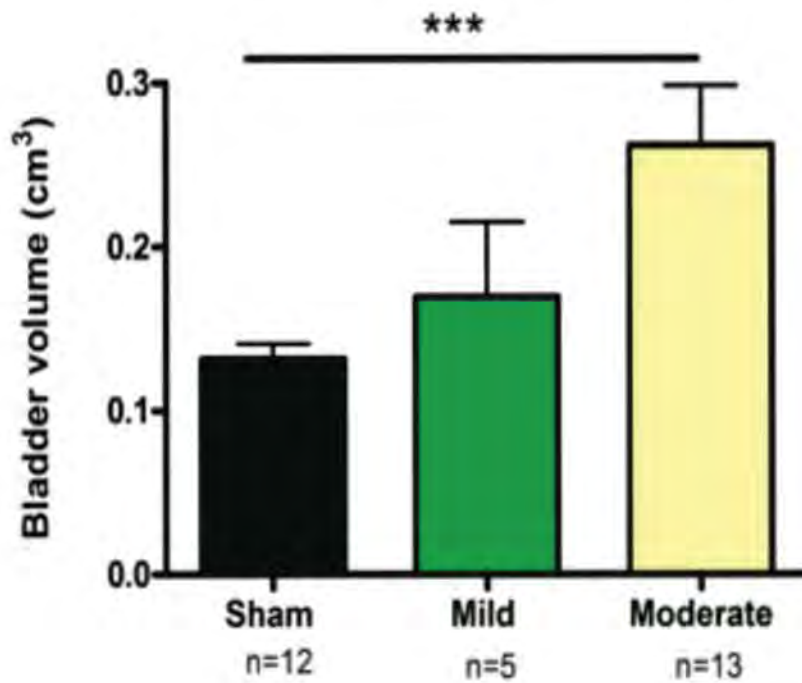


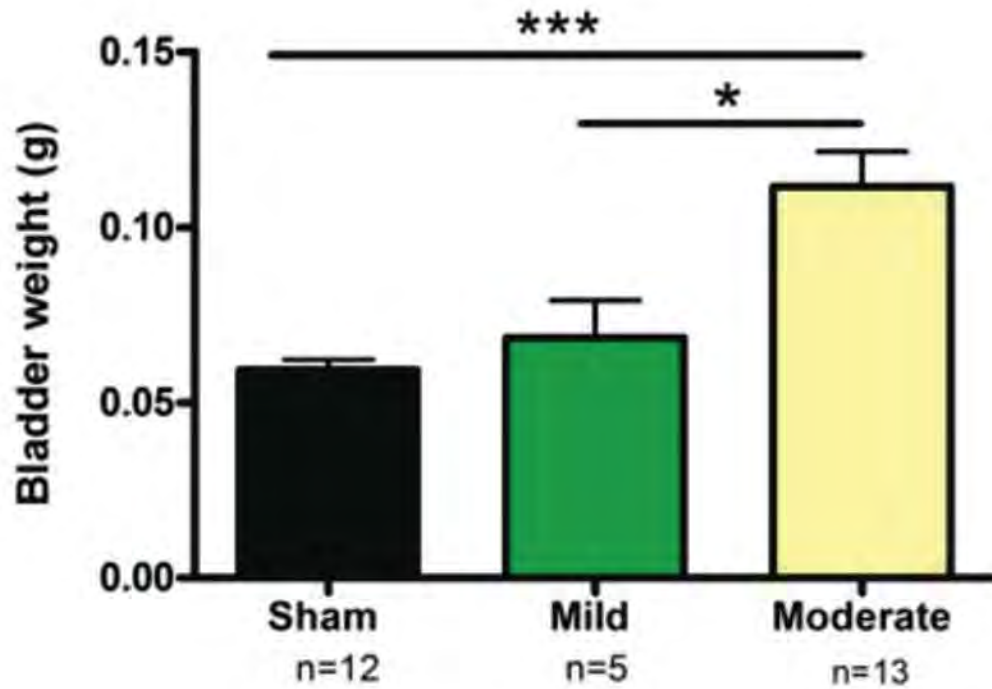
Figure 4: Peak pressure revealed no differences between groups (Kruskal–Wallis test,  $p > 0.05$ ).



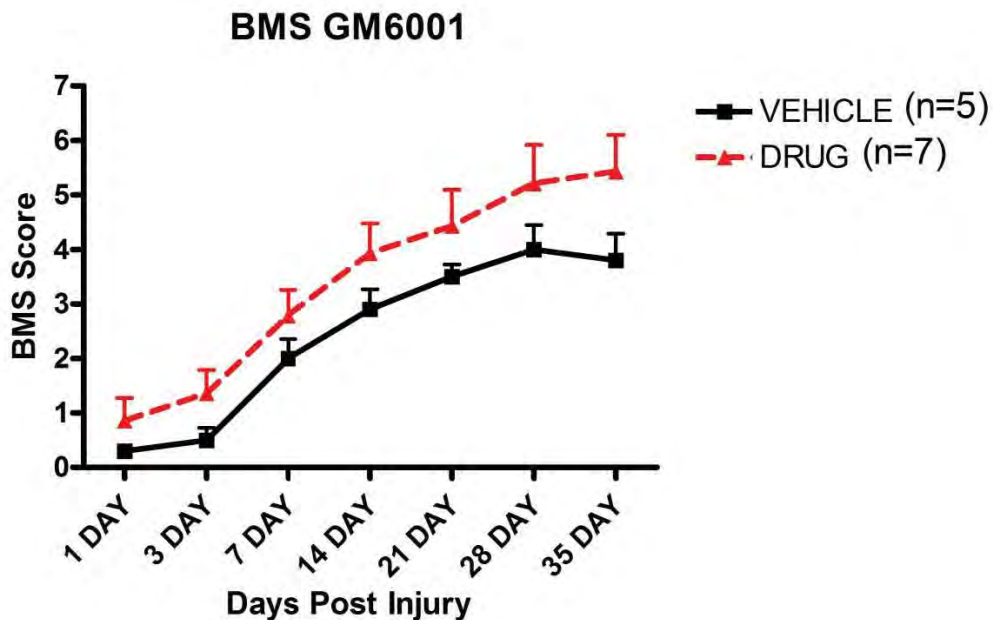
**Figure 5:** Bladder volume significantly increased after a moderate injury whereas there are no differences between mild and sham controls. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, \*\*\*P<0.001



**Figure 6:** Bladder weight incrementally increases with injury severity. Kruskal–Wallis test followed by Dunn's Multiple Comparison Test, \*\*\*P<0.001, \*P<0.05



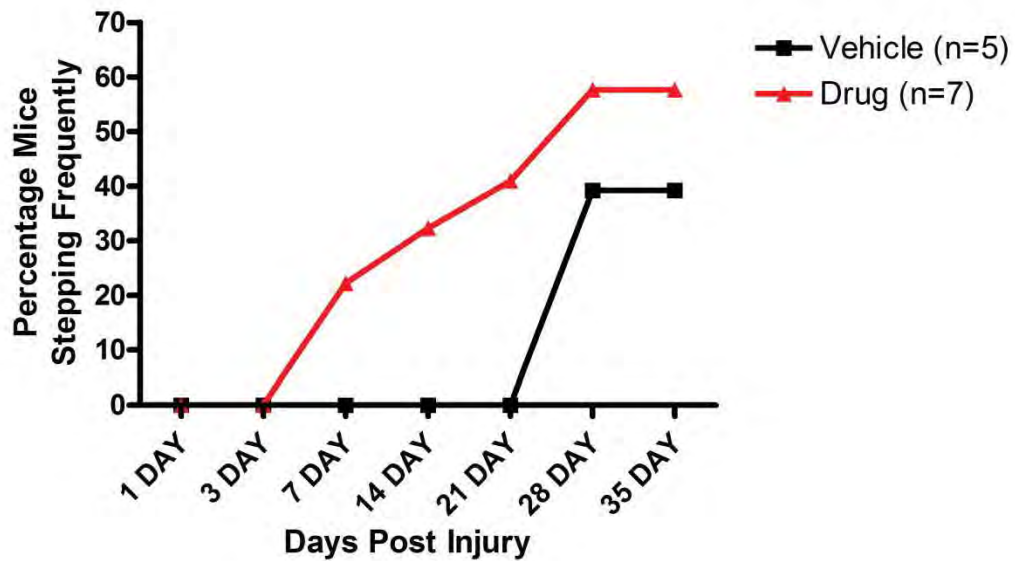
**Figure 7:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or 4% carboxy methyl cellulose beginning 8 hours post injury and every 12 hours thereafter for 3 days (route of delivery, intraperitoneal). While both groups improved locomotor function with time, there were no differences between drug and vehicle, based upon the BMS score. Two-way repeated measures ANOVA of BMS score revealed the following:  $P= 0.58$  for interaction,  $P<0.0001$  for time, and  $P= 0.16$  for treatment.



