Altered vasopressin and natriuretic peptide levels in a rat model of spinal cord injury - Implications for the development of polyuria

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Abstract

Urinary dysfunction is a common complaint following spinal cord injury (SCI), and is a leading issue for individuals with SCI that impacts their quality of life. One urinary complication that has received little attention is SCI-induced polyuria, even though SCI individuals will significantly restrict their fluid intake to decrease urine production leading to a sequela of medical complications. Understanding the mechanisms instigating the development of polyuria will allow us to target interventions which may alleviate polyuria symptoms leading to significant improvements in the quality of life and urinary health of SCI individuals. In a rat SCI contusion model, an increase in the amount of urine excreted over a 24 hour period ($p \leq 0.001$) was found at two weeks post-injury. The urine excreted was more dilute with decreased urinary creatinine and specific gravity ($p \leq 0.001$). Several factors important in fluid balance regulation – vasopressin (AVP), natriuretic peptides, and corticosterone (CORT) also changed significantly post-injury. AVP levels decreased ($p = 0.042$) while atrial natriuretic peptide (ANP) and CORT increased ($p = 0.005$; $p = 0.031$, respectively) at two weeks post-injury. There was also a positive correlation between the increase in ANP and urine volume post-injury ($p = 0.033$). The changes in AVP, ANP and CORT are conducive to producing polyuria, and the timing of these changes coincides with the development of SCI-induced polyuria. This study identifies several therapeutic targets that could be used to ameliorate polyuria symptoms and improve quality of life in SCI individuals.

Keywords: urinary dysfunction, bladder, neurotrauma, corticosterone
Introduction

Spinal cord injury (SCI) leads to a myriad of acute and chronic urinary complications that have detrimental effects on overall health and quality of life (1). Urinary complications after injury are viewed by the SCI population as one of the most significant disruptions negatively impacting quality of life (1). One important yet poorly understood urinary complication following SCI is polyuria – the excess production of and/or passage of urine (9, 47). Amplification of urine production after SCI further burdens an already difficult daily bladder management program with a need for more catheterizations especially at night, contributing to disruption of sleep and thus daily activities. Countering this excess urine production with the restriction of fluid intake, including in the evening prior to bedtime, poses a significant health concern. The prevalence of polyuria in the SCI population has not been reported, although studies investigating nocturnal polyuria (excessive nightly urine production) within the SCI population has shown that the prevalence of this condition may be up to 81% (6).

Although several studies have documented the occurrence of polyuria in human SCI and rodent spinal contusion models (9, 14, 17, 42, 47), little research has been done to identify underlying causes/mechanisms. One potential contributor is the hormone arginine vasopressin (AVP), a major regulator of fluid balance in the body (19). AVP (also known as antidiuretic hormone) is released from the posterior pituitary in response to changes in blood osmolality. It acts in the kidney by binding to V2 receptors in the collecting ducts, which then leads to the transposition of aquaporin 2 receptors to the serum membrane thereby allowing water to be reabsorbed back into the blood. AVP levels follow a diurnal cycle, with levels increased during the night (45) to prevent the need to awaken for urination. Several human SCI studies (17, 42) have shown that increased urine production occurs at night (nocturia) along with evidence that the normal nightly increase in AVP levels is absent at chronic post-injury stages. Increased urine production in the rodent contusion model on a 12 hour light-dark cycle occurs throughout the 24 hour period (as measured using metabolic cages) (14, 47), suggesting that AVP should be decreased throughout the day if AVP levels contribute to SCI induce polyuria in our model.
Although there is initial strong evidence for SCI-induced polyuria and a potential role of changing serum AVP levels, the factor(s) that precipitate the development of this condition have yet to be elucidated. Potential mechanisms by which AVP levels may be decreased following SCI are also unknown. Blood osmolality is the primary stimulator for AVP release (7, 31), but there are a number of non-osmotic stimuli that also influence the release of AVP into the blood stream including glucocorticoids, norepinephrine (NE), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) (8, 26, 27, 35, 48, 49). Some of these factors that can effect AVP release, such as glucocorticoids and natriuretic peptides, are known to increase urine output (11, 13, 15, 43). Increased levels of glucocorticoids significantly increase glomerular filtration rate (11) as well as decreasing AVP levels and enhancing the renal responsiveness to natriuretic peptides by up-regulating Natriuretic Peptide Receptor A (NPRA) in the kidney (20) and increasing ANP release (5). Natriuretic peptides have been shown to increase urine production not only through suppression of AVP release but also through direct effects on the kidneys (13, 15, 39, 50) where they increase glomerular filtration rate and inhibit the actions of AVP on V2 receptors. Little is known about ANP and BNP levels following SCI. One study in a cohort of paraplegic and quadriplegic individuals has shown that ANP levels are increased significantly following injury (37). This data, however, was collected from SCI individuals at chronic time points and it is not clear how early after injury these changes in natriuretic peptides occur. Thus, to begin elucidating mechanisms underlying SCI-induced polyuria and identifying potential pharmacological targets, serum and/or urinary levels of AVP and several known non-osmotic factors (ANP, BNP, and glucocorticoids) that influence AVP and can also increase urine output were measured in this initial experiment in samples obtained both pre-injury and again two weeks after a clinically relevant contusion injury from the same set of animals.
Methods

