The Effect of Osmotic Stimulation of Copeptin on Glucose Metabolism in Healthy Adults

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The Effect of Osmotic Stimulation of Copeptin on Glucose Metabolism in Healthy Adults

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Kinesiology

by

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ABSTRACT

Diabetes is currently affecting 30 million Americans. To combat disease onset, lifestyle interventions have been focused on. Low water intake, alongside elevated concentrations of circulating copeptin, has been linked to increased risk for. However, it is unknown whether this effect on glucose regulation is acute or chronic. Furthermore, no study to date assessed the effects of copeptin independent of the renin-angiotensin-aldosterone system (RAAS). Purpose: The purpose of these studies was therefore two-fold: 1) to establish high endogenous copeptin concentrations independent of RAAS, and 2) to assess glucose metabolism following a standardized oral glucose tolerance test (OGTT) during high concentrations of copeptin.

Methods: 60 subjects participated in this randomized, crossover design study. On two trial days, subjects were infused for 120-min with either 0.9 % sodium chloride (NaCl, ISO) or 3.0 % NaCl (HYPER) (Study 1). Post infusion, a 240-min oral glucose tolerance test (OGTT, 75 g) was administered (Study 2). Changes in plasma volume (∆PV), plasma osmolality (POsm), electrolyte, copeptin, glucose, insulin, glucagon, crh, acth and cortisol concentrations were measured every 30 min. Results: During HYPER, plasma osmolality (POsm) and copeptin increased (P<0.05) and remained elevated during the entire six-hour protocol, while RAAS hormones were within the lower normal physiological range at baseline, and declined following infusion. Fasting plasma glucose did not differ between trials (P >0.05) at baseline and during the 120-min of infusion. During the OGTT the positive integer of the area under the curve (AUC) for glucose was greater during HYPER (401.5±190.5 mmol·L⁻¹·min) vs. the ISO trial (354.0±205.8 mmol·L⁻¹·min, P < 0.05). The positive integer of the AUC for insulin during OGTT did not differ between trials (HYPER: 55,850±36,488 vs. ISO: 57,205±31,119 pmol·L⁻¹·min). Baseline values of serum glucagon were not different between the two trials, however,
the AUC of glucagon during the OGTT was also significantly greater in HYPER (19,303±3,939 ng·L⁻¹·min) vs. the ISO trial (18,600±3,755 ng·L⁻¹·min; P < 0.05). **Conclusion:** The present data indicate that acute osmotic stimulation of copeptin induced greater hyperglycemic responses during the oral glucose challenge possibly due to greater glucagon levels.
ACKNOWLEDGEMENTS

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I.

INTRODUCTION

The global burden of diabetes is increasing at alarming rates. Whereas in 2010, 285 million people were reported as diabetic, this number has reached an estimated 415 million in merely 5 years (1). The Center for Disease Control and Prevention estimates, that, following current trends, as many as 1 in 3 American adults could suffer from diabetes by 2050 (2). This rampant rise in prevalence urgently calls for a change in programs and policies targeting awareness, improving diagnosis and identification of additional risk factors for T2DM with the ultimate goal to contain this major health crisis (3).

Risk factors for diabetes are categorized as non-modifiable and potentially modifiable factors. With age, family history/genetic predisposition, ethnicity, a history of gestational diabetes (GDM), and polycystic ovary syndrome being considered non-modifiable, it is the modifiable category that allows for intervention and delay of T2DM onset in at-risk populations (4, 5). So far, the most important potentially modifiable risk factors for T2DM have been identified as sedentary lifestyle, poor dietary habits, and sleep deprivation (6). In clinical trial settings, lifestyle interventions targeting reduction in central obesity (7) via changes in diet and increased physical activity, have shown efficacy in combating the pathophysiological cascade leading to T2DM with outcomes exceeding those resulting from pharmaceutical approaches (8, 9). While we seem to have identified efficacious methodologies improve modifiable risk factors such as reducing central obesity via increase in physical activity and improved nutrition, barriers such as poor motivation; lack of time; environmental, societal and social pressures; socioeconomic constraints; along with health and physical limitations prevent these methodologies to be adapted successfully in free living adults, outside of the clinical trial setting.
Therefore, the search for additional, potentially more easily modified risk factors must continue.

Recently, epidemiological studies have associated low water intake with increased risk for new-onset hyperglycemia (11); while overall higher plain water intake has been associated with lower overall T2DM risk (12) and lower glycated hemoglobin (HbA1C) values in males (13). During states of low water intake, an imbalance of fluid gain to fluid loss results in an increase in plasma osmolality (P\text{Osm}) which stimulates the release of arginine vasopressin (AVP) from the posterior pituitary (14). Via activation of AVP specific V2 receptors, aquaporin channels at level of the kidney open and reabsorption of water into the vasculature is facilitated (15, 16). Furthermore, AVP has been shown to elicit hyperglycemic effects in rat and mouse models, presumably via hepatic glucose production (17, 18). Inconsistent findings in regards to AVP specific receptor involvement and signaling hierarchy during glucose and lipid metabolism however, has deemed further investigations into pharmacological approaches to AVP receptor antagonist/blocking therapy not yet feasible (19-22).

Lack of investigation of potential hyperglycemic effects of AVP in humans is likely explained by the very short half-life of ~24min. However, in 2008, the group around Nils Morgenthaler discovered the c-terminal peptide of 39-amino acids, copeptin, to be cleaving off the same neurophysin II moieties as AVP and therefore being co-secreted from the posterior pituitary (23, 24). Copeptin has since been shown to be more stable than AVP, while showing similar responses to water intake and deprivation, terming it a valid surrogate marker (25). Studies from European cohorts have since defined copeptin as a biomarker for T2DM risk in European cohorts (26, 27).
To our knowledge, only three human intervention trials to date have assessed the effect of acute water intake interventions on lowering circulating copeptin and fasting glucose concentrations (28-30), showing efficacy in those participants with habitually elevated copeptin concentrations alone. However, only one study to date has assessed glycemic responses following an acute elevation of copeptin concentrations. Interestingly, findings from this study show no effect on glycemia (31).

While these studies are the first human interventional studies addressing this topic, no methodology has been able to single out the potential interplay of circulating copeptin concentrations on glucose metabolism as suggested by animal models.

The aim of the following investigation was therefore 2-fold: a) to utilize a method of dehydration, which will allow for an acute rise in copeptin concentrations compared to control independently of renin-angiotensin-II-aldosterone-system (RAAS) activation; b) to assess glucose metabolism responses to a standardized oral glucose tolerance test during states of high circulating copeptin concentrations compared to control while controlling for RAAS activity in both conditions.
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II.

STUDY 1:
THE EFFECTS OF HYPERTONIC SALINE INFUSION ON INDEPENDENT STIMULATION OF COPEPTIN

ABSTRACT

**Background:** While epidemiological studies have shown positive associations between the AVP surrogate marker copeptin and risk for Type 2 diabetes mellitus (T2DM), early interventional trials have yielded mixed results, which may be due to perturbations elicited by renin-angiotensin-II-aldosterone-system (RAAS) activation.

**Objective:** To achieve RAAS-independent stimulation of AVP measured via surrogate marker copeptin.

**Design:** 60 subjects (30 females) participated in this cross-over design study. On two trial days, separated by at least seven days (males) or one menstrual cycle (females), subjects were infused for 120-min with either 0.9 % sodium chloride (NaCl, ISO) or 3.0 % NaCl (HYPER).

**Results:** During HYPER, plasma osmolality (POsm) and copeptin increased ($P < 0.05$) and remained elevated during the entire two-hour protocol, while renin-angiotensin-aldosterone system (RAAS) hormones were within the lower normal physiological range at the beginning of the protocol and declined following infusion.