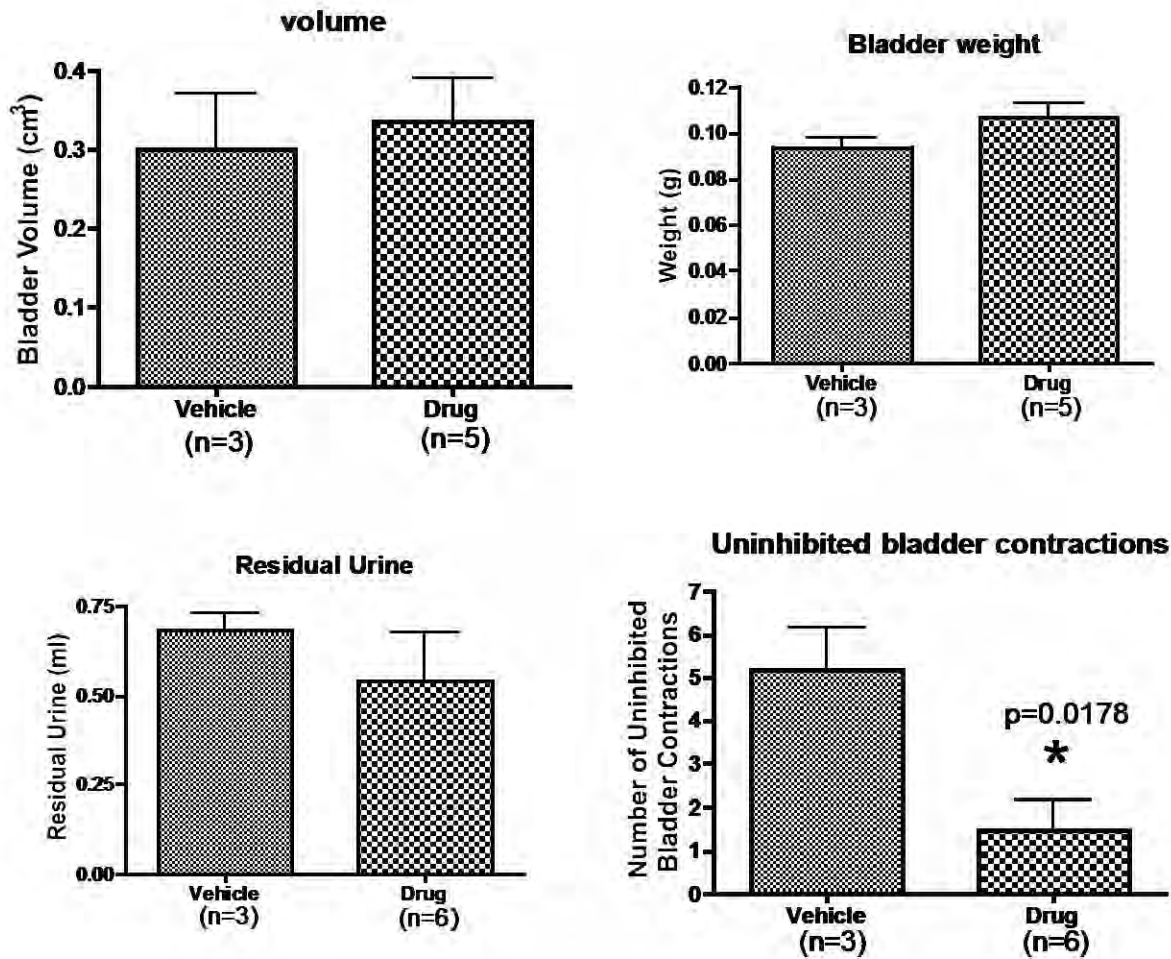
**Figure 8:** Comparison of initial BMS scores at 1 day versus 35 days, revealed a significant improvement in the GM6001-treated group. Student T-test, \* P=0.025



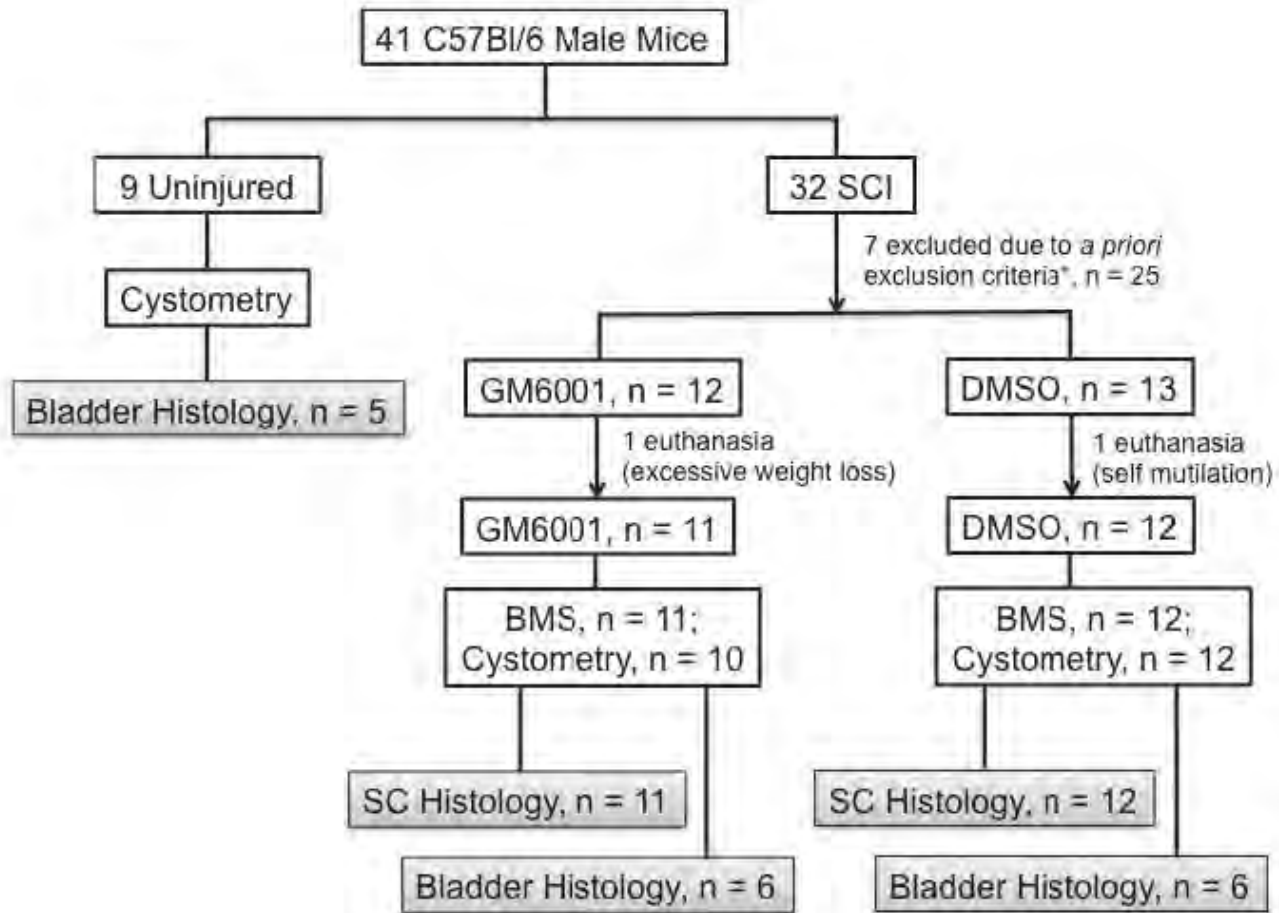
**Figure 9:** Evaluation of percentage of mice, subjected to a moderate spinal cord injury and GM6001 treatment show hindlimb stepping over time. Two way ANOVA, no significant interaction, significant effect of both treatment ( $P= 0.017$ ) and time ( $P= 0.015$ ).



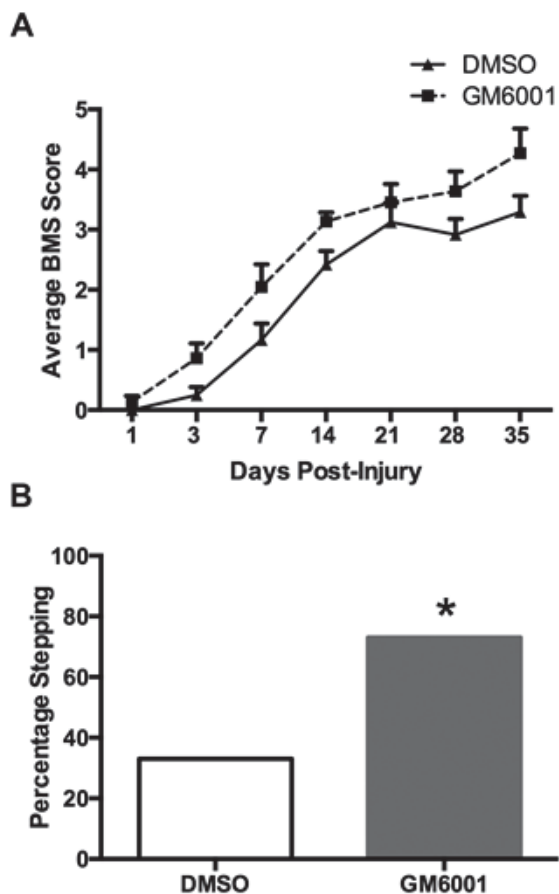
**Figure 10:** Analysis of bladder function after drug treatment in moderate spinal cord injury in mice. Cystometry was conducted in those animals that were also evaluated for neurologic function (Refer to Figures 7-9). There were signature reductions in number of uninhibited bladder contractions (Unpaired Students T-test).