Ethical Approval -

All experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Spinal Contusion Injury –

Male Wistar rats (n=48) were obtained from Harlan (Harlan Laboratories Inc, Indianapolis, IN) weighing 200-250 g at the start of the study. Animals were single housed with a 12 hour light/dark cycle with water and food ad libitum. A number of the animals included in this study received activity based training similar to work previously done in this lab (14); however this training did not begin until after samples and data were collected for this portion of the study.

Following pre-injury assessments (see below), 42 animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals then received a T9 laminectomy to expose the T8 spinal cord. A T8 contusion SCI of 215 kdyn impactor force with the Infinite Horizon device (IH) was then delivered and subsequently the muscle layers and skin closed with 4-0 ethilon suture (Ethicon, Cornelia, GA) and wound clips (9 mm AutoClips, Braintree Scientific Inc., Braintree, MA), respectively. After closure, all rats recovered in a clean cage on a heating pad and given a postoperative course of antibiotics (5 mg/kg gentamycin) for 5 days and analgesia (2 mg/kg Meloxicam) for 3 days. Manual crede of the urinary bladder was performed three times a day for the first 4-5 days and then twice daily until reflexive bladder function returned.

A smaller cohort of animals (n=6) served as sham surgical controls. As with the SCI rats, control animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and given a T9 laminectomy; however no contusion was delivered. Following the laminectomy, the incision was closed and the animals were recovered as outlined above. Control animals received the same postoperative antibiotic and analgesic regimen as the SCI group. Bladders were also checked for
retention following the laminectomy. However, no emptying was necessary in the
control animals as they maintained full bladder function following surgery.

**Behavioral Testing**

Animals received pre-injury and post-injury behavioral testing to monitor urine
output and drinking volume over 24 hours using the Comprehensive Lab Animal
Monitoring System (CLAMS - Columbus Instruments, Columbus, OH). Animals
underwent two baseline measurements to allow them to acclimate to the CLAMS
system and baseline levels were measured from the second baseline testing session.
Specialized software recorded drink and voiding events over 24 hours (Oxymax v 5.14,
Columbus Instruments). Overground locomotor assessment was also performed pre-
operatively and post-operatively using the 21-point Basso, Beattie and Bresnahan
(BBB) scale (2).

**Blood and Urine Sampling**

Blood was collected in a BD microtainer (Becton, Dickinson and Company,
Franklin Lakes, NJ) from animals via tail vein draw pre-injury (as a baseline measure)
and then post-injury (10 days following injury) for measurement of serum AVP levels.
Samples were centrifuged for 15 minutes at 14000 g and then the serum was removed.
Sample osmolality was tested by freezing point depression with an Advanced model
3320 Micro-osmometer (Advanced Instruments Inc., Norwood, MA). Samples were
then stored at -20°C.

Measurement of stress hormones (corticosterone and norepinephrine) and
natriuretic peptides (ANP and BNP) were done using urine collected during 24 hour
metabolic cage (CLAMS system - Columbus Instruments, Columbus, OH) behavioral
testing to avoid artificially inflated levels of these hormones that can occur during the
blood drawing procedure (61). Additionally, measuring levels from 24 hour urine
collection provided a more accurate measurement of these biological factors especially
those that exhibit variation throughout the day (such as corticosterone). Urine samples
following the 24 hour behavioral assessments were centrifuged for 10 minutes at 10000
g and then aliquoted. Urinalysis was performed on samples using the CLINITEK
Status+ analyzer (Siemens Healthcare Diagnostics Inc, Tarrytown, NY) to record specific gravity, urine creatinine, and pH. Samples were then stored at -20°C.