**Conclusions:** The present data indicate that acute osmotic stimulation via hypertonic saline infusion results in stimulation of copeptin while reducing circulating RAAS hormone concentrations. This method may be the preferred methodology to assess independent effects of copeptin concentration on metabolic processes.
This study was registered at ClinicalTrials.gov as NCT02761434.

**Keywords:** vasopressin; OGTT; dehydration; hydration; diabetes
INTRODUCTION

Recent literature has linked low water intake and elevated concentrations of copeptin, a surrogate marker of the fluid regulatory hormone arginine vasopressin with increased risk of new onset hyperglycemia and an increased risk for type 2 diabetes mellitus (1-5). While analysis of epidemiological data has found associations that support the hypothesis of prescribed hydration practices as a preventative measure for the development of impaired glucose intolerance (1, 6), results reported from interventional trials are mixed. These varying findings in interventional trials are likely due to differing methodologies.

Studies investigating the efficacy of chronic changes in hydration practices assessed the effect of increased water intake for interventions of 6 weeks (7) and 1 week (8) in healthy adults. While the 6-week intervention prescribed 2 L·day⁻¹ for men and 1.5 L·day⁻¹ for women to match the EFSA dietary reference values for fluid intake, the 1-week intervention trial included 3 L of water in addition to habitual fluid intake. Findings however differed, as that the 6-week intervention reported a reduction in circulating copeptin concentrations in individual with low to moderate total fluid intake, while the 1-week intervention, despite a higher water intervention protocol, reported a response for individuals with habitually low water intake only.

In a follow-up study enrolling habitual low-drinkers with circulating copeptin concentrations of 10.7 pmol·L⁻¹ for males and 6.1 pmol·L⁻¹ for females identified via a population-based survey, Enhorning et al. reported a significant reduction in fasting plasma glucose and copeptin concentrations following a 6-week 1.5 L·day⁻¹(9). These findings, while reporting a beneficial effect of increased water intake on fasting glucose and copeptin levels, also underline that these effects are solely seen in individuals with elevated circulating copeptin concentrations (2).
Studies investigating the effect on glycemic responses following acute manipulation of copeptin concentrations are few and stand in contrast to findings from the chronic intervention protocols. Administering a model of acute hypohydration to assess its effect on glycemic regulation in healthy adults, Carroll et al. utilized a passive heating protocol with subsequent 24-h fluid restriction to stimulate increased circulating concentrations of AVP, measured via surrogate copeptin. When comparing oral glucose tolerance test (OGTT) responses to the fluid replenished control trial however, no effect on glycemic regulation was detected. The lack of findings could be due to trial design: the passive heating protocol administered to induce hypohydration. Passive heating per se has previously been reported to affect glucose and insulin concentrations (10). Further, aldosterone, another fluid regulatory hormone sensitive to plasma volume losses as they occur during, for instance, passive heating, has been linked to decreased insulin sensitivity in normal and obese individuals (11). Therefore, utilization of the passive heating protocol could have caused perturbations, ultimately resulting in null-findings.

Johnson et al. tested the effect of a 3-day fluid restriction and hypothesized upregulation of the renin-angiotensin-II-aldosterone-system (RAAS) and hypothalamic pituitary hormones on glycemic responses in type 2 diabetics. While not measuring concentrations of copeptin, the assessed impaired glucose tolerance to OGTT was attributed to elevated concentrations of cortisol alone. With previous studies assessing acute effects of hypohydration while relying on methodologies resulting in hypovolemic dehydration and subsequent stimulation of all fluid regulatory pathways (12), we propose that the hypervolemic dehydration approach should be utilized to allow for focus on the effect of copeptin on metabolic processes alone.

Hypertonic saline infusion protocols have been widely used to induce an osmotic stimulus by raising plasma osmolality to study metabolic processes in a simulated dehydrated
state (13-16). The infusion protocol with a hypertonic solution allows for an acute rise in POsm and hence AVP stimulation that can be achieved within hours, without the inconvenience of tedious water restriction protocols that place stress on the participant and often yield in non-compliance due to discomfort caused by symptoms of under-hydration such as intense thirst, dry mouth, headache and impaired cognitive performance (17-20).

With intravenous fluid administration, volume loading occurs. Since RAAS is more sensitive to activation of unloaded baroreceptors during states of decreased blood volume (21), infusion protocols should therefore silence RAAS activity. Utilization of hypertonic solution results in an increase in POsm dependent upon the solute load of the solution and duration, with subjects considered to be entering hypertonicity at POsm values ≥300 (22). With induction of hypertonicity, an increase in osmotic pressure gradient originating from the extracellular fluid compartment will draw fluid from the inter-, and intracellular fluid compartments and a cellular dehydration effect alongside a stimulation of arginine vasopressin release has been described (23, 24) in efforts to reach osmotic equilibrium.

The aim of this study was therefore to induce hypertonic or isotonic states via intravenous infusion, while assessing cellular dehydration via proxy measurements of plasma volume expansion, total plasma protein and plasma potassium concentration, respectively. As a secondary aim, this study sought to assess the effect of utilizing hypervolemic hypernatremia as a means to suppress fluid regulatory hormones attributed to RAAS, while keeping AVP stimulation, measured via surrogate marker copeptin, active.
METHODS

Subjects

Out of 77 volunteers recruited between April 2016 and March 2017, 12 candidates failed to meet inclusion criteria, while 5 subjects voluntarily withdrew from the study after enrollment. Sixty adult volunteers (30 females) participated in this study [age, 39.0±8.0 y; weight, 78.2±15.2 kg; height, 1.70±0.09 m; BMI, 26.9±4.0 kg·m\(^{-2}\)]. The Consolidated Standards of Reporting Trials (CONSORT) flow diagram is shown in Supplemental Figure 1.

During the screening process, subjects were required to complete a medical history questionnaire which was reviewed by an advanced nurse practitioner to exclude patients with diabetes, kidney disease, metabolic disorders, cardiovascular disease, and other potential fluid balance covariates such as habitual use of non-steroidal anti-inflammatory drugs or serotonin reuptake inhibitors. For female participants, the oral hormonal contraception method was permitted as long as it included a seven-day wash-out period during their monthly cycle. Pregnancy, lactation, or use of injectable contraceptives however, were additional exclusionary factors. All participants signed an informed consent statement prior to enrollment. The study was approved by the institutional review board for human experimentation in accordance with the Helsinki Declaration of 1975 as revised in 1983. This trial was registered before the onset of subject recruitment at www.clinicaltrials.gov as NCT02761434.

Experimental Design

In this counter-balanced, cross-over, and single-blind design study, each participant completed two separate trials. To determine the acute effect of hypertonic saline infusion on induction of cellular dehydration and hypertonic hypervolemia, participants received a 120-min
saline infusion (Figure 1). Both trials differed only in saline concentration administered during the infusion, with each subject receiving 0.1 ml·kg⁻¹·min⁻¹ of either hypertonic saline (HYPER, 3% NaCl), to raise POsm, thereby generating a transmembrane osmotic gradient, or isotonic saline (ISO; 0.9% NaCl), to serve as control (15).

