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EXAMINING THE EFFECTS OF NPRA ANTAGONIST ANANTIN ON SCI-INDUCED POLYURIA

By

Lindsey Elaine Zipperer
B.S., Appalachian State University, 2020

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A Thesis Approved on

December 2022

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ABSTRACT

EXAMINING THE EFFECT OF NPRA ANTAGONIST ANANTIN ON SCI-INDUCED PLOYURIA

Lindsey Elaine Zipperer

December 9, 2022

Polyuria, or the over production of urine, is a prevalent condition that significantly impacts the quality of life in individuals suffering from a spinal cord injury (SCI). Post-SCI induced polyuria is thought to have been associated with altered levels of vasopressin (AVP), atrial natriuretic peptide (ANP), and natriuretic peptide receptor A (NPRA). In the present study, the function of NPRA was analyzed in the rat contusion model using anantin, an NPRA antagonist. A daily dose of either 100 µg of anantin or vehicle was administered intraperitoneally immediately following the SCI surgery and continued until termination, 4 weeks post injury (wpi). The animals were housed in metabolic cages for a 24-hour period every week to measure urine and drink volumes. Other assessments included mean arterial pressure (MAP) and serum potassium/sodium concentrations. Metabolic cage data showed a significant increase in void volume regardless of treatment when compared to pre-injury baselines. There was a significant decrease in MAP post-SCI, which was significantly less for anantin-treated rats. Further, there was a significant decrease in serum sodium and no change in serum potassium across all groups. Taken together, these results indicate that targeting NPRA alone may

not be an effective intervention, and further targets should be investigated to provide a more cohesive understanding of SCI-induced polyuria.

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INTRODUCTION

The consequences of sustaining a spinal cord injury (SCI) can be detrimental and lead to whole-body impairments including circulatory, musculoskeletal, immune, respiratory, reproductive, and renal systems. While musculoskeletal deficits are often outwardly seen as the most detrimental consequence of an SCI, the SCI population views urinary complications as one of the most significant disruptions to their quality of life[1]. One of the most important, yet poorly understood, urinary complications following SCI is polyuria, the overproduction and/or passage of urine[2, 3]. Polyuria has been documented in both human SCI and pre-clinical contusion models[4, 5]. Bladder emptying methods include intermittent catheterization, indwelling catheters, straining, and manual compression. Without these methods, patients are at an increased risk of bladder infections, urinary infections, and even sepsis[6, 7]. These methods must often be employed at night due to nocturia and can be highly disruptive to a patient's sleep schedule and thus daily activities. To avoid these persistent overnight bladder emptying methods, patients will often limit their fluid intake during the day. This can cause confounding issues such as dehydration, constipation, and autonomic dysreflexia[8].

While the underlying cause of SCI-induced polyuria is unknown, several studies have associated certain biomarkers - such as arginine vasopressin (AVP), atrial natriuretic peptide (ANP) and its receptor natriuretic peptide receptor A (NPRA) - with the overproduction of urine in the contusive rat model[9, 10]. While the administration of desmopressin (DDAVP), a synthetic analogue of arginine vasopressin, has had limited

success improving the effects of nocturnal polyuria, it is restrictive to certain age groups as it can cause water retention and hyponatremia[4, 9, 11]. Furthermore, a recent study in the rat contusion model found that daily injections of desmopressin reduced 24-hour water intake, and could thereby exacerbate the dehydration already seen in the SCI population [12]. Therefore, other therapeutics targeting these key fluid balancing hormones should be investigated.

The focus of the current study is to target the natriuretic peptide receptor, NPRA, to reduce SCI induced polyuria. The primary function of natriuretic peptides, like ANP and brain natriuretic peptide (BNP), is to promote natriuresis, or the excretion of sodium in the urine. Human ANP is encoded by the Natriuretic Peptide A gene (NPPA) and is primarily expressed in atrial myocytes, which form the muscular walls of the heart[13]. ANP is secreted by myocytes in response to stretching of the atrial wall and high blood pressure. ANP functions to increase renal sodium expression and inhibit sodium/water reabsorption into the kidney. This reduces expanded extracellular fluid volume, thereby increasing urine output[9, 14]. In addition, the acute effects of ANP include a reduction of blood volume, blood pressure, as well as cardiovascular homeostasis[15]. Serum ANP is used as a biomarker in clinical settings for cardiovascular diseases, such as stroke and coronary artery disease[16, 17].