**Enzyme Linked Immunosorbent Assay (ELISA)** –

Pre-injury and post-injury serum vasopressin levels were measured using an Arginine Vasopressin EIA kit (Cayman Chemical, Ann Arbor, MI, catalog # 583951). The AVP detection range for the kit was 3,000 – 23.4 pg/ml. Prior to testing, serum samples were purified using C-18 solid phase extraction (SPE) columns (Thermo Scientific, Rockwood, TN). After purification, 50 µl of each sample was placed in duplicate in a 96 well plate and incubated overnight at 4°C with AVP antibody. The next day, Ellman’s reagent was used as the chromagen to identify AVP levels and the optical density (O.D.) for each well was measured at 405 nm on a Spectra max Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA) using Softmax pro software version 4.3 LS (Molecular Devices, Sunnyvale, CA).

Urinary corticosterone (CORT) levels were measured using a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI, catalog # K014-H1). The corticosterone detection range for the kit was 10,000 – 78.125 pg/ml. Samples were diluted 1:20 and then tested in duplicate. Samples were incubated with Corticosterone antibody supplied with the kit for one hour, then following plate washes 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well for 30 minutes. Once 50 µL of stop solution was added to each well, the O.D. for each well was measured at 450 nm.

Urinary norepinephrine levels were measured using a Noradrenaline ELISA kit (Eagle Biosciences, Nashua, NH, catalog # EA610/96). The noradrenaline detection range for the kit was 500 – 1.5 ng/ml. Standards and samples were first taken through an extraction phase in order to extract the norepinephrine from each solution. The supernatant from each well was then extracted and placed into a 96 well plate and incubated with noradrenaline antiserum overnight at 4°C. Following washes, a secondary antibody was placed in the wells for 30 minutes. After further washing TMB was added to the plate for 30 minutes. The reaction was then stopped and the O.D. for each well was immediately read at 450 nm.
Urinary ANP levels were measured using an Atrial Natriuretic Peptide enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, catalog # K026-H1). The ANP detection range for the kit was 180 - 0.741 ng/ml. Samples were diluted 1:5 and then tested in duplicate in a 96 well plate. Following one hour incubation of samples with ANP antibody supplied with the kit, the plate was washed and TMB was placed in each well for 30 minutes. The TMB reaction was then stopped and the plate was read at 450 nm.

Urinary BNP levels were measured using a Human/Mouse/Rat BNP Enzyme immunoassay kit (RayBiotech, Norcross, GA, catalog # EIAR-BNP). The BNP detection range for the kit was 1000 – 0.1 pg/ml. A BNP primary antibody (supplied in the kit) was allowed to incubate in each well of a 96 well plate for one and a half hours at room temperature. The plate was then washed four times and then the samples (diluted 1:2) and standards were added in duplicate to the plate which was then left on a shaker for two and a half hours. After further washing an HRP-streptavidin solution was added to the plate for 45 minutes. The plate was again washed four times and then TMB was added to the plate which was then left for 30 minutes. The TMB reaction was then stopped and the plate was read immediately at 450 nm.

Data analysis –

Behavioral data - Following CLAMS system data collection, Oxymax data files were exported as text files and opened in Microsoft Excel (Microsoft Corporation, Redmond, WA) for analysis. A void was determined to occur when there was an increase of at least 0.1 g recorded by the urine sensor. The total 24 hour urine volumes were calculated by the summation of the volumes from all void events that occurred over the 24 hour period that the animal was in the metabolic cage. Total drinking volumes was taken directly from the volumes delivered by the Volumetric Drinking Monitor [VDM] system (part of the CLAMS system).

ELISA data - All ELISA plates were read using the SoftMax Pro software system. Data files were saved and exported into a text file format that could be opened in Microsoft Excel for further analysis. Once sample and standard averages were calculated, the
data was transposed into a data analysis software package (SigmaPlot version 10.0, Systat Software Inc., San Jose, CA) where the standard curves were created and used to determine sample concentrations of the given target protein (AVP, corticosterone, norepinephrine, ANP, and BNP).