**Study controls**

Experimental days were separated by one week for males and post-menopausal or ovariectomized females to ensure sufficient wash-out (16). Female subjects with regular menstrual cycle were tested during the early follicular phase of two consecutive menstrual cycles, to control for the effect of reproductive hormones on body fluid balance (25). For the 24-h prior to testing subjects recorded all food and fluid intake into a provided food diary and refrained from exercise, caffeine, and alcohol. In preparation for the second experimental day, subjects received a copy of their first pre-trial dietary record and were asked to replicate it. To standardize the dinner prior to each testing day, subjects consumed a provided frozen meal (two Smart Ones Spaghetti bowls; 506 kcal, 78 g carbohydrates, 10 g fat, 26 g protein), before entering a 10-h fasting period prior to the scheduled trial. During this 10-h fast, subjects were allowed to drink plain water only. Lastly, to ensure adequate hydration, subjects were instructed to consume provided bottled water based on the 80% of the Institute of Medicine reference values for water intake (2 L for females and 3 L for males) for the day prior to each experiment (26). These pre-trial hydration guidelines were based on data from the National Health and Nutrition Examination Survey, which reports fluid intake to account for approximately 80% of total water intake (27).
Study protocol

All subjects reported to the lab following a 10-h fast and provided a urine sample. Hydration status was assessed via urine specific gravity measurement with values <1.020 required to proceed with the protocol (28). Subjects then sat in a cushioned phlebotomy chair (Convalescent Recliner – XL 5291, Winco Mfg., LLC, Ocala, FL, USA) and were not allowed to stand-up or lay down until the end of the experiment to avoid intercompartmental body fluid shifts associated with changes in body posture (29). An intravenous (IV) catheter was placed into an antecubital vein and subjects rested for at least 20 min prior to baseline blood sampling. Following the baseline blood sample collection, saline was infused via an IV pump (Baxter Flo-Guard® 6201, Baxter Healthcare Corporation, Deerfield, IL, USA) for 120 min, at a constant rate of 0.1 ml·kg⁻¹·min⁻¹. Throughout the IV infusion (120 min), blood samples were taken every 30 min for a total of 5 samples.

Biochemical analysis

At each timepoint, blood samples were analyzed for POsm (freezing point depression), plasma sodium (PNa) concentration, plasma potassium (PK) concentration (ion-sensitive electrodes), and total plasma protein (TPP) concentration (refractometry). Plasma volume changes (ΔPV) were calculated based on hematocrit and hemoglobin concentration via the Dill & Costill equation (30). Copeptin was measured with random access immunoanalysis (BRAHMS Kryptor Compact Plus, ThermoFisher, Berlin, Germany). Aldosterone and plasma renin activity (PRA) were assessed by liquid chromatography/tandem mass spectrometry, while angiotensin II (AII) concentrations were determined via radioimmunoassay.

Sample-size calculation
Sample-size calculation was based on pilot data based on four subjects. To detect a difference of \( \geq 3\% \) in POsm, a total study sample of 11 participants would be required to test the null hypothesis with a power of 0.8 and associated type 1 error rate \( \alpha \) of 0.05.

**Statistical analysis**

Data were analyzed via repeated measures analysis of variance to assess within subject responses across conditions, time, and condition by time. Baseline data were assessed for normality via the Shapiro-Wilk test; with Bonferroni correction used as post-hoc. All data are reported as means ± standard deviation unless otherwise noted. Statistical significance was set a priori at an \( \alpha \) of 0.05. Statistical analysis was performed utilizing the software package JMP® Pro 14.1 (SAS Institute Inc. 2018, Cary, NC, USA).

**RESULTS**

**Baseline (pre-infusion)**

The pre-programmed infusion volume did not differ between conditions within subjects (938±183 mL), at a rate of 0.1 ml·kg\(^{-1}\)·min\(^{-1}\). POsm, PNa, PK, TPP as well as the fluid regulatory hormones copeptin, angiotensin II, aldosterone and plasma renin activity at the onset of the infusion (0 min) did not differ between trials (\( P > 0.05 \)).

**Manipulation of Tonicity**

Administration of saline infusion yielded increased PNa and POsm values from baseline for both conditions, respectively (Figure 1, Panel A & B; \( P < 0.05 \)). Starting at 30min however, hypertonic saline infusion resulted in significantly higher measured concentrations of PNa and
POsm compared to isotonic control. These significantly higher POsm and PNa concentrations remained elevated for the remainder of the infusion protocol.

**Plasma Volume Expansion**

Calculated percent changes in plasma volume (ΔPV) were greater during HYPER compared to ISO starting at 30min (Figure 2, Panel A; P < 0.05). In line, beginning at 30min, measured total plasma protein showed significantly lower concentrations during HYPER.

**Inorganic Ions**

Presence of plasma chloride (PCl) increased from baseline during both conditions. However, 30min into HYPER, PCl concentrations were significantly higher than during ISO, with higher PCl concentrations measured during the remainder of HYPER compared to control (Figure 3, Panel A, P < 0.05).

Plasma potassium (PK) concentrations also increased from baseline for both conditions, with significantly higher PK concentration measured at the end of HYPER compared to the end of ISO (Figure 3, Panel B, P < 0.05).

**Fluid Regulatory Hormones**

Plasma Renin Activity, aldosterone and angiotensin II were assessed at baseline and post-infusion 120min. Aldosterone showed a significant decline from baseline following both conditions (Figure 4, Panel B, P < 0.05), while plasma renin activity and angiotensin II showed a significant suppression following HYPER only (Figure 4, Panel A&C, P < 0.05).
Copeptin concentrations increased from baseline starting at 60min for HYPER, while maintaining baseline concentrations during ISO (Figure 4, Panel D, $P < 0.05$).

**DISCUSSION**

The purpose of this study was to assess the effect of hypertonic saline infusion on induction of cellular dehydration with comparison of fluid regulatory hormone responses as a secondary outcome. Saline infusion administration of either 3.0% (HYPER) or 0.9% (ISO) resulted in hypertonic and isotonic $P_{\text{osm}}$ values, respectively.

When assessing indices of cellular dehydration, $P_K$ concentrations serve as a strong indicator of presence of cellular water (31). Since cell membranes do not generate and maintain significant hydrostatic pressure gradients, cell volume can only be regulated via gain or loss of osmotically active solutes primarily via the inorganic ions $\text{Na}^+$, $\text{K}^+$ and $\text{Cl}^-$, as well as small organic osmolytes which yield cellular water efflux or influx (31, 32). As the major intracellular inorganic cation, $\text{K}^+$ exits the cell via activated $\text{K}^+$ channels and K-CL cotransporters during regulatory cellular volume decrease (33). Though extracellular osmolality and therefore presumably intracellular osmotic pressure gradients were increased from baseline during both experimental conditions, as measured via POsm, HYPER resulted in significantly higher POsm values. In turn, this could explain the higher PK concentration measured after 120min of hypernatremia, since no $\text{K}^+$ was infused, unlike $\text{Na}^+$ and $\text{Cl}^-$ respectively, the rise in inorganic $\text{K}^+$ could therefore be interpreted as an indicator of cellular water efflux along the osmotic pressure gradient.

Furthermore, calculations of percent changes in plasma volume (30), along with measurements of total plasma protein concentration depict a heightened percent change in
plasma volume, alongside a larger dilution of plasma protein content. Keeping in mind that infusion boluses administered for both conditions were identical within subjects, we argue that this is a further indication for fluid from the intra-and intercellular compartment entering the extracellular vasculature along the acutely generated transmembrane osmotic gradient, ultimately resulting in cellular shrinkage/dehydration (34-36).