Under normal physiological conditions, NPRA functions to promote vasodilation and the production of urine. It is located in the lungs, kidney, adipose tissue, and heart/blood vessels. The NPRA receptors located within the kidneys are crucial receptors to regulating water retention/excretion through the hormone ANP. ANP will activate NPRA to increase the excretion of salt into the collection ducts of the kidney, increase

glomerular filtration rates, and inhibit the reabsorption of water. Further, the ANP/NPRA system is directly related to circulatory disorders, hypotension, and modulation of cardiac hypertrophy seen in the SCI population[18-20]. While it is understood that the significant increase of kidney NPRA and urinary ANP together likely exacerbate the SCI induced polyuria[10, 21], there have been few therapeutic interventions targeting these hormones.

One therapy that has had limited success in reducing polyuria is the drug anantin. Anantin is the first microbially produced competitive peptide antagonist of natriuretic peptides and has been investigated as a possible therapy *in vitro* or acutely (less than 24-hours)[22, 23]. Anantin has been shown to acutely reduce urine output and oppose ANP-dependent blood pressure decrease in non-SCI settings[24, 25]. A recent study examined the effect of anantin on SCI-induced polyuria in the contusive rat model. An initial dose-response experiment found that intraperitoneal injections of a 60 µg dose of anantin resulted in significantly lower 24-hour void volumes when compared to lower doses (15 µg and 30 µg) over a 2-week period[26]. However, the same results were not seen after an 8-week period, perhaps suggesting that the dose of anantin was too low to sustain a prominent effect on urine volume over a longer time period.

The objective of this thesis is to determine the effect of the NPRA antagonist anantin on SCI-induced polyuria. Cardiovascular function will also be examined, given that blocking NPRA could potentially increase blood pressure[15, 20]. Additionally, serum sodium and potassium levels were measured, as the inhibition of NPRA may precipitate a sodium/solute imbalance[27, 28]. It is hypothesized that a higher dose of anantin, 100 µg, will be more effective at reducing polyuria in the SCI rat contusion model and may have secondary outcomes on blood pressure and serum solute levels. This

study will provide further insight into the underlying mechanisms of polyuria following SCI and provide a potential therapeutic intervention that would hopefully contribute to this population's quality of life.

METHODS

Animals

All animal experimental protocols were approved by the University of Louisville School of Medicine Institutional Animal Care and Use committee and all procedures were carried out in accordance with National Institutes of Health (NIH) guidelines. Adult male Wistar rats were obtained (n= 24) and were single-housed with a 12:12-h light/dark cycle. Animals were randomly assigned to either vehicle (veh) or anantin (A) groups following baseline data collection and spinal cord injuries. Experimenters assessing outcomes were blinded to which rats were given drug or vehicle.

Spinal Cord Injuries

After one week of acclimation to the vivarium and one week of pre-injury assessments (blood pressure recordings, blood draws, metabolic cage data), the animals were anesthetized with an intraperitoneal injection of 80 mg/kg Ketoset® (Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10mg/kg, AnaSed; Lloyd Laboratories, Shenandoah, IA). The animals then underwent a moderate contusion injury at the T9 spinal level using the Infinite Horizon (IH) impactor as previously described[9, 10]. The IH impactor can be positioned at different locations along the spinal cord to create both compression and/or contusion SCIs. Ocular reflexes and toe pinch techniques were used to confirm the surgical plane of anesthesia. The surgical site was shaved and disinfected using 4% chlorhexidine soap (Henry Schein). Ocular lubricant was applied (OptixCare,

Aventix) after animals were placed on heating pad (low heat) to maintain normal body temperature. Animals then received a T8 laminectomy to expose the T9 spinal cord. A moderate contusion injury was administered using the IH impactor with a 215 kilodyne impactor force. No dwell time was used. Sutures were used to close the muscular layer. The skin was subsequently closed with surgical wound clips. After closure, animals were transferred to a clean cage on a heating pad per standard post-operative care. Antibiotic (penicillin G, PenJect; Henry Schein Animal Health, Dublin, OH) and analgesic (meloxicam, Eloxiject; Henry Schein Animal Health) were administered subcutaneously [29, 30]. To account for fluid loss during surgery, 5mL of physiological saline was administered subcutaneously pre- and post- operatively (total 10mL). Manuel Credé maneuver of the urinary bladder was performed twice daily (early morning and late afternoon) until reflexive bladder function was restored at 4-6 days post-injury.