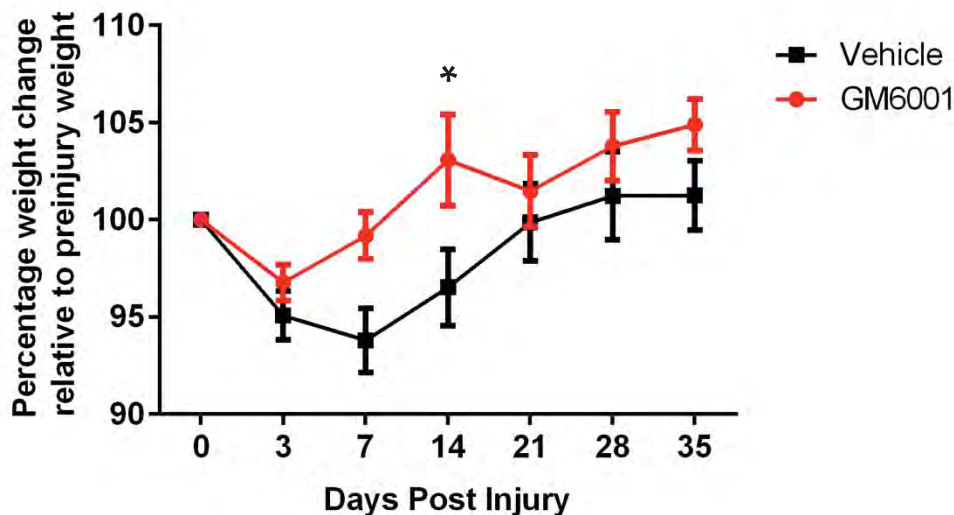
**Figure 11:** Number of mice used for neurologic and urologic behavior testing and for immunohistochemistry. \*We had set a priori exclusion criteria: any animal showing extensive ankle movement of either one or both hind limbs (average score >0.5) at eight hours post-injury would be removed from the study. Seven mice fell into this category. Two additional animals with adverse events were removed from the study, one due to weight loss and another one due to self-mutilation. In the drug treatment group, one mouse did not undergo cystometry for technical reasons, but was included in all other outcome measures. Abbreviations: SCI (spinal cord injury), BMS (Basso Mouse Scale), and SC (spinal cord).



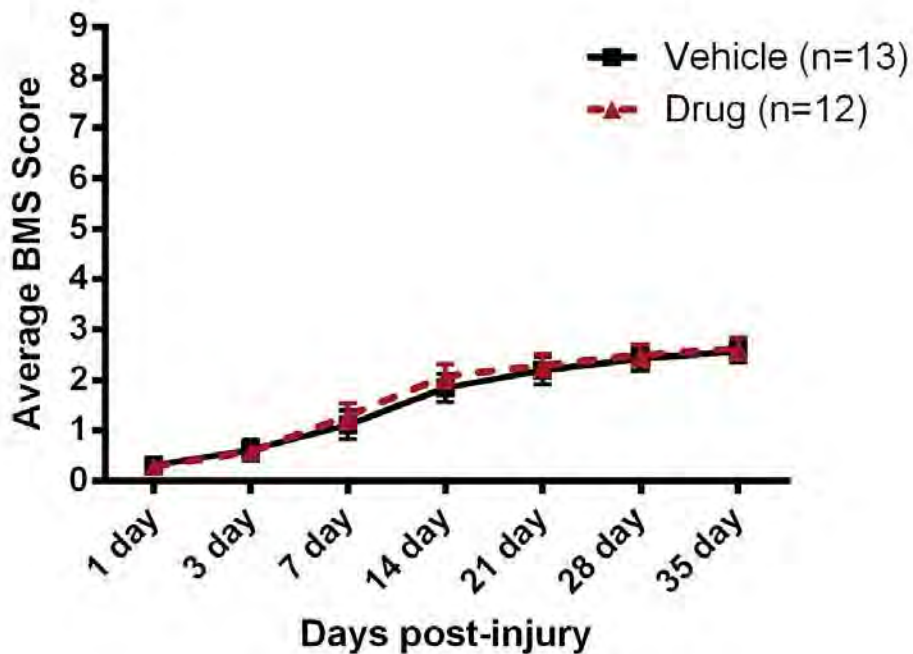
**Figure 12:** Delayed GM6001 treatment improves locomotor function after moderate SCI. **A**, Basso mouse scale (BMS) score shows that both groups improve locomotor function over time; nonetheless, GM6001 treatment results in improved locomotor function as compared to vehicle treated group [effect of treatment ( $p=0.0397$ )]. At 35 days post-injury, vehicle treated mice on an average had a score of 3.3 (ability to plantar place but no plantar stepping). The GM6001 treated group had an average score of 4.3 (mice display occasional plantar stepping). Values are mean + SEM. **B**, Higher percentage of GM6001-treated mice had the ability to step as compared to vehicle-treated mice, chi square analysis,  $*p=0.0294$ , DMSO:  $n = 12$ ; GM6001:  $n = 11$ .



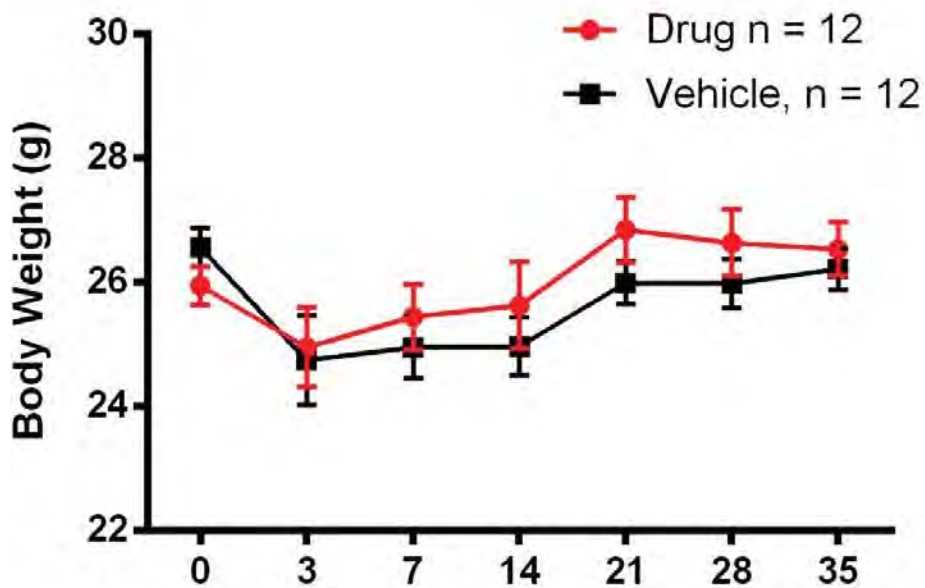
**Figure 13:** Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Repeated measures Two-way ANOVA of percentage weight change revealed the following:  $P = 0.0317$  for interaction, followed by Sidak's multiple comparisons test. Within group comparisons showed that GM6001 treated group did not have a significant weight loss as compared to pre-injury values, whereas, the vehicle treated group had significant weight loss over the first week. Between group comparisons showed that GM6001 treated mice showed a greater weight gain as compared to vehicle treated group ( $*p < 0.05$ ). DMSO:  $n = 12$ ; GM6001:  $n = 11$ .



**Figure 14:** Male C57Bl/6 mice were subjected to a severe spinal cord injury and treated with either GM6001 or DMSO (vehicle) beginning 8 hours post injury and every 12 hours thereafter for 3 days. While both groups improved with time, there were no differences between drug and vehicle treated groups based upon BMS scale. Two-way repeated measures ANOVA of BMS score revealed the following:  $P= 0.9468$  for interaction,  $P<0.0001$  for time, and  $P= 0.7530$  for treatment.



**Figure 15:** Male C57Bl/6 mice were subjected to a severe spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Both gained weight with time but GM6001 treatment did not affect weight gain as compared to vehicle treated group. Repeated measures Two-way ANOVA of weight revealed the following:  $P= 0.2662$  for interaction,  $P<0.0001$  for time, and  $P= 0.5362$  for treatment.