The secondary aim of this study was to show a successful suppression of fluid regulatory hormones involved with RAAS (37), while maintaining arginine vasopressin activity (38), measured via the surrogate marker copeptin (39). RAAS affiliated hormones plasma renin activity, angiotensin II and aldosterone all showed a significant suppression in concentration from baseline following HYPER. This is likely explained due to the heightened percent change in plasma volume, indicating hypervolemia. Expansion of blood volume results in the loading and therefore silencing of baroreceptors specific to activation of aldosterone and the plasma renin activity-angiotensin II cascade (21, 40). Copeptin concentrations on the other hand, measured as a surrogate for arginine vasopressin, rose in response to the rise in POsm. Though also responsive to losses in blood volume, arginine vasopressin release is highly sensitive to changes in POsm; which yield in an activation of osmoreceptors in the hypothalamus and ultimately result in the pituitary release of arginine vasopressin. With the utilization of a 120min infusion of hypertonic saline we therefore show a suppression of RAAS activity, while not interfering with the osmosensitive action of arginine vasopressin.

Our study comes with limitations. Hypertonic hypervolemia was achieved via acute changes in extracellular osmolality and was therefore an anisomotic stimulus placed on the participant. This demands caution when interpreting the findings, as naturally occurring dehydration is most likely isosomotic and more gradual, yielding in regulatory cell volume
changes different from those reported here. Further, due to limitations in laboratory instrumentation as well as the \textit{in vivo} nature of the study, we were not able to directly measure changes in cellular volume. Methodologies for assessment include ion-sensitive microelectrode, as well as radioactive tracer methods, none of which were feasible within our current laboratory setting. However, upon studying of the current literature we are confident that the proxy procedures used to assess cellular fluid shifts as well as establishing an acute model of dehydration are adequate.

In conclusion, administration of a 120-min bolus of 3.0\% NaCl infusion resulted in acute hypertonic hypervolemia. Cellular dehydration was likely achieved, marked by a significant rise in PK at the end of infusion compared to isotonic control. Utilization of hypertonic saline infusion was successful in suppressing RAAS while not interfering with osmotic activation of arginine vasopressin release measured via surrogate marker copeptin.
REFERENCES


FIGURE LEGENDS

Figure 1.1
Protocol schematic. Abbreviations: NaCl, sodium chloride infusion.

Figure 1.2
Panel A: Measured plasma sodium (PNa) concentrations during administration of 0.9% and 3.0% NaCl. Panel B: Measured plasma osmolality (POsm) during administration of 0.9% and 3.0% NaCl. Levels not connected by the same letter are significantly different at \( P < 0.05 \). Data are mean ± SE.

Figure 1.3
Panel A: Measured percent change in plasma volume (ΔPV) during administration of 0.9% and 3.0% NaCl. Panel B: Measured total plasma protein (TPP) during administration of 0.9% and 3.0% NaCl. Levels not connected by the same letter are significantly different at \( P < 0.05 \). Data are mean ± SE.

Figure 1.4
Panel A: Measured plasma potassium (PK) concentration during administration of 0.9% and 3.0% NaCl. Panel B: Measured plasma chloride (PCl) during administration of 0.9% and 3.0% NaCl. Levels not connected by the same letter are significantly different at \( P < 0.05 \). Data are mean ± SE.

Figure 1.5
Panel A: Measured plasma renin activity during administration of 0.9% and 3.0% NaCl. Panel B: Measured aldosterone concentration during administration of 0.9% and 3.0% NaCl. Panel C: Measured angiotensin II concentration during administration of 0.9% and 3.0% NaCl. Panel D: Measured copeptin concentration during administration of 0.9% and 3.0% NaCl. Levels not connected by the same letter are significantly different at \( P < 0.05 \). Data are mean ± SE.
Figure 1.1.
Figure 1.2.
Figure 1.3.
Figure 1.4.
Figure 1.5.
III.

STUDY 2:

OSMOTIC STIMULATION OF COPEPTIN ACUTELY IMPAIRS GLUCOSE REGULATION

ABSTRACT

Background: Epidemiological studies in humans show increased levels of copeptin, a surrogate marker of arginine vasopressin (AVP), to be associated with increased risk for type 2 diabetes (T2D).

Objective: To examine the acute and independent effect of osmotically stimulated AVP, measured via the surrogate marker copeptin, on glucose regulation in healthy adults.

Design: 60 subjects (30 females) participated in this cross-over design study. On two trial days, separated by at least seven days (males) or one menstrual cycle (females), subjects were infused for 120-min with either 0.9 % sodium chloride (NaCl, ISO) or 3.0 % NaCl (HYPER). Post infusion, a 240-min oral glucose tolerance test (OGTT, 75 g) was administered.

Results: During HYPER, plasma osmolality (POsm) and copeptin increased (P<0.05) and remained elevated during the entire six-hour protocol, while renin-angiotensin-aldosterone system (RAAS) hormones were within the lower normal physiological range at the beginning of the protocol and declined following infusion. Fasting plasma glucose did not differ between trials (P >0.05) at baseline and during the 120-min of infusion. During the OGTT the positive integer of the area under the curve (AUC) for glucose was greater during HYPER (401.5±190.5 mmol·L⁻¹·min) vs. the ISO trial (354.0±205.8 mmol·L⁻¹·min, P< 0.05). The positive integer of the AUC for insulin during OGTT did not differ between trials (HYPER: 55,850±36,488 vs. ISO: 57,205±31,119 pmol·L⁻¹·min). Baseline values of serum glucagon were not different between the two trials, however, the AUC of glucagon during the OGTT was also significantly
greater in HYPER (19,303±3,939 ng·L⁻¹·min) vs. the ISO trial (18,600±3,755 ng·L⁻¹·min; P < 0.05).

Conclusions: The present data indicate that acute osmotic stimulation of copeptin induced greater hyperglycemic responses during the oral glucose challenge possibly due to greater glucagon levels.

This study was registered at ClinicalTrials.gov as NCT02761434.

Keywords: vasopressin; OGTT; dehydration; hydration; diabetes
INTRODUCTION

Prevalence of chronic metabolic dysfunction is dramatically increasing worldwide and has become both a major public health issue and a global economic burden (1). Impaired glucose regulation is the hallmark for metabolic dysfunction; that could eventually lead to diabetes and associated comorbidities. Most recent reports from the World Health Organization indicate that the global number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014, representing 8.5% of adults (2). In the United States alone, an estimated 84.1 million people (33.9% of adults) had pre-diabetes in 2015 (3). While genetic make-up, age and sex have been defined as non-modifiable risk factors for new onset of type 2 diabetes mellitus (T2D), population-based studies revealed increased diabetes incidence occurring in parallel with major and rapid changes in lifestyle behaviors (4). Therefore, there is an increasing and urgent need to identify modifiable risk factors that may represent potential levers for the prevention of the onset of metabolic dysfunctions and that may help to blunt the T2D epidemic. In addition to the major modifiable contributors to this disease such as obesity, poor diet, and physical inactivity (5), recent evidence suggests that the hormone AVP may be another key modifiable risk factor in the development of diabetes (6-8).

AVP, also known as antidiuretic hormone, is the key hormone in the regulation of body fluid balance; with one of its functions being the maintenance of plasma osmolality within a narrow range. However, stimulation of AVP secretion, for instance in conditions of low water intake, appears to be a risk factor for the development of diabetes (9). Indeed, preclinical studies involving vasopressin injection, water intake manipulation, and the use of a vasopressin V1a receptor-specific blockade, have collectively demonstrated that vasopressin and hydration play a significant role on basal glycemia, glucose tolerance and liver steatosis in Zucker rats (10, 11).
Similarly, research in mice lacking the V1b vasopressin receptor has shown to improve insulin sensitivity (12).