Locomotor Assessment

Post-injury motor behavior was assessed using the Basso-Beattie-Bresnahan (BBB) [31] locomotor test. The test was administered once per week on every rat. Two experimenters blinded to the study would rank the animal's mobility based on average of the right and left hindlimb BBB scores. This evaluation is used as an indicator of injury severity and spontaneous regeneration, per our previously published data [5].

Metabolic Cages

Animals were housed in a 12-station metabolic cage to monitor 24-hour drink volume and urine output volume [32]. To obtain reliable baseline measurements, the animals were housed in metabolic cages for two separate 24-hour time periods, but only

the second 24-h period was used for analysis. Following SCI, metabolic cage assessments were performed once-weekly until termination [9, 10]. Food and water were available *ad libitum.* Total 24-hour urine volumes were centrifuged for 15 minutes to separate food remnants before being logged. Total drink volume was recorded.

Anantin Treatment

The NPRA antagonist anantin (United States Biological; Salem, MA) was used to determine the effect of NPRA inhibition on urine volume after SCI. Previous studies have implicated that anantin may be effective at reducing urine production in non-SCI-induced settings[25, 33]. A recent study has shown some efficacy of anantin in SCI-induced settings at 60 µg/animal[26]. To determine if a higher dose would be more effective at reducing urine output, aliquots 100 µg/animal were administered, allowing for approximately a 25% increase in anantin concentration per animal. The anantin was dissolved in ddH2O per manufacturing instructions and stored at -20 ºC until use. Injections with either 100 µg or vehicle (ddH2O) began on the injury date and continued daily until termination (4 wpi). Injection administration occurred at the same time daily, between 14:00-16:00 hours.

Blood pressure

Heart rate, systolic, diastolic, and mean arterial pressure (MAP) were recorded in conscious animals using a non-invasive tail cuff method (Harvard Apparatus, LE 5002)[34]. Animals underwent an initial acclimation period to the environment and apparatus to ensure accurate blood pressure readings are not impacted by stress related to testing. To begin, the animals were acclimated in the quiet testing room for 15 minutes.

The animals were then placed in testing tubes, each of which were of adjustable size to limit movements. The tubes were placed on a heating pad at the low setting to promote relaxation and vasodilation per manufacturer instructions. The cuff was placed on the tails to allow the animals to become accustomed to the pressure. The animals remained in the tubes for 15-minutes before being placed back in the cages. For each data collection session, animals would acclimate to the room for a 15-minute period before being placed in the tubes for another 15 minutes. The blood pressure cuff was placed in the same general area, mid-tail, to assure consistency across the weekly blood pressure reading. A minimum of three measurements, but no more than five, were recorded per rat during each weekly session.

Blood Sampling

Blood collections were performed on both anantin and vehicle animals at three time points: pre-injury (baseline), 2 weeks post-injury (wpi), and terminal (4 wpi). Isoflurane (2%) was used to briefly anesthetize animals for blood draws. Ocular reflexes were tested prior to blood collection to assure the anesthetic plane was reached. The base of the tail was then shaved, and the animal was placed on a heating pad set to low heat. The lateral vein was punctured using an 18g needle and 05.-0.7 mL of blood was collected into serum separator tubes (BD microcontainer, Becton, Dickinson and Co)[35]. Sterile gauze was applied to stop the bleeding, and styptic powder with benzocaine was used as needed (Kwik-Stop, ARC Laboratories). The tubes were then centrifuged for 15 minutes at 14,000 rpm. The separated serum was then collected and stored at -20 ºC until analysis.