**Table 1:** Cystometric parameters

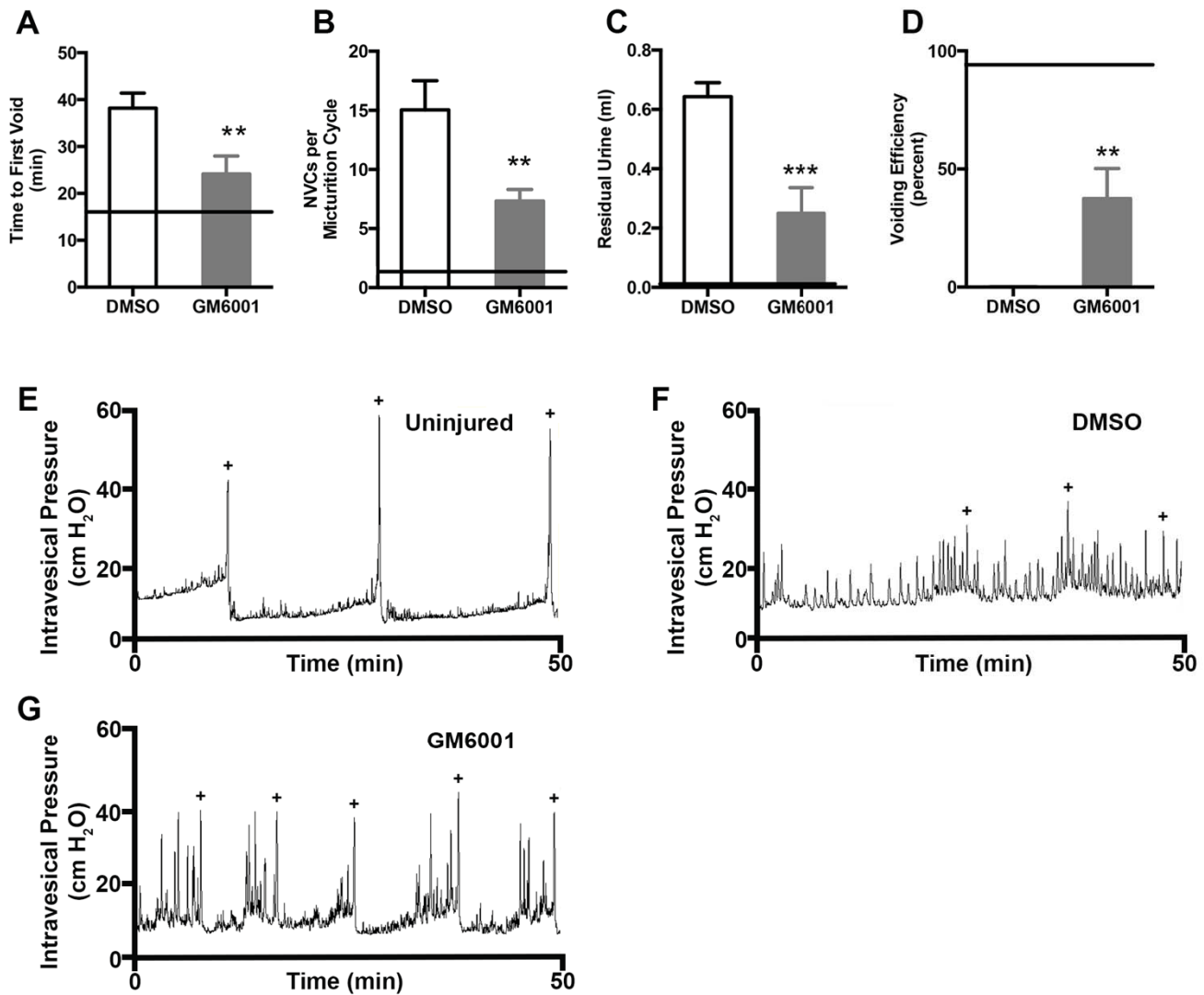
	Uninjured <sup>a</sup> (n = 9)	SCI DMSO (n = 12)	SCI GM6001 (n = 10)
Baseline pressure (cm H <sub>2</sub> O)	7.51 ± 1.21	10.68 ± 0.70	7.95 ± 0.83 <sup>#</sup>
Threshold pressure (cm H <sub>2</sub> O)	18.41 ± 1.99 <sup>##</sup>	13.40 ± 1.24	13.32 ± 0.92
Opening pressure (cm H <sub>2</sub> O)	30.07 ± 1.82	24.97 ± 2.75	24.51 ± 2.15
Maximum pressure (cm H <sub>2</sub> O)	49.46 ± 2.93 <sup>###</sup>	34.38 ± 2.60	34.14 ± 1.96
Δ bladder pressure (cm H <sub>2</sub> O)	41.94 ± 2.57 <sup>###</sup>	23.70 ± 2.13	26.19 ± 2.43
Voiding duration (s)	31.19 ± 2.57 <sup>*</sup>	44.31 ± 3.50	38.44 ± 2.93
NVCs/ voiding cycle	1.07 ± 0.24 <sup>####</sup>	15.04 ± 2.46	7.32 ± 0.99 <sup>##</sup>
Δ NVCs increase (cm H <sub>2</sub> O)	6.23 ± 0.35 <sup>****</sup>	12.58 ± 0.58	10.56 ± 0.66 <sup>*</sup>
Time to first voiding/leak (min)	15.83 ± 2.27 <sup>****</sup>	38.19 ± 3.20	24.14 ± 3.84 <sup>*</sup>
Intermicturition interval (min)	17.94 ± 2.38	13.48 ± 1.29	13.86 ± 2.35
Residual urine (ml)	0.006 ± 0.004 <sup>****</sup>	0.643 ± 0.047	0.250 ± 0.086 <sup>***</sup>
Voiding efficiency (%)	92.9 ± 4.1 <sup>####</sup>	0.2 ± 0.1	37.5 ± 12.8 <sup>##</sup>

<sup>a</sup> Uninjured animals served to establish baseline values. Data for all groups shown as mean ± SEM. Δ bladder pressure is the difference between baseline pressure and maximum pressure. NVCs: non-voiding contractions.

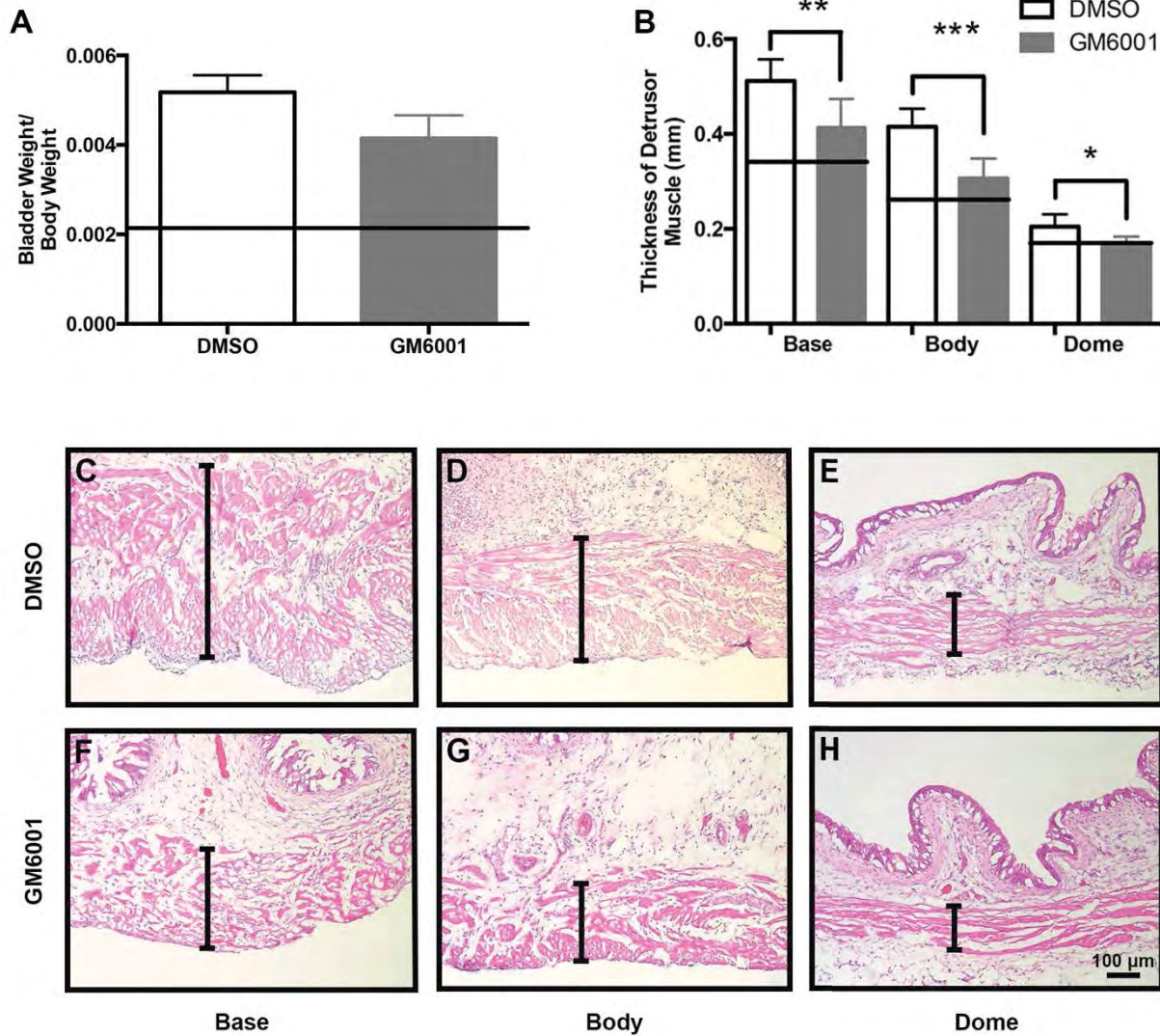
\*<sup>#</sup>:p<0.05, <sup>##</sup>:p<0.01, <sup>###</sup>:p<0.001, <sup>\*\*\*\*</sup>/<sup>####</sup>:p<0.0001 vs. DMSO treatment

Asterisks denote unpaired t tests, while hash-tags denote Mann-Whitney tests.