Once sample concentrations were determined, values were then adjusted to account for sample dilution or concentration that occurred during sample preparation for the ELISA. Values from urine samples were then divided by the urinary creatinine (Cre) concentration to adjust for the urine concentration.

Statistical Analysis - For all outcome measures (urine volumes, urinary Cre levels, AVP/corticosterone/norepinephrine/ANP/BNP levels) for the SCI group, paired t-tests were carried out to compare pre-injury and post-injury levels using SigmaStat version 3.5 (Systat Software Inc., San Jose, CA). A Pearson correlation was performed to determine the influence of drink volume, ANP, BNP, and AVP on 24 hour urine volume, and a principle components analysis (PCA) was performed to identify interrelationships between these same variables using SPSS version 24 (IBM corp, Armonk, NY). For the control group, Wilcoxon signed rank tests were undertaken to compare pre-injury and post-injury levels due to the small sample size in this group. Data was checked for outliers using the extreme studentized deviate method. One subject was an extreme outlier in the 24 hour urine measurement. As this was the primary measurement establishing the validity of the polyuria model, it was decided to remove this value from all analysis. Pre-injury and post-injury values were considered significant when $p \leq 0.05$. Probability and standard errors are reported for each of the outcome measures. Graphs show the individual data points with a bar showing the mean.

Percent change was calculated for the means pre and post injury for each group (SCI or sham). Post SCI values were subtracted from the pre-injury values. The difference between the two values was then divided by the post SCI value and this was then multiplied by 100.
**Results**

At two weeks following SCI, all injured animals demonstrated significant locomotor impairment as measured by the BBB locomotor scale. Prior to injury, all animals scored 21 on the BBB. At two weeks following SCI/sham surgery, the average BBB score was 8.42 +/- 0.3 (t = 42.22, p ≤ 0.001) for the SCI group and 21 for the non-injured sham surgical (laminectomy) controls.

**Urine output after SCI** –

Following SCI, the total urine volume excreted over a 24 hour period was significantly increased (t = -9.82, p ≤ 0.001) compared to baseline pre-injury levels (Fig. 1A). The average 24 hour urine volume was more than double at two weeks following SCI (increase of 118%), compared to just a 9% increase for the sham surgical control group. Total drink volume over 24 hours was then examined to identify if changes in this measure could account for this increase in urine output. For the SCI group, although there was a significant increase in the total 24 hour drink volume (t = -4.32, p ≤ 0.001; Fig. 1B), there was no correlation with 24 hour urine output (Fig. 1C), indicating that some of the consumed water is being lost through other means. An increase in water content of feces collected with metabolic cages was shown previously for a contused group of rats having a similar extent of injury (47). Interestingly, drink volume actually trended down after surgery alone (laminectomy), although the difference was not significant (P > 0.05).

**Urinary Creatinine and Specific Gravity following SCI** –

A urinalysis of samples collected over a 24 hour period before and after SCI was used to identify the effect of injury on urine composition, most importantly urine concentration and water content. Urine was more dilute following SCI as measured by urinary creatinine (Cre) and specific gravity. Urinary Cre levels two weeks following SCI were on average half of pre-injury baseline levels (pre-injury = 91.43 mg/dL +/- 9.412 and post-injury 46.43 mg/dL +/- 3.934; t = 4.098, p ≤ 0.001). There was no significant change in urinary Cre levels in the control group, although the trend was toward a more
concentrated volume following sham surgery (possibly a reflection of the decreased fluid intake seen in Fig. 1B).

The urine specific gravity (SG) was also significantly decreased following SCI (pre-injury = 1.028 +/- 0.001 and post-injury = 1.020 +/- 0.001; t = 6.036, p ≤ 0.001). SG is the ratio of urine density compared to water and is determined by the concentration of solutes, including sodium, in urine (8). It is often used as a measure of urine concentration, and the closer the value is to 1 the more water-like and less concentrated it is. There was no change in SG in the surgical sham control group relative to baseline.