Flame Photometry

Flame photometry is used for the quantitative concentration of solutes (in this case sodium and potassium) in an aqueous solution[36]. Serum sodium is directly regulated by the ANP/NPRA system. Before analyzing the samples, standards were diluted and used to calibrate the system per manufacturer instructions. Serum samples were acclimated to room temperature and diluted 1:150 per manufacturer recommendations. The diluted samples are then aerosolized via a nebulizer. The wavelength of color emitted by the flame after the samples aerosolization corresponds to a specific metal ion concentration. The concentration is displayed on the screen and logged in a study record.

Statistical Analyses

One-way repeated ANOVA measures were performed to compare 24-hour drink/urine volume, MAP, and serum sodium/potassium concentrations between the anantin and vehicle groups across all timepoints. Whereas the ANOVA tests distinguished statistical significance when compared to baseline values, T-test analyses were used to distinguish statistical significance between the groups at specific timepoints. All data was checked for outliers using the online GraphPad calculator. SigmaStat v3.5 (Systat Software) was used for all statistical analyses, where significance was set at $p<0.5$ for all tests utilized.

RESULTS

Experimental groups:

Complete data sets were obtained for 23 out of 24 rats. Details about each group - including baseline body weight, impactor force and displacement, and BBB scores - are summarized in Table 1. No significant differences between treatment and vehicle groups were found, confirming effective randomization. One rat was eliminated from the study due to an early BBB outlier (indicative of a mild injury).

Metabolic cage:

Both the vehicle and treatment groups demonstrated significant increases in 24-hour urine volumes at all timepoints following SCI injury (Figure 1A). However, there were no significant differences between the anantin and vehicle group at any timepoint across the 4 weeks. Of note, both the anantin and vehicle groups demonstrated a significant increase in 24-hour drink volume across all timepoints (Figure 1B). There were no significant differences in drink volume between the two groups at any timepoint. Interestingly, correlation of urine output and water intake showed statistical significance and moderate positive correlation (Figure 2). However, water intake displayed a 1.5-fold increase from pre-injury baseline values to 1 wpi, whereas urine output displayed a 2.0 fold increase from pre-injury baseline values to 1 wpi. This indicates that there are other physiological mechanisms besides water intake influencing the overproduction of urine.

Blood pressure

A non-invasive tail cuff apparatus was used once weekly to measure at rest heart rate (measured in beats per minute (BPM)), systolic blood pressure, diastolic blood pressure, and mean arterial pressure (MAP). The vehicle group showed a significant decrease in blood pressure at every time point (Figure 3). The anantin group demonstrated a significant decrease in MAP at 2 wpi and 3 wpi but was not statistically different from baseline values at 1 wpi or 4 wpi. Further, there were significant differences in MAP between the anantin and vehicle groups at 1 wpi and 4 wpi.

Serum sodium and potassium

There was a significant decrease in relative serum sodium levels in both the vehicle and anantin groups at all timepoints when compared to baseline values (Figure 4). There was a statistically significant difference between the anantin and vehicle serum values at 2wpi, but not at the terminal timepoint (4 wpi). There was no significant change in relative serum potassium levels in the vehicle or anantin treatment groups at any timepoint when compared to baseline values. However, there was a significant increase in the anantin serum potassium when compared to the vehicle group at 2 wpi, but not at the terminal timepoint (4 wpi).

Table 1: SCI Impactor Parameters and Assessment Outcome Values

Group	n	Injury force (kdyne)	Displacement (μm)	Baseline weight	14 dpi BBB	Terminal BBB
Anantin	12	228 ± 18.4	1428.4 ± 175	453 g ± 21	8.7 ± 2.67	10.3 ± 1.23
Vehicle	11	227 ± 18.5	1279.8 ± 107	449 g ± 30	10.3 ± 1.49	10.9 ± 0.54