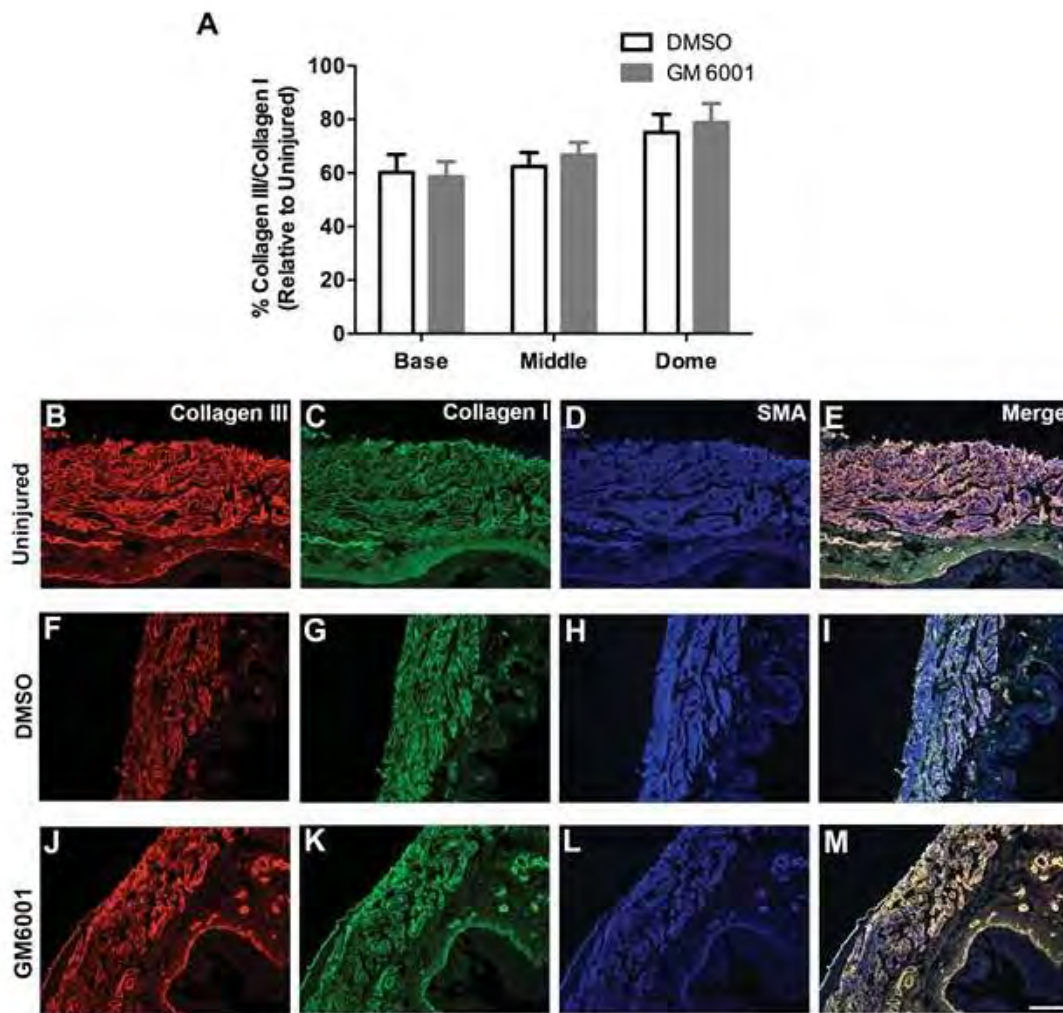
**Figure 16:** GM6001 treatment improves urodynamic measures after moderate SCI. **A – D**, Cystometry results demonstrate that drug treatment decreases the development of large bladder capacities, the emergence of non-voiding bladder contractions per micturition cycle, and the retention of urine. Overall, voiding efficiency is improved after drug treatment. Black line indicates the baseline value for uninjured animals. Values are mean + SEM; DMSO: n = 12; GM6001: n = 10, Uninjured: n = 9 (\*\*p<0.01, \*\*\*p<0.001). **A, C**: unpaired t tests; **B, D**: Mann-Whitney tests. **E – G**, Representative voiding and bladder pressure recordings (+ indicates voiding/ urine leakage) were recorded for each group at 6 weeks post-injury. Drug treatment resulted in fewer non-voiding bladder contractions per micturition cycle and a better voiding efficacy, signified by the return of pressure to baseline values after voiding.



**Figure 17:** GM6001 treatment decreases bladder weight and bladder wall thickness after moderate SCI. The bladder increases in size in response to injury, as depicted by weight and wall thickness. **A**, Drug treatment does not significantly reduce bladder weight; however, there is a strong trend of reduced bladder weight in the drug-treated group as compared to the vehicle-treated group (Mann-Whitney test:  $p = 0.0509$ ); DMSO:  $n = 12$ ; GM6001:  $n = 11$ . **B**, There is a significant reduction in wall thickness in each separate bladder region in the drug-treated group as compared to the vehicle-treated group (unpaired t tests:  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). Black line indicates the baseline value for uninjured animals. Values are mean + SEM; DMSO:  $n = 6$ ; GM6001:  $n = 6$ ; Uninjured:  $n = 5$ . **C – H**, H&E staining of representative bladder sections. The muscle layer is shown in pink. Note the decreased detrusor muscle thickness in each bladder region following drug treatment as compared to vehicle treatment.

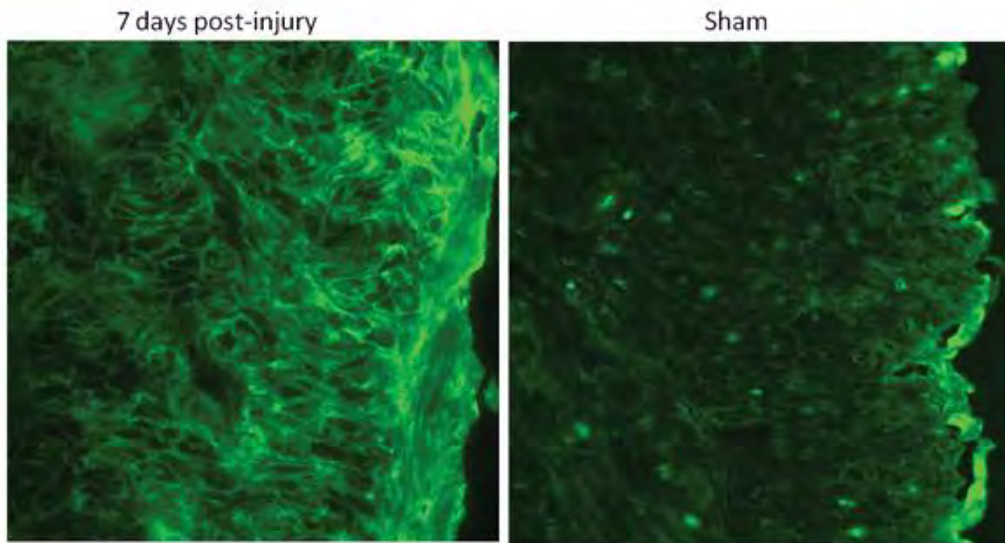


**Figure 18:** GM6001 treatment does not affect collagen III/collagen I ratio within the detrusor muscle. Morphological changes in bladder wall at six weeks post-injury reveal **A**, injury results in a reduction in collagen III/collagen I ratio, and this is not rescued by GM6001 treatment. **B-M**, representative images from the body region of the bladders of uninjured (**B-E**), vehicle-treated (**F-I**) and GM6001-treated (**J-M**) mice. Bars represent mean + SEM; DMSO: n = 6; GM6001: n = 6; Uninjured: n = 5. Scale bar in M denotes 100 $\mu$ m.