**AVP levels and serum osmolality after SCI**

AVP, which is amongst several groups of hormones that control fluid balance and maintain homeostasis in the body, was found to have significantly lower serum levels (by 9.5%) after SCI (Fig. 2A, t = 1.78, p = 0.0415). There was no significant change in AVP levels however in the control group relative to baseline levels. Note that the comparison is between pre and post-surgery (SCI or laminectomy) values using a paired t-test to minimize potential variations between animals (as sham and SCI rats were from different batches).

The strongest regulator of AVP levels is serum osmolality. To begin to elucidate the mechanisms which may influence AVP levels following SCI, serum osmolality was also measured in pre- and post-injury samples. Although serum osmolality was significantly different after injury (t = -3.48, p = 0.001), the change was in a direction that would lead to an increase in AVP levels under normal physiological conditions (Fig. 2B). Serum osmolality did not change significantly pre and post-surgery in the control group (W = 2, p = 0.271).

**Non-osmotic Regulators of AVP Post-SCI**

Non-osmotic factors that can influence AVP levels include corticosterone (CORT), a stress-related hormone (30). Increased CORT levels are known to suppress the release of AVP from the hypothalamus and thus an increase in CORT after SCI could be a contributing factor to the suppression of AVP that was observed in these animals post-SCI (30, 35). Urinary CORT levels were observed to be increased by
47.3% at two weeks following SCI (Fig. 3A, t = -1.93, p = 0.031). Increased CORT levels can also increase urine output (11, 43), however no correlation was found between CORT levels and 24 hour urine output post-injury. There was no significant change in the CORT/Cre ratio in the control group (19.4% lower on second measurement).

Another stress related hormone that can influence AVP secretion is norepinephrine (NE) (36). Increased NE levels has been shown to suppress AVP release from the hypothalamus leading to lower circulating AVP levels (35). Thus an increase in NE after SCI may contribute to the decreased AVP levels demonstrated in this experiment. However, no increase in NE was seen in these subjects with neither the control and SCI animals showing a significant change in NE levels after surgery or injury (Fig 3B, control group W = -13, p = 0.219; SCI group t = - 0.613, p = 0.54).

Another potential regulator of AVP levels are natriuretic peptides (ANP and BNP). As with CORT and NE, an increase in ANP and BNP can suppress AVP release from the hypothalamus leading to decreased serum AVP levels (8, 27, 49).

Interestingly, in our cohort of subjects, the urinary levels of atrial natriuretic peptide was significantly elevated relative to pre-injury baseline at the two week post-SCI time-point (ANP: t = -2.73, p = 0.005) (Fig. 4A), whereas ANP levels in the control animals was not significantly different post-injury. Brain natriuretic peptide (BNP) trended toward higher values following injury, although this increase did not reach significance (p = 0.06). Apart from their influence on AVP levels (increased ANP and BNP levels can suppress AVP release), these peptides can also have a direct diuretic effect (50). A Pearson correlation indicated that there was a significant positive correlation between ANP levels post-injury and 24 hour urine output (r = 0.351, p = 0.033, Fig. 4C). There was no significant correlation between AVP or BNP levels post-injury and 24 hour urine output.

Due to observed changes in a number of physiological substrates post-injury, many of which interact with each other to influence urine output, a PCA was carried out to identify interrelationships between these substrates at two weeks following SCI. When all factors were added to the PCA, four components accounted for 81% of the variance seen in the analysis (Fig. 5). The first two components accounted for over 50% of the variance (53.8%). In Component 1, 24 hour urine volume, CORT and ANP
levels at two weeks post-injury had a strong positive influence on this component with Cre and SG levels having a strong negative influence within the same component. Component 2 involved an inter-correlation between 24-hour drink volume and NE levels post-injury. Component 3 (osmolality) and 4 (oliguria) accounted for 27.2% of the variance (Fig. 5).