Nonetheless, given the instability and low concentration of AVP in blood, the measurement of copeptin, the stable C-terminal cleavage product of the vasopressin precursor secreted in equimolar amounts with AVP, has become a surrogate and widely used proxy of AVP secretion (13, 14). This has facilitated the measurement of antidiuretic activity in large-scale population studies in which direct measurement of AVP would be unfeasible, and has provided compelling epidemiological evidence linking copeptin, as a surrogate of AVP, to metabolic health outcomes. Evidence from prospective population studies in multiple countries report a strong, independent association of copeptin with hyperglycemia, higher risk of developing T2D, diabetic cardiomyopathy, impaired insulin sensitivity and even death (6, 7, 15, 16). These findings have been further supported by a mendelian randomization approach showing that at least in men, allelic variants increasing AVP (copeptin) concentration also appear to increase incidence of impaired fasting glucose (17).

Yet, in humans, short-term studies designed to test the underlying mechanisms for AVP, and metabolic dysfunction remain scarce. Furthermore, many pathways, including the secretion of AVP, the Hypothalamic-Pituitary-Adrenal (HPA) axis, and the Renin-Angiotensin-Aldosterone System (RAAS), are interrelated (18-20) and are implicated with dysregulation of glucose homeostasis (21-23). Additionally, certain traditional experimental methods of increasing AVP via dehydration (exercise or exposure to heat) may have independent effects on glucose homeostasis. Thus, the independent role of elevated AVP on glycemic control in human remains unclear.
Therefore, the aim of the present study was to examine the acute and independent effect of osmotically stimulated AVP, measured via the surrogate marker copeptin, on glucose regulation in healthy adults. An intravenous infusion protocol of hypertonic saline was employed to osmotically stimulate AVP; while suppressing RAAS system via plasma volume expansion and hypernatremia. We hypothesized that acute osmotic stimulation of AVP secretion, measured via the surrogate marker copeptin, would lead to greater hyperglycemia during an oral glucose tolerance test.

METHODS

Subjects

Out of 77 volunteers recruited between April 2016 and March 2017, 12 candidates failed to meet inclusion criteria, while 5 subjects voluntarily withdrew from the study after enrollment. Sixty adult volunteers (30 females) participated in this study [age, 39.0±8.0 y; weight, 78.2±15.2 kg; height, 1.70±0.09 m; BMI, 26.9±4.0 kg·m⁻²; glycosylated hemoglobin (HbA1c), 5.2±0.3%]. The Consolidated Standards of Reporting Trials (CONSORT) flow diagram is shown in Supplemental Figure 1.

During the screening process, subjects were required to complete a medical history questionnaire which was reviewed by an advanced nurse practitioner to exclude patients with diabetes, kidney disease, metabolic disorders, cardiovascular disease, and other potential fluid balance covariates such as habitual use of non-steroidal anti-inflammatory drugs or serotonin reuptake inhibitors. To avoid enrollment of subjects with undiagnosed diabetes, HbA1c was measured from capillary blood. For female participants, the oral hormonal contraception method was permitted as long as it included a seven-day wash-out period during their monthly cycle.
Pregnancy, lactation, or use of injectable contraceptives however, were additional exclusionary factors. All participants signed an informed consent statement prior to enrollment. The study was approved by the institutional review board for human experimentation in accordance with the Helsinki Declaration of 1975 as revised in 1983. This trial was registered before the onset of subject recruitment at www.clinicaltrials.gov as NCT02761434.

**Experimental design**

In this counter-balanced, cross-over, and single-blind design study, each participant completed two separate trials. To determine the acute effect of osmotically stimulated AVP, measured via copeptin, on glucose regulation, participants received a 120-min saline infusion followed by a 240-min oral glucose tolerance test (OGTT, Figure 1). Both trials differed only in saline concentration during the infusion, with each subject receiving 0.1 ml·kg⁻¹·min⁻¹ of either hypertonic saline (HYPER, 3% NaCl), to osmotically stimulate AVP (copeptin) secretion, or isotonic saline (ISO; 0.9% NaCl), to serve as control (24, 25).

**Study controls**

Experimental days were separated by one week for males and post-menopausal or ovariectomized females to ensure sufficient wash-out (26). Female subjects with regular menstrual cycle were tested during the early follicular phase of two consecutive menstrual cycles, to control for the effect of reproductive hormones on body fluid balance (27). All subjects were instructed to consume ample amount of carbohydrates (> 150 g per day) for the three days leading up to test day to improve the accuracy of the OGTT for glucose tolerance classification (28). For the 24-h prior to testing subjects recorded all food and fluid intake into a provided food...
diary and refrained from exercise, caffeine, and alcohol. In preparation for the second experimental day, subjects received a copy of their first pre-trial dietary record and were asked to replicate it. To standardize the dinner prior to each testing day, subjects consumed a provided frozen meal (two Smart Ones Spaghetti bowls; 506 kcal, 78 g carbohydrates, 10 g fat, 26 g protein), before entering a 10-h fasting period prior to the scheduled trial. During this 10-h fast, subjects were allowed to drink plain water only. Lastly, to ensure adequate hydration, subjects were instructed to consume provided bottled water based on the 80% of the Institute of Medicine reference values for water intake (2 L for females and 3 L for males) for the day prior to each experiment (29). These pre-trial hydration guidelines were based on data from the National Health and Nutrition Examination Survey, which reports fluid intake to account for approximately 80% of total water intake (30).

**Study protocol**

All subjects reported to the lab following a 10-h fast and provided a urine sample. Hydration status was assessed via urine specific gravity measurement with values <1.020 required to proceed with the protocol (20). Subjects then sat in a comfortable chair and were not allowed to stand-up or lay down until the end of the experiment to avoid intercompartmental body fluid shifts associated with changes in body posture (31). An intravenous (IV) catheter was placed into an antecubital vein and subjects rested for at least 20 min prior to baseline blood sampling. Following the baseline blood sample collection, saline was infused via an IV pump (Baxter Flo-Guard® 6201, Baxter Healthcare Corporation, Deerfield, IL, USA) for 120 min, at a constant rate of 0.1 ml·kg⁻¹·min⁻¹. Following the infusion, a blood sample was drawn, and subjects ingested within five min a 239 mL standardized glucose beverage (Azer Scientific,
Morgantown, PA, USA) containing 75 g of glucose. Throughout the IV infusion (120 min) and subsequent OGTT (240 min), blood samples were taken every 30 min for a total of 13 samples. Blood pressure was recorded right after each blood draw from the non-catheterized arm in duplicate (Tango+, SunTechMedical Inc, Morrisville, NC, USA). Mean arterial pressure (MAP) was calculated based on diastolic (DBP) and systolic blood pressure (SBP) with the following equation: MAP (mmHg) = DBP + [(SBP – DBP) / 3], (32, 33).

**Biochemical analysis**

At each time point, blood samples were analyzed for POsm (freezing point depression), electrolyte concentration (ion-sensitive electrodes), total plasma protein (TPP) concentration (refractometry). Plasma volume changes (∆PV) were calculated based on hematocrit and hemoglobin concentration with the Dill & Costill equation (34). Copeptin was measured with random access immunoanalysis (BRAHMS Kryptor Compact Plus, ThermoFisher, Berlin, Germany). Glucagon, insulin, C-peptide, human corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol were measured with enzyme-linked immunosorbent assay. Blood glucose, free fatty acids, and triglycerides were quantified by spectrophotometry. Aldosterone and plasma renin activity (PRA) were assessed by liquid chromatography/tandem mass spectrometry, while angiotensin II (AII) concentrations were determined via radioimmunoassay. Insulin resistance was assessed with the homeostatic model assessment index (HOMA-IR) at the end of the saline infusion (35), and insulin sensitivity during the first 120 min of the OGTT test based on the Matsuda index (36).
Statistical analysis

Data were analyzed via repeated measures analysis of variance to assess within subject responses across conditions, time, and condition by time. Bonferroni correction was used as post-hoc. Baseline data were assessed for normality via the Shapiro-Wilk test. Positive integers for the area under the curve (AUC) were calculated (37, 38) using MATLAB (MathWorks Inc. 2018, Natick, MA, USA). All data are reported as means ± standard deviation unless otherwise noted. Statistical significance was set a priori at an alpha of 0.05. Statistical analysis was performed utilizing the software package JMP® Pro 14.1 (SAS Institute Inc. 2018, Cary, NC, USA).