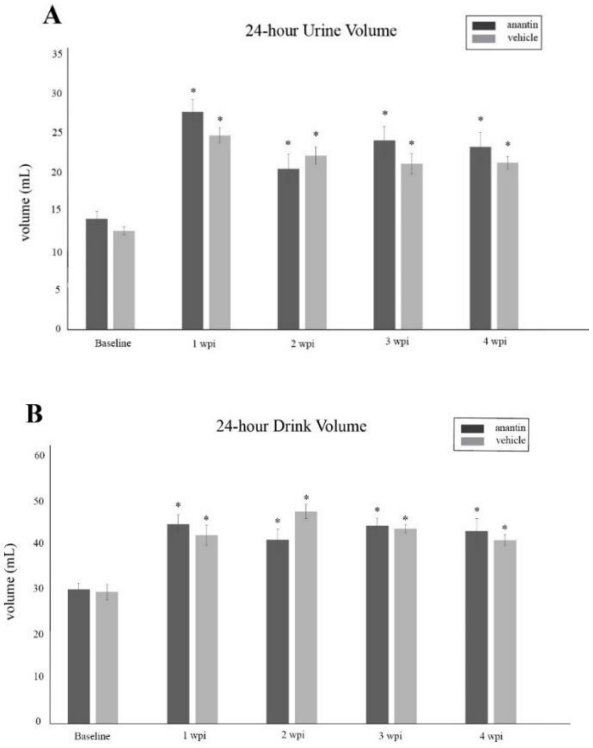

Figure 1:

Metabolic Cage Data Summary. The average 24-hour void volume output demonstrates a statistically significant increase (*$p<0.05$) in urine output at every timepoint for both the treatment and vehicle groups when compared to pre-injury baselines (average 13.66 mL)(Fig 1A). Drink volume also showed a statistically significant increase when compared to pre-injury baselines (average 29.95mL)(Fib 1B). There was no difference

between the anantin or vehicle treatment group at any timepoint across drink intake or urine output. Values represent average volume with standard error.

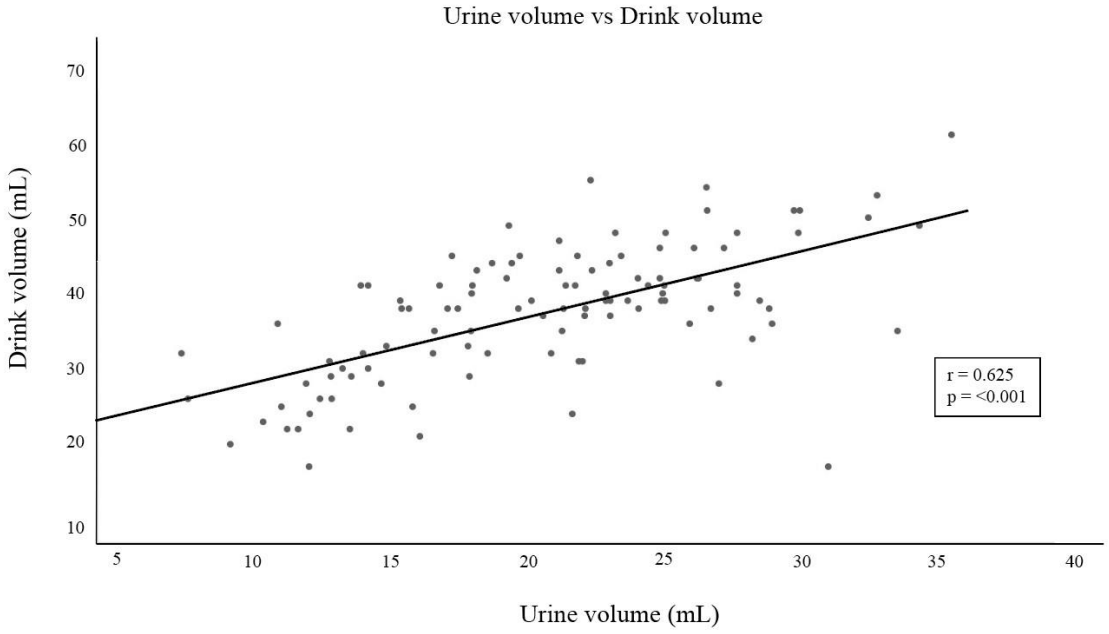

Figure 2:

Urine Volume vs Drink Volume: The correlation of urine output and water intake showed statistical significance and moderate positive correlation ($p<0.001$, $r=0.625$).