Discussion

The results of this study further demonstrates the occurrence of polyuria following SCI and for the first time reveals that this increase in urine production is concomitant with changes in the levels of several important peptides that regulate fluid balance. Not only do these peptides directly affect urine output, they are known to influence one another (see Discussion below). Thus, although the changes in ANP levels following SCI have the strongest direct influence on urine output as revealed with a Pearson correlation, significant changes to AVP and CORT likely contribute indirectly to the overall development of polyuria following SCI, supporting the hypothesis that the mechanism involved in the development of polyuria after SCI is multifactorial (Fig. 6). PCA analysis also revealed this combinatorial effect, with positive interactions existing between several measures (CORT and ANP) and 24-hour urine output. The decrease in AVP and increase in ANP levels two weeks after SCI likely sets in motion a physiological cascade that limits water retention by decreasing the resorption of water in the kidneys leading to polyuria. Urine samples in this study exhibited evidence that this was indeed the case, with samples post SCI registering significantly decreased Cre levels and decreased SG, both indicators of decreased urine concentration. None of these changes in urine content or AVP/ANP/BNP levels was observed in the control group, suggesting that the surgical procedure itself (laminectomy without contusion) was not enough to initiate these changes. Instead, it appears that the SCI itself is in some way influencing AVP and ANP levels.

SCI leads to changes in levels of hormones controlling fluid balance -

As revealed in the Results section, there are significant changes in the levels of CORT, AVP and ANP at two weeks following SCI. As illustrated in Fig. 6, the direction
of these changes (a decrease in AVP, and an increase in ANP) is such that the tightly regulated fluid balance system is driven in the direction of polyuria (11, 13, 15, 43). The maintenance of fluid homeostasis in the uninjured body is tightly regulated by AVP (19). Changes in blood osmolality and/or blood volume are the most common triggers that influence the secretion of AVP from the posterior pituitary (7, 31). When blood osmolality is low (often due to over-hydration), secretion of AVP decreases (40), leading to less resorption of water in the kidneys and an increase in the amount of urine excreted (polyuria). When blood osmolality is high (often associated with dehydration) secretion of AVP increases leading to oliguria (decrease in urine production). In the current study SCI animals did increase their 24 hour water intake which may have decreased AVP secretion. Interestingly though, blood osmolality increased in these animals which should have led to an increase in AVP levels. Future experiments will need to investigate whether there are changes in blood electrolyte levels, in particular sodium, after SCI that may explain why blood osmolality increases even in the presence of significantly increased water intake.

With changes in blood osmolality and fluid intake presenting a contradictory picture regarding their influence on AVP secretion following SCI, a focus on non-osmotic factors that can also influence AVP secretion was necessary. Most notably, an increase in CORT and NE levels are known to suppress AVP secretion (3, 30, 35, 36). CORT levels have been shown to increase following SCI (21, 29) and such was the case for the contused rats at a two-week post-SCI time-point in the present study. This increase did not appear to be a result of surgery as the control animals that underwent a surgical laminectomy without a SCI had no significant increases in CORT levels at the same time-point. In contrast, NE levels were not significantly different following SCI or laminectomy surgery. Thus, the increase in CORT levels following SCI may contribute to the decreased levels of AVP seen at this same time point following SCI. Additionally, increased CORT levels can directly increase urine output (11, 43), therefore increased CORT levels post SCI may also contribute to SCI-induced polyuria independent of its influence on AVP levels, although no correlation between CORT levels and urinary output was found in this study. Future studies involving the administration of anti-
glucocorticoids post-injury would begin to address the influence that increased CORT has on SCI-induced polyuria and AVP levels following SCI.

The current results also reveal an increase in natriuretic peptide levels at two-weeks following SCI, although only ANP elevation reached statistical significance. As with CORT, this increase in ANP does not appear to be related to the surgery itself as the control animals did not show a change in ANP levels at the same time-point following surgery. Changes in natriuretic peptides are interesting because they can contribute to polyuria through several different mechanisms. Firstly, they increase the glomerular filtration rate by causing dilation of afferent blood vessels and constriction of efferent blood vessels (13, 23). Also at the level of the kidney, natriuretic peptides can bind directly to natriuretic peptide receptors (NPR-A) in the kidney to decrease the ability of AVP to bind to the V2 receptor (8, 15, 26, 50), thus interfering with water resorption in the collecting duct. Natriuretic peptides can also indirectly lead to polyuria through the suppression on AVP secretion from the posterior pituitary (8, 27, 38).

Although a relationship was found between an increase in ANP and urine volume following SCI, it is unclear whether this is due to direct effects of ANP on the kidney or indirect effects of ANP on suppressing AVP secretion which in turn leads to polyuria. The effect of ANP increase on the development of polyuria is most likely multifactorial, involving both its direct actions on the kidney and its actions on AVP release from the posterior pituitary.