RESULTS

Hypertonic Saline Infusion

POsm, plasma sodium (PNa), and copeptin at the onset of the infusion (-120 min) did not differ between trials (P>0.05). During the HYPER trial, POsm, PNa, and copeptin increased and remained elevated during the entire six-hour protocol (Figure 2, Panel A, B, and D). Plasma volume was significantly expanded in both trials; however, the expansion was greater during HYPER compared to the ISO trial (Figure 2, Panel C), despite utilizing the same infusion volume for both trials (938±183 mL), at a rate of 0.1 ml·kg⁻¹·min⁻¹. Due to plasma volume expansion in both protocols, measured concentrations were affected. Results with values corrected for plasma volume expansion are shown in supplemental figures 1 & 2 and in the supplemental tables 1, 2 & 3. Aldosterone, AII, and PRA were within lower normal physiological range at the beginning of both protocols and declined following infusion (Table 1). MAP did not change in response to infusion during the HYPER (pre: 86.2±8.6 mmHg, post: 82.7±7.2 mmHg) or the ISO trial (pre: 84.5±7.9 mmHg, post: 83.3±6.85 mmHg, P>0.05).
Glycemic Response to Oral Glucose Tolerance Test

Fasting plasma glucose concentrations did not differ between trials ($P>0.05$) at baseline (-120 min) and remained unchanged during the 120-min of saline infusion for both trials (Figure 3). The positive integer for the glucose AUC during OGTT (primary outcome) was greater in HYPER ($401.5\pm190.5$ mmol·L$^{-1}$·min) vs. the ISO trial ($354.0\pm205.8$ mmol·L$^{-1}$·min, $P<0.05$). Also, glucose concentration was significantly greater for the HYPER than the ISO trial ($P<0.05$) at 60 and 90 min of the OGTT (Figure 3). Baseline serum insulin concentrations did not differ between conditions ($P>0.05$) and remained unchanged during the saline infusion. During OGTT plasma insulin was significantly lower at 30 min for HYPER when compared to the ISO trial (Figure 3, $P<0.05$). The positive integer for the insulin AUC during OGTT (primary outcome) did not differ between trials (HYPER: $55,850\pm36,488$; ISO: $57,205\pm31,119$ pmol·L$^{-1}$·min, $P>0.05$). However, when the positive integer of the AUC was calculated for the first 60 min of OGTT, it was lower during the HYPER vs. the ISO trial ($11,490\pm6,659$ vs. $15,442\pm10,295$ pmol·L$^{-1}$·min; $P<0.001$). C-peptide values did not differ between the two trials during the 120-min of infusion ($P>0.05$). During OGTT C-peptide was lower at 30 and 60 min for HYPER trial (1.06±0.51 and 1.95±0.65 nmol·L$^{-1}$) compared to the ISO trial (1.41±0.66 and 2.19±0.85 nmol·L$^{-1}$; $P<0.05$). The positive integer for the C-peptide AUC during OGTT was smaller in HYPER ($305.1\pm123.5$ nmol·L$^{-1}$·min) vs. the ISO trial ($324.4\pm120.1$ nmol·L$^{-1}$·min; $P<0.05$). HOMA-IR (HYPER: $1.58\pm0.97$, ISO: $1.61\pm1.09$; $P>0.05$) and the Matsuda index of insulin sensitivity (HYPER: $9.2\pm5.2$, ISO: $9.0\pm4.9$) did not differ between trials ($P>0.05$).

Serum glucagon concentration did not differ before saline infusion onset between the two trials (HYPER: $80.1\pm17.3$, ISO: $79.9\pm15.7$ ng·L$^{-1}$). However, it decreased significantly as a
response to the isotonic saline (72.6±14.4 ng·L⁻¹, \(P<0.05\)), which was not seen during the hypertonic infusion (75.0±16.2 ng·L⁻¹). No statistically significant differences for glucagon at any particular timepoint were observed between the two trials during OGTT (\(P>0.05\)). However, the positive integer for the AUC of glucagon during OGTT was significantly greater in HYPER (19,303±3,939 ng·L⁻¹·min) vs. the ISO trial (18,600±3,755 ng·L⁻¹·min; \(P<0.05\)). Serum free fatty acids and triglycerides decreased as a response to the 75 g of glucose ingestion during OGTT (see Supplemental Table 3), however, no statistical difference was observed between two trials (\(P>0.05\)).

**Hypothalamic-Pituitary-Adrenal Axis**

The HPA axis hormonal responses appear in Table 2. CRH did not change during the experiment and no statistical difference was observed between trials (\(P>0.05\)). ACTH did not differ between the two trials at any timepoint (\(P>0.05\)). Compared to the baseline value, ACTH was significantly lower at 120-min of OGTT during the HYPER trial and at 0 and 120 min of OGTT in the ISO trial (\(P<0.05\), Table 2). Cortisol concentrations decreased from baseline (\(P<0.05\)), but no significant differences between the trials were observed (\(P>0.05\), Table 2).

**DISCUSSION**

The aim of the present study was to examine the acute and independent effect of osmotically stimulated AVP, measured via surrogate marker copeptin, on glucose regulation in healthy adults. Our main finding was that the glycemic response during OGTT under the hypertonic condition was significantly greater compared to the isotonic control. These data suggest that osmotically stimulated AVP (copeptin) may acutely impair glucose homeostasis.
The study design was successful in inducing a steady increase in POsm during hypertonic infusion, and in response, a sustained elevated concentration of AVP, measured via copeptin, which lasted for the entirety of the 6-h protocol. Furthermore, our experiment attained a suppression of RAAS as a response to baroreceptor loading due to volume increase in both trials via saline infusion. Our data showed that subjects started the experiment well hydrated, with levels of renin, aldosterone, and angiotensin II within the low physiological ranges which were then further reduced during the saline infusion protocol for both conditions. Hence, we were able to osmotically stimulate the secretion of AVP while keeping RAAS suppressed, therefore enabling the assessment of its independent impact on glycemic response during an OGTT. Copeptin concentrations observed during both trials were comparable to higher physiological ranges assessed in free-living individuals (6).

The AUCs for both glucose and glucagon, assessed during the 240-min OGTT period, were significantly greater during the hypertonic trial compared to isotonic control. This builds upon previous observations by Spruce et al., who infused exogenous vasopressin to yield moderate and high levels of circulating vasopressin respectively, with the aim to examine its effects on glucose kinetics via use of tritium-labeled glucose in healthy adults (39). In their findings, Spruce et al. reported that rates of glucose appearance and glucagon concentration were significantly increased in response to the moderate vasopressin infusion with no further increases detected when increasing the AVP infusion load, suggesting a sensitive interlink between circulating vasopressin concentrations and glucose kinetics. In line, Keller et al. also reported increased rates of glucose appearance assessed via stable isotope tracer methodology in a similar experiment employing infusion of hypertonic saline to shrink cell volume (45). Even though circulating AVP concentrations were not measured by Keller et al., we can infer that the increase
in plasma osmolality beyond 300 mmol·kg⁻¹ as a response to the 5% saline infusion employed, would have also yielded an increased stimulation of endogenous AVP secretion.