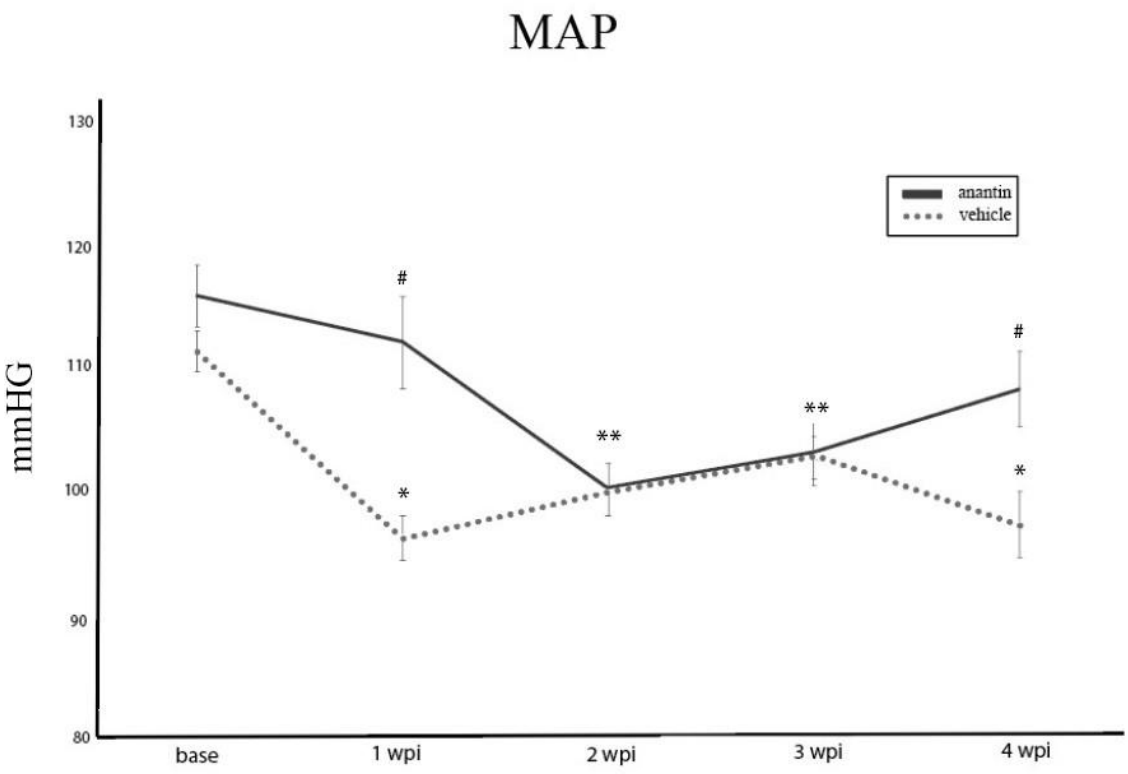

Figure 3:

Change in MAP following SCI: There was a significant decrease in MAP from baseline in the vehicle group at every timepoint following SCI (*p<0.05). The anantin treated animal group showed a significant decrease in MAP from baseline at 2wpi and 3wpi (*p<0.05). There was a statistically significant difference between the anantin and vehicle treatment groups at 1 wpi and 4 wpi (#p<0.5). Values represent means of MAP with standard error.