Although the suppression of AVP release likely involves an increase in multiple non-osmotic factors (CORT, ANP/BNP), it is not known how SCI contributes to these changes. Natriuretic peptides are found predominantly in cardiac tissue (18) although there are also smaller concentrations of these peptides in the nervous system as well (10, 16, 46). Natriuretic peptides are released from cardiac tissue in response to atrial or ventricular wall stretch (4, 18). It has also been shown that their levels increase following neurotrauma (37, 41, 44) although it is not clear whether this increase is due to acute cardiovascular changes that lead to cardiac wall stretch or other mechanisms related to the trauma to the nervous system itself.

Apart from cardiac wall stretch, there are several molecules which can also stimulate natriuretic peptide release. An increase in glucocorticoids, for example, has
been shown to increase ANP release (4, 5, 25), and CORT levels were significantly increased at two weeks after SCI in the current study. The PCA analysis indicated that there were interactions between ANP, CORT, and 24-hour urine output post-injury, suggesting that increased CORT post-injury leads to increased ANP levels that in turn leads to increased urine output (polyuria). However, future studies, using anti-glucocorticoids to dampen the effects of the increased CORT post-injury, would be needed to provide further evidence in support of or against this hypothesis. An increase in endothelin-1 (ET-1) has also been shown to be a potent stimulator for natriuretic peptide release (12, 22, 32, 33). Although we did not measure ET-1 levels in this study, there is evidence that ET-1 levels do increase after SCI (24, 28, 34). Future studies will investigate the extent to which systemic ET-1 levels change after SCI and whether manipulation of ET-1 levels following SCI can influence natriuretic peptide levels post-injury.

Limitations and Considerations -
With a number of biological factors (AVP, CORT, and ANP) changing after SCI that may influence urine output it is possible that in some animals one or two factors may be more influential in the development of polyuria, whereas in other animals another factor or combination of factors may be more influential in the development of polyuria. This caveat may be the reason why there is some variability within our data. Despite this, there is clear evidence that polyuria develops after SCI, a condition that leads to disruptions in quality of life for individuals with SCI and increases the risk of other morbidities such as bladder over-distension and infections. The fact that changes within several biological factors important for fluid balance occur concomitantly with the development of polyuria provides a guide for future studies that can look at interventions which may lead to the amelioration of this condition and improve the lives of SCI individuals.

Conclusions
The results of this study provides evidence in support of the development of polyuria relatively early following SCI and has shown that concomitant with the
development of polyuria there are also changes in several metabolic peptides that play a vital role in maintaining water balance (AVP, ANP, and CORT). These changes in AVP, CORT and ANP are consistent with increasing urine production and output (i.e. polyuria). It remains unclear how much each of these peptides contributes to SCI induced polyuria, although it appears that the cumulative effect of a decrease in AVP along with increases in ANP and CORT is significant enough to effect urine output early after SCI. Future studies will investigate whether interventions targeting AVP and/or ANP can ameliorate SCI induced polyuria and thus provide future avenues for therapeutic interventions that may improve urinary function following SCI and improve the quality of life for SCI individuals.

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Author Contributions –
L.R.M and C.H.H contributed conception and design of research, performed experiments, analyzed data and interpreted experimental results. L.R.M and C.H.H also prepared figures, drafted, revised, and edited manuscript, and approved the final version of the manuscript.
References


Figure Legends -

Figure 1 – 24 hour urine volume increases after SCI. (A) The total volume of urine excreted over a 24 hour period at two weeks following SCI (n=41) was significantly increased compared to preinjury baseline urine output (preinjury = 7.16 ml +/- 0.47, post-injury = 15.63 ml +/- 0.74; p ≤ 0.001). This significant increase was not seen in the control group (p > 0.05). (B) Along with increased urine output the 24 hour drink volume was also increased following SCI (pre-injury = 29.77 ml +/- 1.91, post-injury = 40.91 ml +/- 1.74; p ≤ 0.001). As shown in (C), despite the increase in drink volume post-injury, there is no significant correlation between increased drink volume and increased urine output following injury (r = 0.190, p = 0.267). Bars in A and B represent group means and each circle represents an individual subject.