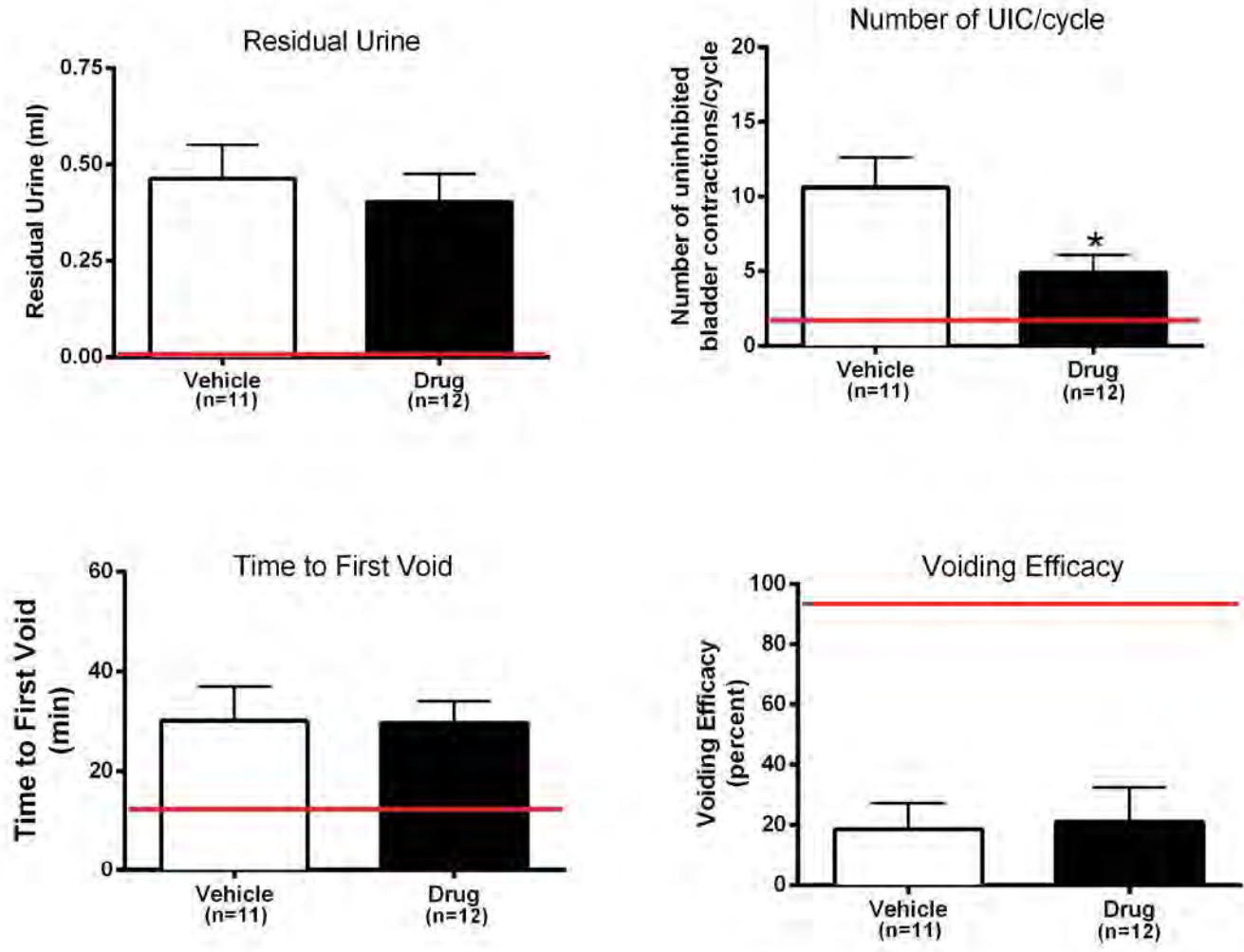


**Figure 19:** *In situ* gelatinolytic activity in the bladder in response to spinal cord injury. Unfixed bladders from mice (uninjured or at 7 days after injury) were frozen, and cryosections were prepared for *in situ* gelatin zymography. Fluorescence is indicative of gelatinolytic activity. In the uninjured bladders, small amount of cellular gelatinase activity is identified within the urothelium. Seven days after spinal cord injury, gelatinase activity is prominent within the bladder urothelium.

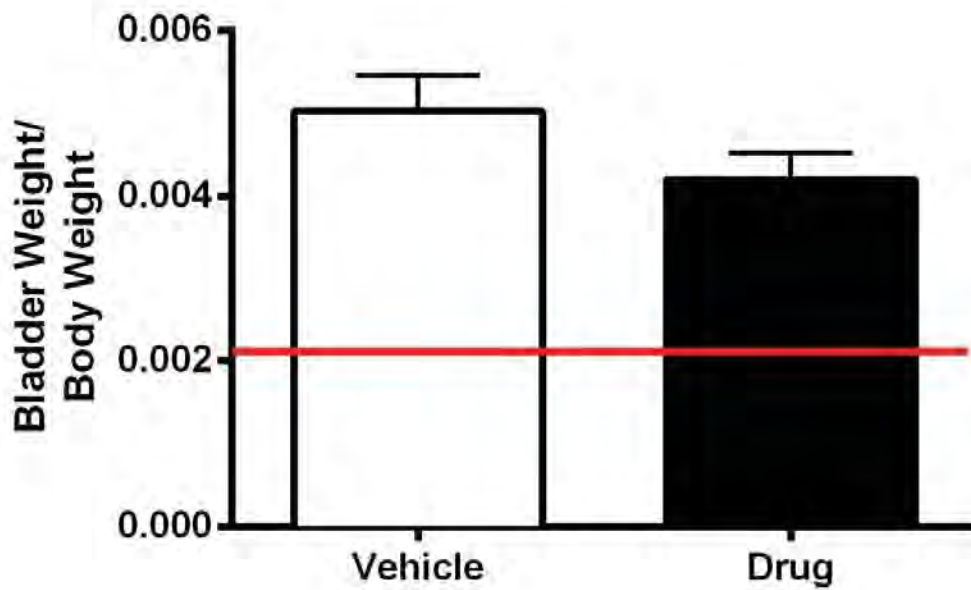
### In Situ Zymography



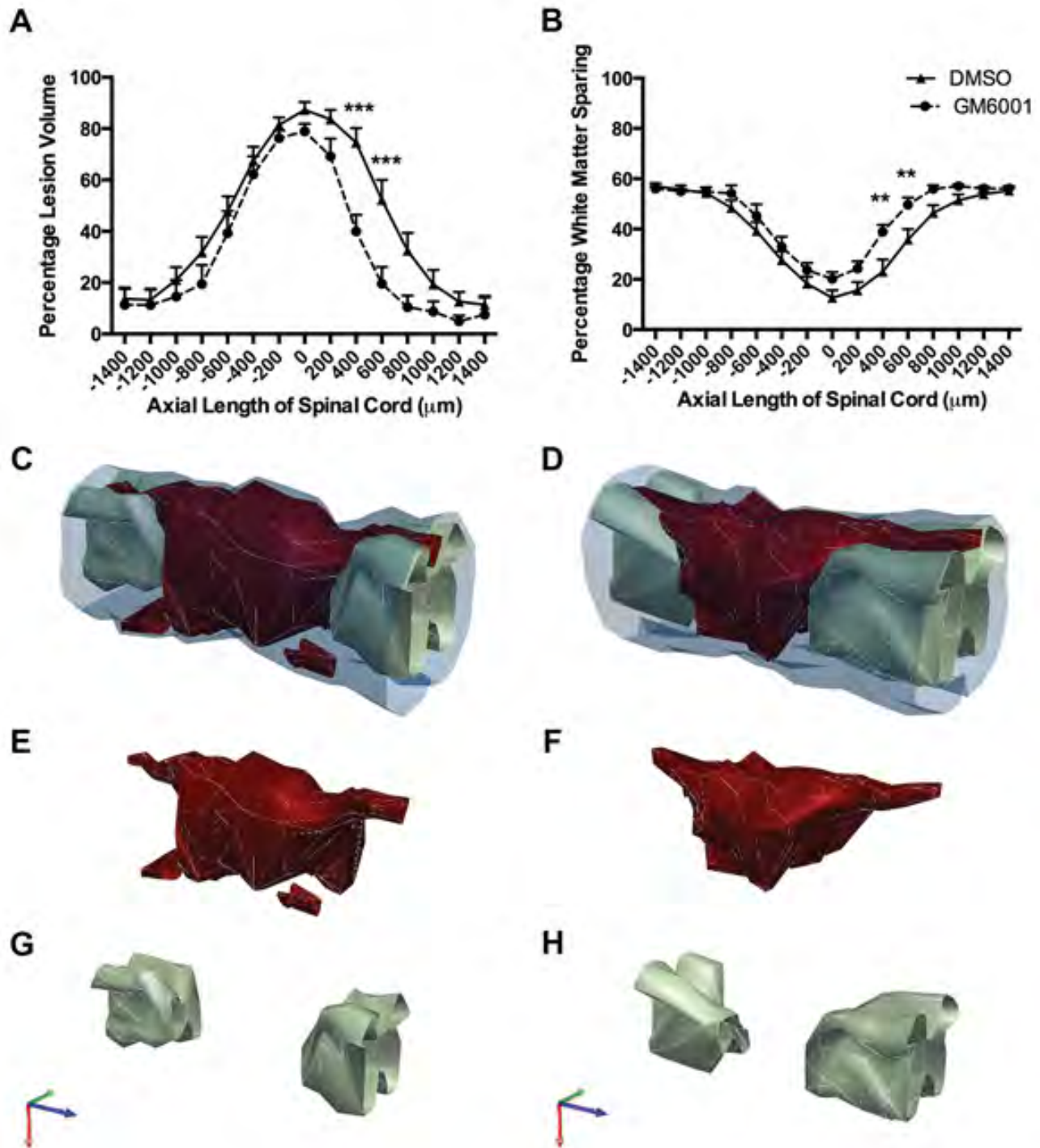
**Figure 20:** Urodynamic outcomes from awake cystometries of severely injured mice treated with GM6001 or vehicle. Drug treatment did not affect urine retention (Unpaired two-tailed T- test,  $p=0.6038$ ), decreased number of uninhibited bladder contractions per cycle (Unpaired two-tailed T- test,  $p=0.0210$ ), but had no effect on time to first void (Unpaired two-tailed T- test,  $p=0.9573$ ), or voiding efficacy (Mann Whitney test,  $p=0.4401$ ). Red lines in each graph indicate baseline values of uninjured male C57Bl/6 mice. Bars represent mean+SEM.



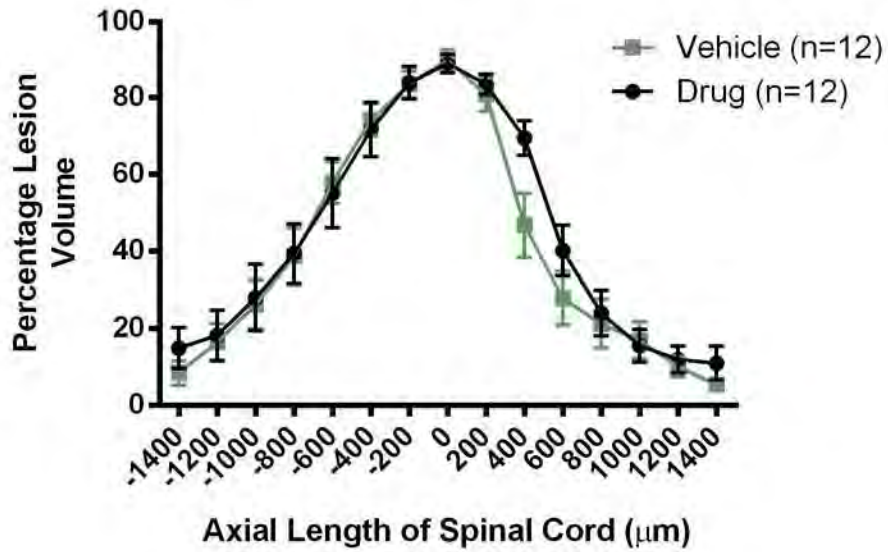
**Figure 21:** Bladder weights were analyzed from severely injured mice, treated with either vehicle or drug. Drug treatment did not lead to significant decrease in bladder weight (Unpaired two-tailed T test,  $p=0.1390$ ). Bars represent mean+SEM.