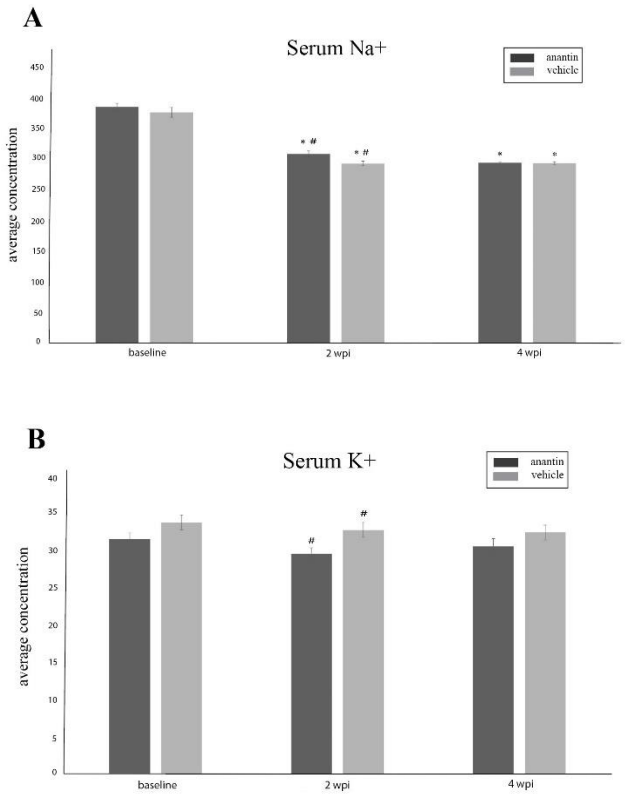

Figure 4

Average serum sodium and potassium following SCI: The average concentration of serum sodium levels in both the anantin and vehicle groups were statistically lower at all timepoints when compared to pre-injury baseline values (*$p<0.05$)(Fig 4A). There was a statistically significant difference between the anantin and vehicle serum sodium values at 2wpi (#$p<0.5$). There was no change of serum potassium concentration of either group when compared to pre-injury baseline values (Fig 4B). There was a significant increase in the anantin serum potassium when compared to the vehicle group at 2 wpi (#$p<0.5$). Values represent means of groups with standard error.

DISCUSSION

SCI-induced polyuria is one of the most persistent disturbances in individuals suffering from spinal cord injuries. The most common intervention includes restricting fluid intake, which can lead to dehydration, constipation, and a myriad of other health issues. Desmopressin has been investigated as a possible therapeutic intervention, however it has restricted use and may exacerbate the dehydration already seen in these patients [12]. While the underlying causes of SCI-induced polyuria remains incompletely understood, many biomarkers have been previously identified as possible targets[3, 10, 21]. The current study targets the biomarker NPRA via the antagonist anantin as a possible means to reduce SCI induced polyuria.

A previous study investigating the effects of anantin on SCI-induced polyuria found that while it was unsuccessful at 60 µg/animal, a higher dose may exhibit more efficacy in future studies[26]. Therefore, aliquots of 100 µg/animal of anantin were administered in this study, allowing for an approximately 25% increase in dosage per animal. This increase did not have a significant effect on urine output post SCI. There are many possible explanations as to why anantin was not effective at returning urine output to pre-injury baseline volumes. An ideal route of drug administration has not been determined, and therefore the once daily dose of 100 µg/animal was not enough to elicit a notable physiological response. Previous studies have utilized intravenous, intraperitoneal, or direct drug application with varying results[24-26]. Further, the

bioavailability of anantin (or the extent and rate at which it is active) is unknown, and therefore the dose of 100 µg/animal may not have been enough to cause a significant change to urine production. A recent study has also shown that the levels of kidney NPRA fluctuate following SCI, whereas the polyuria seen remains consistent[21]. This suggests that NPRA alone may not be a significant driver in SCI-induced polyuria, and thus anantin would have little effect.

The significant decrease in MAP was another notable finding of this study. The decrease in MAP within the vehicle treated animal group is consistent with the hypotension seen in the SCI population[37, 38]. However, the anantin treated animal group was significantly closer to baseline values at the first timepoint (1 wpi) and terminal timepoint (4 wpi). Similar findings were found in a recent study where the vehicle treated animal group demonstrated a significantly lower MAP than the anantin treated group post-injury (1-2 wpi)[26]. One possible explanation for this is the fluctuations in NPRA density following SCI. A recent study found that kidney NPRA expression was significantly higher at 3, 7, and 42 days post injury (dpi) relative to 14 dpi values[21]. NPRA causes a decrease in blood pressure following SCI, which is consistent with responses seen in the vehicle group. It is possible that an increased concentration of NPRA at these timepoints increased capacity for binding of anantin, allowing it to inhibit the activity of NPRA and thereby increase MAP. Moving forward, it would be worthwhile to examine a more long-term effect of anantin on MAP.