In contrast, a recent study by Carroll et al. reported no differences in glycemic regulation assessed via OGTT in acutely hypohydrated adults (40). In this study, subjects underwent two passive heating trials followed by either fluid replacement (control) or fluid restriction (hypohydration) to induce the desired testing condition before an OGTT assessment followed the subsequent day. However, passive heating per se has previously been reported to increase glucose and insulin concentrations (41). Thus, it is possible that the heat-stress subjects were exposed to in preparation for both conditions could have interfered with their findings.

Although in the current study the overall AUC for insulin did not differ between conditions during OGTT, the AUC was lower for HYPER when assessed during the first 60-min of OGTT. Similar to insulin, C-peptide values were lower during HYPER at 30 and 60 min of OGTT, with the positive integer AUC for C-peptide also being lower for the HYPER than the ISO trial. No differences were seen on insulin resistance/sensitivity at the end of saline infusion nor during the OGTT assessed via HOMA-IR and Matsuda index, respectively. These data are in contrast with previous studies indicating that elevated copeptin and hyperosmolality were factors contributing to insulin resistance (16, 26). One possibility for a difference in findings is the employed infusion model, resulting in hypervolemic hyperosmolality. While in natural occurring hypovolemic dehydration baroreceptors unloading results in activation of RAAS (18), by design, our study meant to keep RAAS deactivated. With activity of aldosterone and AII kept low, their reported interference on glucose uptake at the level of the skeletal muscle may, therefore, also be minimized (42-44), which could explain the lack of effect seen in Matsuda and HOMA-IR assessments when comparing hyperosmotic to iso-osmotic conditions.
Although AVP is well-known for its anti-diuretic and pressor effects, widespread AVP receptors would suggest a broad range of central and peripheral effects. Specific to metabolic dysfunction, both V1a and V1b receptors have been implicated in glycemic control and insulin resistance. V1a receptors have been previously described to increase hepatic glycogenolysis, and gluconeogenesis *in vitro* and during animal experiments, thus elevating plasma glucose (10, 11, 45). Moreover, glucagon can induce the stimulation of hepatocytes and increase glycemia, while AVP can induce glycolysis independently. Lastly, alpha and beta cells of the islets of Langerhans both inhabit V1b receptors which have been implicated with stimulating both insulin and glucagon (10, 45-47). Based on these previous findings, it can be hypothesized that in our experiment, the osmotic stimulation of AVP seen during HYPER could have induced greater glycemic responses during OGTT, via a two-fold effect: the V1a receptor activation yielding in direct hepatic release of glucose, along with activation of V1b receptors, resulting in an increase in circulating glucagon concentrations.

Previous studies in humans have suggested an impaired suppression of glucose production induced by cortisol infusion (23). Recently, Johnson et al., reported glycemic impairment following three days of decreased water intake in T2D subjects during an OGTT to be associated with elevated cortisol (48). AVP also has been shown to stimulate ACTH secretion directly, in the absence of CRH stimulus, which in turn elevates cortisol secretion (49). However, in the present study no differences in ACTH, CRH, or cortisol were observed between the trials showing that the AVP-driven stimulation of the HPA axis might not be an acute but rather a long-term stress response (50).

During the last decade, epidemiological evidence has shown that low water intake is associated with higher blood glycemia (9, 51, 52). Interestingly, small interventional trials have
shown that days or weeks of increased water intake can lower circulating copeptin (53, 54) or AVP (55). In particular Enhorning et al. examined the glycemic responses of healthy adults following a one-week increased water intake intervention, and reported that the increased water intake in low drinkers with high baseline copeptin led to lower fasting glucagon without any changes in insulin (53). Furthermore, a recent study by Asferg et al. measured higher circulating concentrations of copeptin and glucagon in obese men than in normal control subjects, with copeptin showing significant association with glucagon concentrations independent of weight status (56). Therefore, these data might suggest water intake as a potential intervention strategy to lower AVP, since elevated glucagon per se has been identified as a risk factor for impaired glucose tolerance and T2D (53, 57-60).

The present study comes with several limitations. Even though our data did not indicate non-compliance, preparation of subjects for each OGTT trial relied on written instructions and self-reported compliance. Secondly, utilization of hypertonic saline causes the “unnatural” state of hypertonic hypervolemia. With RAAS suppressed, potential interplay with AVP and synergistic effects on glucose regulation naturally occurring during states of negative water balance in free living adults cannot be assessed. Also, based on previous data, both cell volume alterations and vasopressin have been linked to glucose regulatory impairments. Use of the hypertonic infusion protocol does not render a way to distinguish whether the observed hyperglycemic effect was driven by cell shrinkage and/or AVP. However, both stimuli co-exist during naturally occurring states of dehydration. Furthermore, data both in experimental animals (10, 11) and in humans where exogenous vasopressin infusion was employed indicate that AVP seems to have a direct effect on hyperglycemia (39). Another limitation of the osmotic stimulation protocol is that even though the same amount of fluid was infused in both trials, the
osmotic load of the hypertonic fluid led to greater plasma volume expansion when compared to the isotonic infusion. The isotonic fluid could move from the vascular to the interstitial space while the hypertonic fluid could possibly draw fluids from the interstitial and intracellular space into the vasculature via osmosis. As a result, the greater plasma volume present in the vasculature could have diluted the concentration of blood measures leading to lower measured concentrations. However, since systemic receptors are sensing concentration and not total content, the uncorrected values are presented in the manuscript. The corrected data which take into account the dilution effect associated with the occurred plasma volume expansion in both trials, appear online as supplemental figures and tables, presenting a very similar response to the uncorrected data.

With recent data reporting a positive association between copeptin and glucagon in healthy obese men (56), our study adds to the literature by showing elevated concentrations of glucagon in response in the presence of high circulating concentrations of copeptin during the OGTT. Our findings are novel, as potential noise rendered from the normally occurring interplay of RAAS was controlled for, therefore allowing for an estimation of impact stemming from the stimulation of AVP concentration alone on metabolic function.

In summary, the present data indicate acute osmotic stimulation of copeptin, measured as a surrogate marker of AVP, led to greater hyperglycemic responses during an oral glucose challenge, potentially associated with greater glucagon response.
AUTHORS CONTRIBUTION

The authors’ responsibilities were as follows:

SAK, GL and ETP designed the study; LTJ, HS, JDA, CAS, ADS, DMS, CLB, TMK, OM conducted data collection and/or sample analysis; SAK, LTJ, HS, and JDA analyzed the data; LTJ, SAK, ETP, AD, and TV wrote the paper; SAK was the principal investigator and had primary responsibility for the final content. All authors read, critically revised, and approved the final manuscript.

Acknowledgements

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Conflict of Interest Statement

ADS is a scientific consultant for Gatorade Sports Science Institute, TV, AD, GL and ETP are employed by Danone Research, OM has received grants form Danone Research, SAK has served as scientific consultants for Quest Diagnostics, Standard Process and Danone Research and has received grants from Danone Research.
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FIGURE LEGENDS

Figure 1
Protocol timeline. Infusion protocol started at -120 min ending at time point 0 min. The oral glucose tolerance test (OGTT) of 75 g was administered at 0min, sample collection continued until 240 min.

Figure 2
Mean plots of plasma concentrations during the hypertonic and isotonic conditions for: A – plasma osmolality (POsm), B – plasma sodium (PNa), C – changes in plasma volume and D – copeptin. Error bars depict standard error of the mean.
* denotes statistically significant difference between conditions for the same timepoint (P<0.05).