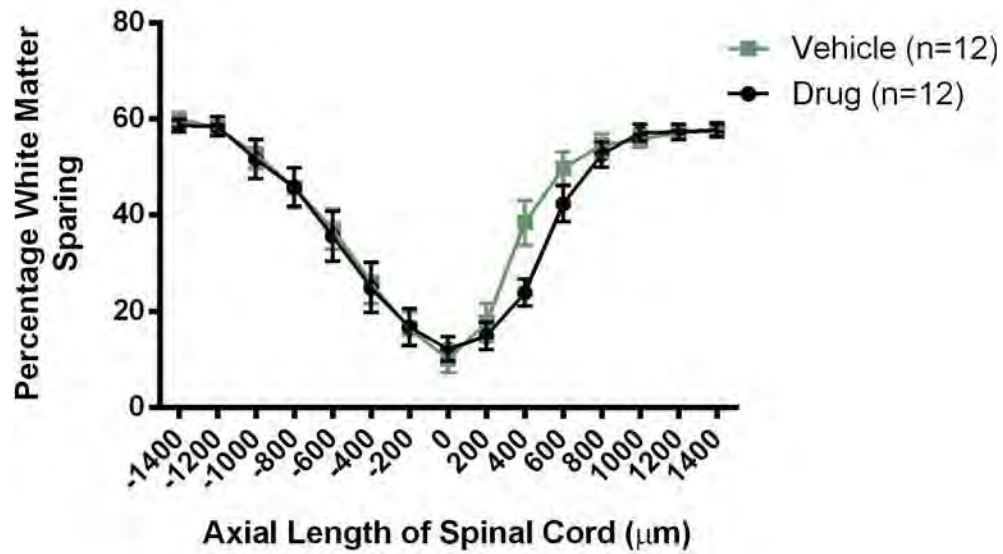
**Figure 22:** Delayed treatment with GM6001 reduces lesion volume and increases white matter sparing in moderately injured mice. **A**, In both groups, lesion volume decreases over a distance of 3 mm at regions removed from the lesion epicenter. Drug treatment significantly decreases lesion size at 400 and 600  $\mu\text{m}$  caudal to the epicenter ( $***p < 0.001$ ). **B**, White matter sparing increases at regions removed from the lesion epicenter. Drug treatment significantly increases white matter sparing at 400 and 600  $\mu\text{m}$  caudal to the epicenter ( $**p < 0.01$ ). Stereological 3D reconstruction of representative animals from the vehicle (**C**) and drug-treated (**D**) groups illustrate the differences between groups exhibited histologically. Red represents lesion, blue represents white matter and green represents gray matter. The isolated vehicle-treated injury lesion (**E**) is larger than that of an animal given GM6001 (**F**). These differences are also shown in the isolated 3D reconstruction of the gray matter from the vehicle-treated animal (**G**), which is smaller than that of an animal that received drug treatment (**H**). Values are mean  $\pm$  SEM, DMSO:  $n = 12$ ; GM6001:  $n = 11$ .



**Figure 23:** The rostral and caudal extent of lesion volume in severely injured mice treated with GM6001 or vehicle was quantified at 200- $\mu$ m intervals spanning from 1400  $\mu$ m rostral to 1400  $\mu$ m caudal to the epicenter. Percentage lesion decreased at distances further removed from the epicenter in both groups but no differences were found between the groups in lesion volume. Two-way ANOVA, interaction,  $p=0.7712$ ; effect of distance,  $p<0.0001$ ; effect of treatment,  $p=0.1064$ .

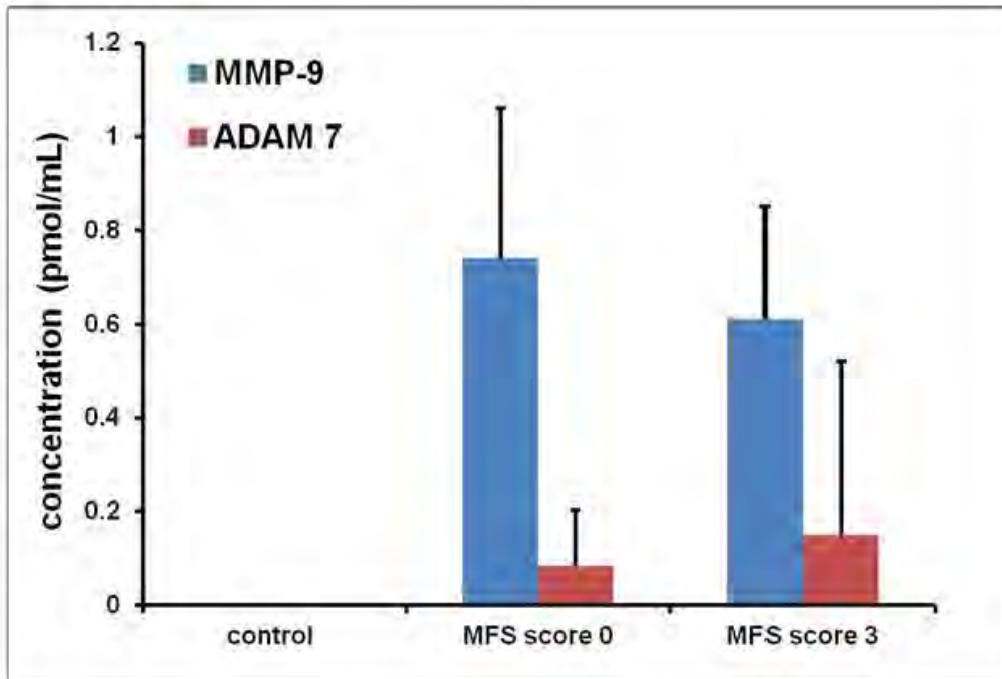


**Figure 24:** The rostral and caudal extent of spared white matter in severely injured mice treated with G M6001 or vehicle was quantified at 200- $\mu\text{m}$  intervals spanning from 1400  $\mu\text{m}$  rostral to 1400  $\mu\text{m}$  caudal to the epicenter. Percentage spared white matter increased at distances further removed from the epicenter in both groups but no differences were found between the groups in spared white matter. Two-way ANOVA, interaction,  $p=0.6069$ ; effect of distance,  $p<0.0001$ ; effect of treatment,  $p=0.1235$ .