Flame photometry findings indicated a significant decrease in serum sodium concentrations from baseline at all timepoints in both the anantin and vehicle treated animal groups. We have previously reported an acute increase (2wpi) of serum

osmolality, which suggests that other electrolytes may have been involved [9]. Hyponatremia, or low sodium levels, is found clinically with both acute and chronic spinal cord injuries [38-40]. Further, SCI individuals with hyponatremia show greater evidence of neurogenic hypotension. This combination of low BP and hyponatremia has been shown to complicate the descending renal pathway, thus contributing to further urinary complications in this population. Investigating MAP and serum sodium levels in future studies could be useful in determining therapeutic interventions for these potentially deleterious conditions.

The results of this study suggest that SCI-induced polyuria is a complex issue involving many other multilayered systems, and therefore NPRA alone may not be an ideal target. Additional targets that influence urinary and cardiovascular function, such as renin and angiotensin, may be a useful direction for future studies. The renin-angiotensin-aldosterone system (RAAS) is a critical regulator of blood volume and modulates sodium reabsorption, potassium secretion, and vascular tone [41]. The release of renin within the kidneys is inhibited by the release of ANP, which has been found to vary at different timepoints post-SCI [21]. Once it is released, renin will increase the production of angiotensin and activate multiple systemic effects, such as stimulating thirst, sodium reabsorption in the kidneys, and prompting the release of vasopressin and aldosterone. While previous studies have investigated the changes in the RAAS system following SCI [42, 43], there has yet to be a study examining the possible association between the RAAS system and SCI-induced polyuria.

While anantin exhibited a small but significant effect on decreased MAP, it was not an effective therapeutic intervention for SCI- induced polyuria. Further studies

investigating both urinary function and cardiovascular function in the SCI animal model will help provide insight into possible therapeutic interventions for this population.

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CURRICULUM VITAE

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Education

2020 – present	Graduate, Department of Anatomical Sciences and Neurobiology University of Louisville, Louisville KY
2015-2020	Undergraduate, Department of Health and Exercise Science Major in Exercise Science Minor in Biology, Minor in Nutrition and Foods Appalachian State University, Boone NC

Academic Experience

2020 – present	Graduate Student, Spinal Cord Injury Research Lab
Fall 2021	Teaching Assistant for Neuroanatomy
Spring 2020	Teaching Assistant for Advanced Head and Neck Course
2018 - 2020	Dissection Assistant in Cadaver Lab
Fall 2019	Undergraduate Research in Integrative Muscle Physiology Lab
Fall 2018	Teaching Assistant for Anatomy Lab
2016-2018	Undergraduate Research Assistant in Human Performance Lab

Work Experience

2022 – present	ER Technician at University of Louisville Hospital
2018-2020	Veterinary Technician at Boone Animal Hospital
2016-2020	Holmes Convocation Center Supervisor

Volunteer/Outreach

2020 – present	UofL Diversity and Inclusion Book Club Member
2020 - present	Member of Science Policy and Outreach Group
2020 – present	Regular Blood Donation for American Red Cross
07/16/2022	Volunteer at West Louisville Math and Science Project: "Exploring Human Anatomy"
03/24/2022	Volunteer at Louisville Science Pathways "Brain Days"
03/02/2022	Judge at Louisville Regional Science & Engineering Fair
01/26/2022	Guest lecture for Waggener HS: Basics of Anatomy and Physiology
01/11/2022	Guest lecture for UofL Undergraduates: Embryological Development of the CNS
Summer 2021	Scribe at Family Community Clinic
05/17/2021	Guest lecture for Olmstead MS: Basics of Anatomy and Physiology

2018 – 2020	President of Treble Attraction Acapella
2016 - 2017	Volunteer in ASU Animal Research Facility

Achievements

2016 - present	Dean's list (3.45 GPA or higher)
	Chancellor's list (3.85 GPA or higher)

Scholarships

2018-2019	Marlee Foundation Scholarship Recipient
2015 - present	Unit Scholarship Fund Recipient (received every year)

Publications

Spring 2021	Jason H. Gumbel MS1, Lindsey E Zipperer BS1, Charles H. Hubscher PhD1,2, *Effect of anantin on polyuria in spinal cord injured rats* [Manuscript submitted for publication], Department of Anatomical Sciences and Neurobiology, University of Louisville