Figure 2 – Vasopressin levels decrease after SCI but are not associated with changes in plasma osmolality. (A) Serum vasopressin (AVP) levels decreased significantly at two weeks following SCI (n = 41) compared to preinjury levels (pre-injury = 9.35 pg/ml +/- 0.680, post-injury = 8.46 pg/ml +/- 0.497; p = 0.042). AVP levels did not however, change significantly in the control group (p > 0.05). (B) Osmolality changed significantly following SCI compared to baseline (pre-injury = 299.15 mOsm +/- 0.690 and post-injury = 301.95 mOsm +/- 0.452; p ≤ 0.002). This increase in osmolality however, should stimulate the release of AVP leading to an increase in AVP levels not the decrease in AVP levels which was observed. There was however no correlation between AVP levels and serum osmolality (p > 0.05). In the control group, there was no significant change in osmolality (p > 0.05). Note that at the post-surgery time-point, three of the six sham animals had the same blood Osm (300 mOsm) and two other shams had the same blood Osm (297 mOsm). Bars in A and B represent group means and each circle represents an individual subject.

Figure 3 – Corticosterone but not norepinephrine levels increase following SCI. (A) Increased corticosterone levels are known to lead to a suppression of AVP release. Urinary corticosterone (CORT) levels (measured as a ratio of CORT/Cre to account for
the more dilute urine post-SCI) were increased significantly at two weeks following SCI (n = 41) compared to pre-injury levels (pre-injury = 625.31 +/- 97.034 and post-injury = 921.19 +/- 126.678; p=0.03). There was no significant change in CORT levels in the surgical control group (p > 0.05). (B) There was no significant change in urinary norepinephrine levels at two weeks following SCI (p > 0.05). Bars in A and B represent group means and each circle represents an individual subject.

**Figure 4** – *Natriuretic peptide levels increase following SCI*. (A) Urinary atrial natriuretic peptide (ANP) levels (measured as a ratio of ANP/Cre) were significantly increased at two weeks following SCI (n = 38) compared to baseline pre-injury levels (pre-injury = 0.57 +/- 0.065 and post-injury 1.07 +/- 0.164; p = 0.005). Changes in ANP levels were not observed in the control group (p > 0.05). (B) Urinary brain natriuretic peptide (BNP) levels (measured as a ratio of BNP/Cre) trended toward higher values as well following SCI, although this change did not reach statistical significance (p = 0.06). As with ANP, BNP levels were not significantly changed in the control group (p > 0.05). (C) ANP levels were also positively correlated with 24 hour urine output at two weeks following injury (p = 0.0329). Bars in A and B represent group means and each circle represents an individual subject.

**Figure 5** – *Relationship between various biological factors and urine output post-SCI*. PCA analysis was performed to identify inter-correlations between the various biological markers assessed. (A) Four components accounted for 81% of the variance seen in the PCA with Component 1 (polyuria) accounting for 31.5% of the variance. Post SCI 24 hour urine volume, CORT and ANP levels at two weeks post-injury had a strong positive relationship to component 1 with Cre and SG levels having a strong negative relationship to this component. Component 2 accounted for 22.3% of the variance and involved an inter-correlation between 24-hour drink volume and NE levels post-injury. Component 3 accounted for 17.2% of the variance and involved a strong negative relationship of BNP to Osm. Finally, component 4 demonstrated a weak relationship between AVP and urine output, although urine output was more highly related to component 1 than component 4.
Figure 6 – SCI induced changes in fluid balance that can contribute to the development of polyuria. Summary diagram conceptualizing how changes in AVP, CORT, and natriuretic peptide levels can influence the delicate fluid balance system after SCI. In the uninjured physiological state fluid balance is tightly regulated through the control of release of various peptides, most notably AVP, ANP, and BNP. A change in the levels of these peptides can lead to a state of oliguria (decreased urine) or polyuria depending on the direction of change. For example, increased nocturnal AVP levels results in oliguria during sleep that prevents sleep disruption due to the need to urinate. Following SCI increases in ANP and CORT, and decreases in AVP appear to alter the homeostatic control of fluid balance within the physiological system driving the system towards a state of polyuria.
PCA analysis (varimax)

Factors in Components 1-4
1 - 24 hour urine output, ANP, CORT, Cre and SG
2 - 24 hour drink volume and NE
3 - BNP and osmolality
4 - AVP and 24 hour urine volume