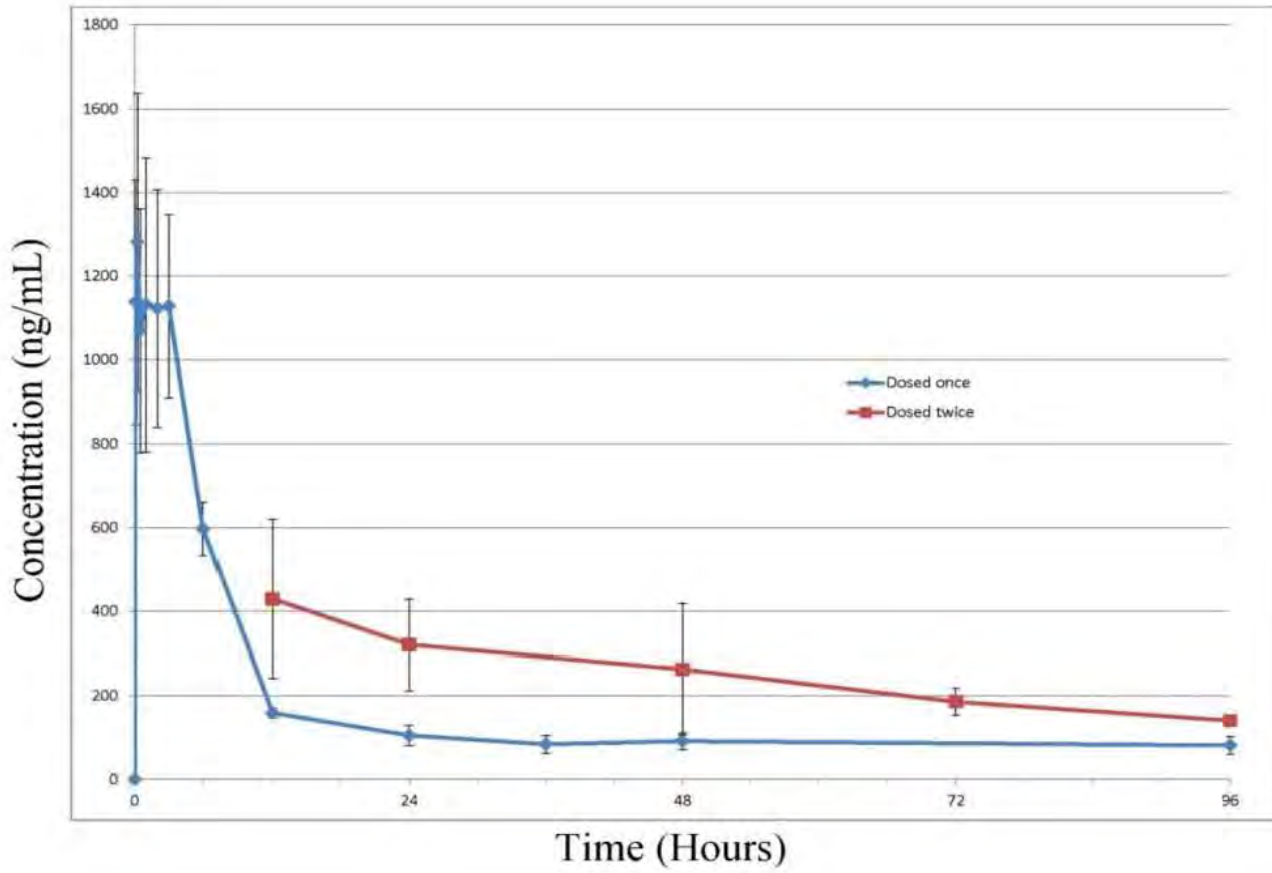


## SUPPORTING DATA: - TAMU

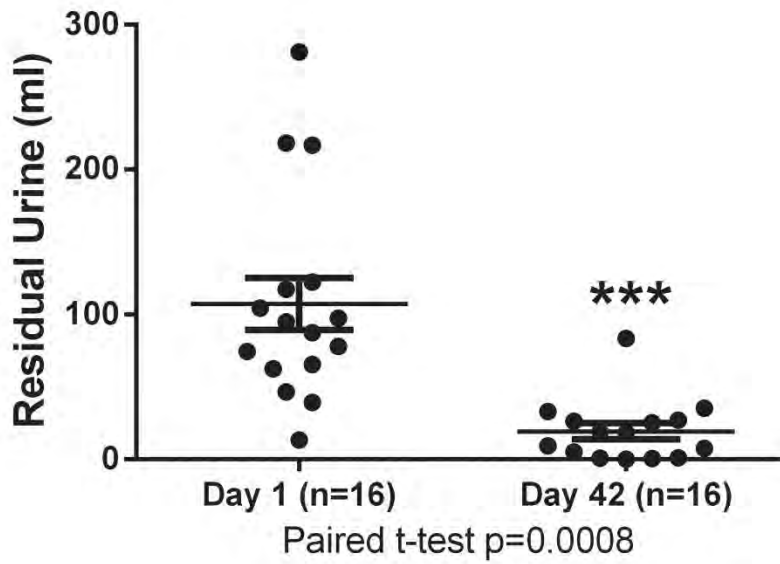
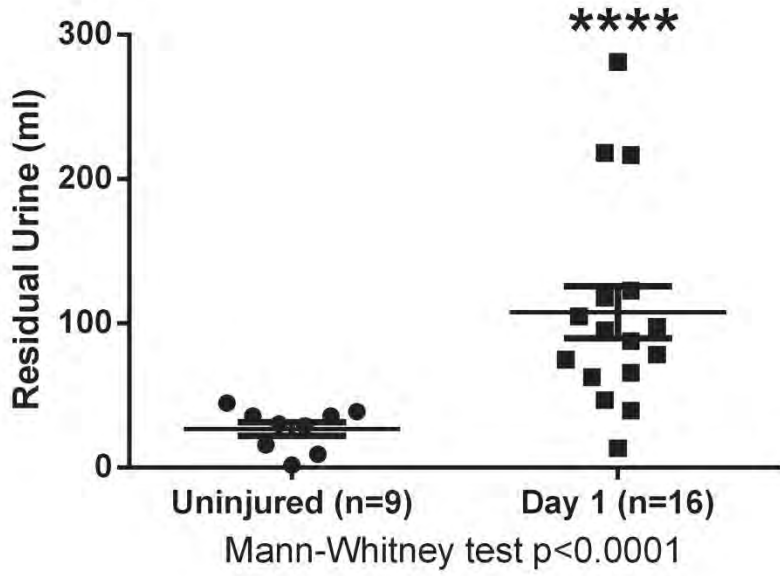
**Figure 1:** Quantification of MMP and ADAM activity in normal and SCI dogs. Dogs with SCI have significantly greater MMP-9 and ADAM-7 expression compared with healthy controls. No other MMPs or ADAMs were detected in the CSF of normal or SCI dogs. Dogs with severe SCI (MFS = 0) did not have significantly greater expression of MMP-9 or ADAM-7 than those with mild-to-moderate SCI (MFS = 3)



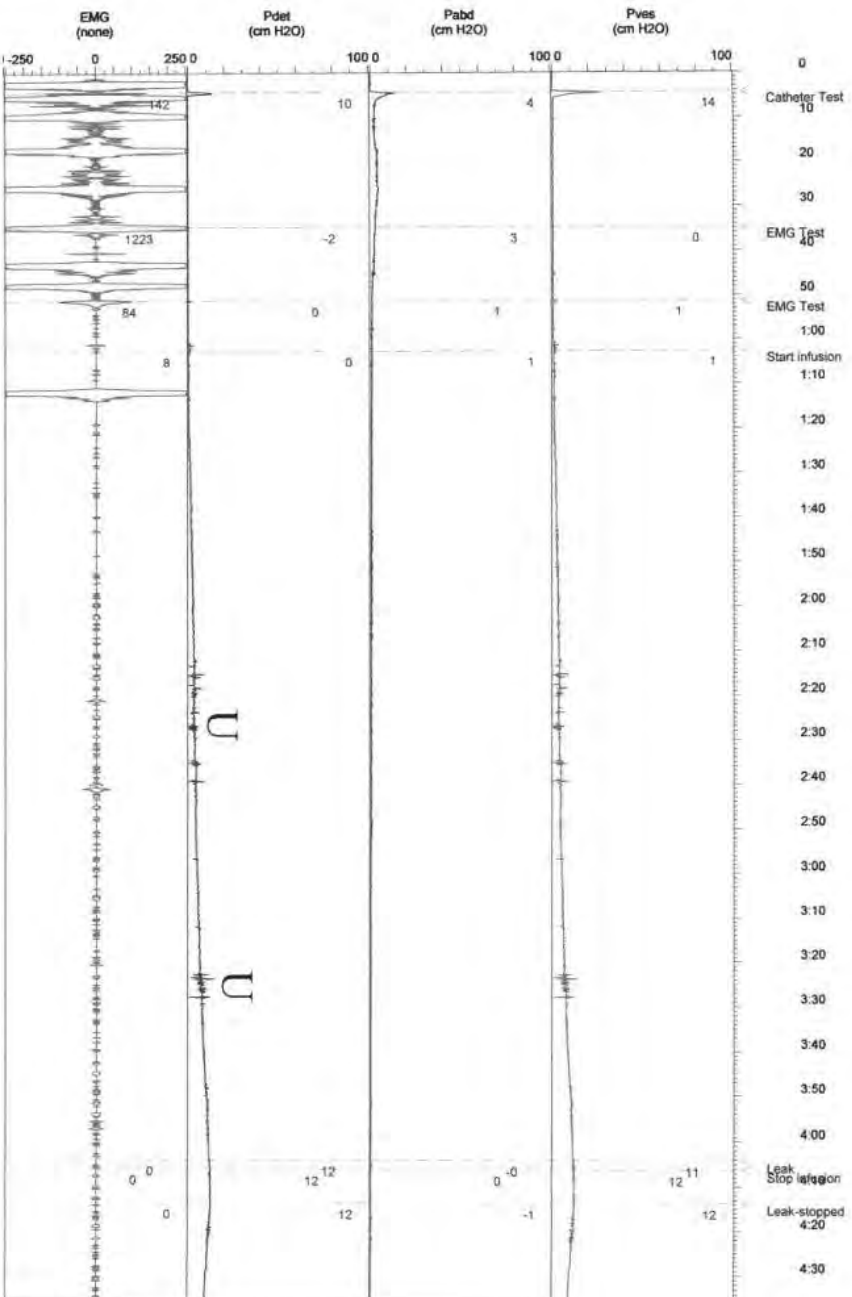
**Figure 2:** Plasma concentration of GM 6001 in 5 dogs dosed once at 100 mg/kg S.C. and 5 dogs dosed twice at a 12-hour interval. Data for the two-dose cohort was collected only at time points following the second dose.



**Figure 3:** Residual urine volume in un-injured dogs and dogs with SCI. Compared to dogs with SCI, uninjured dogs had significantly lower residual urine volume (upper panel). Residual urine volume was significantly lower day 42 following injury compared to day 1, suggesting recovery of voiding mechanisms (lower panel)



**Figure 4.** Cystometry 24 hours following spinal cord injury in a dog with intervertebral disk herniation. Note the absence of voiding, the presence of un-inhibited bladder contractions (U), and the low leak point pressure (12 mm H<sub>2</sub>O)



**Figure 5.** Cystometry 3 days following Figure 2. Note the presence of a voiding reflex, the absence of un-inhibited bladder contractions, and the presence of anal EMG activity.