Figure 3
Mean plots for glycemic responses during both conditions for glucose (Panel A) and insulin (Panel B). Error bars depict standard error of the mean.
* denotes statistically significant difference between conditions for the same timepoint (P<0.05).
TABLE 2.1 Fluid regulatory hormone responses

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<td></td>
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<td>Aldosterone pmol·L⁻¹</td>
<td>144±96</td>
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<td>22.0±10.7</td>
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<td>Plasma Renin Activity ng·mL·h⁻¹</td>
<td>0.86±0.57</td>
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</table>

* significant difference between conditions at given timepoint, † significant difference from baseline of the same trial (-120 min, P<0.05)
### TABLE 2.2 Hypothalamic-pituitary-adrenal axis hormonal response

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<td>-120 0 60 120 240</td>
</tr>
<tr>
<td><strong>CRH ng·L⁻¹</strong></td>
<td>518±388 461±363 493±390 505±413 455±338</td>
<td>519±467 484±427 513±461 509±428 523±437</td>
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<td><strong>ACTH pmol·L⁻¹</strong></td>
<td>4.0±2.5 3.6±2.2 3.3±1.8 3.2±1.6† 3.5±2.0</td>
<td>3.8±2.5 2.8±1.6† 3.4±2.0 3.0±1.8† 3.8±2.2</td>
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<tr>
<td><strong>Cortisol nmol·L⁻¹</strong></td>
<td>367±205 259±146† 292±163† 223±118† 292±145†</td>
<td>410±186 228±130† 248±116† 249±124† 276±146†</td>
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</table>

† significant difference from baseline of the same trial (-120 min, P<0.05)
Figure 2.1
Figure 2.2.
Figure 2.3.
IV.

CONCLUSIONS

These studies sought to determine whether states of acute elevations in circulating vasopressin, assessed by copeptin, would impair glucose kinetics following an oral glucose challenge in healthy adults with no reported history, or laboratory indices of diabetes. In order to investigate this, we first established a methodology potent in achieving acute increases in copeptin concentrations, while silencing RAAS affiliated hormones. Infusion with hypertonic saline achieved an endogenous stimulation of copeptin, which resulted in concentrations within physiological ranges comparable to those reported in epidemiological studies to present a higher risk for T2DM. Due to the volume load in both infusion protocol conditions, RAAS hormones were either kept at the low range baseline concentrations during the isotonic trials, or concentrations were even further reduced following hypertonic infusion, as a result of intra and intercellular water entering the extracellular periphery, following the acutely increased osmotic pressure gradients. With either newly induced high, or maintained from baseline concentrations of copeptin, we were then able to assess the effect of circulating copeptin concentrations on glucose metabolism responses following a standardized oral glucose tolerance test. Our data showed that during states of high copeptin concentrations, more glucose was present in the circulation compared to states of normal to low copeptin concentrations following an administration of an identical glucose bolus for both conditions. While insulin responses were similar for both conditions, the incremental area under the curve for glucagon was significantly larger during high circulating copeptin concentrations.
This data suggests that during acute states of high circulating copeptin concentrations, glucagon is also stimulated, resulting in a heightened glucose appearance in the circulation presumably from hepatic glucose production. While our findings report measurements taken during acute states of high copeptin, a recent study found a positive correlation between fasting glucagon and copeptin concentrations in free living adults, suggesting that these acute findings are valid in chronic states as well. Furthermore, current knowledge in type 2 diabetes pathophysiology touts glucagon dysregulation, more specifically, hyperglucagonemia as a precursor to T2DM onset. While defining the exact mechanisms involved in stimulation of glucagon via AVP/copeptin is beyond the scope of this study design, future research should seek to examine the interplay of AVP/copeptin further. Moreover, additional data is needed to outline efficacy of hydration status management on circulating copeptin concentrations and preserving long-term health outcomes.
February 14, 2017

MEMORANDUM

TO: Stavros Kavouras  
    Elaine Lee  
    Hyun-Gyu Suh  
    Jordan Smith  
    Adam Seal  
    Alison Schoeder  
    Katherine Montgomery  
    Marshall Ward  
    Chunbo Yang

    Tabatha Teal  
    Lisa Jansen  
    Yasuki Sekiguchi  
    Zachary Lewis  
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    Kyle Cook  
    Cody Shopper  
    Cory Butts  
    Dylan Scott

    Tracie Kirkland  
    J.D. Adams  
    Audrey Smith  
    Zoe McKinney  
    Bryce Wall  
    Alexandria Aldridge  
    Cameron Sprong  
    Jacob Clark

FROM: Ro Windwalker  
IRB Coordinator

RE: PROJECT CONTINUATION

IRB Protocol #: 14-12-360

Protocol Title: The Effect of Vasopressin on Glucose Regulation

Review Type: ☑ FULL IRB

Previous Approval Period: Start Date: 12/19/2014 Expiration Date: 12/15/2016

New Expiration Date: 12/15/2017

Your request to extend the referenced protocol has been approved by the IRB. If at the end of this period you wish to continue the project, you must submit a request using the form Continuing Review for IRB Approved Projects, prior to the expiration date. Failure to obtain approval for a continuation on or prior to this new expiration date will result in termination of the protocol and you will be required to submit a new protocol to the IRB before continuing the project. Data collected past the protocol expiration date may need to be eliminated from the dataset should you wish to publish. Only data collected under a currently approved protocol can be certified by the IRB for any purpose.

This protocol has been approved for 68 total participants. If you wish to make any modifications in the approved protocol, including enrolling more than this number, you must seek approval prior to implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

If you have questions or need any assistance from the IRB, please contact me at 109 MLKG Building, 5-2208, or irb@uark.edu.

109 MLKG • 1 University of Arkansas • Fayetteville, AR 72701-1201 • (479) 575-2208 • Fax (479) 575-6527 • Email irb@uark.edu
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## SUPPLEMENTAL TABLE 2.1. Fluid regulatory hormone responses corrected for plasma volume expansion

<table>
<thead>
<tr>
<th>Time</th>
<th>HYPER</th>
<th>ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>-120</td>
<td>0</td>
</tr>
<tr>
<td>Aldosterone pmol·L⁻¹, Corrected</td>
<td>144±96</td>
<td>69±36†</td>
</tr>
<tr>
<td>Angiotensin II pmol·L⁻¹, Corrected</td>
<td>22.0±10.7</td>
<td>19.1±8.1</td>
</tr>
<tr>
<td>Plasma Renin Activity ng·mL·h⁻¹, Corrected</td>
<td>0.86±0.57</td>
<td>0.53±0.34*†</td>
</tr>
</tbody>
</table>

* significant difference between conditions at given timepoint, † significant difference from baseline of the same trial (-120 min)
### SUPPLEMENTAL TABLE 2.2. Hypothalamic-pituitary-adrenal axis hormonal response corrected for plasma volume expansion

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>HYPER</th>
<th>ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-120</td>
<td>0</td>
</tr>
<tr>
<td>CRH ng·L⁻¹ Corrected</td>
<td>518±388</td>
<td>544±429</td>
</tr>
<tr>
<td>ACTH pmol·L⁻¹ Corrected</td>
<td>4.0±2.5</td>
<td>4.3±2.7*</td>
</tr>
<tr>
<td>Cortisol nmol·L⁻¹ Corrected</td>
<td>367±205</td>
<td>328±208†*</td>
</tr>
</tbody>
</table>

* significant difference between conditions at given timepoint, † significant difference from baseline of the same trial (-120 min).

*P*<0.05
### SUPPLEMENTAL TABLE 2.3. Fatty Acid Response

<table>
<thead>
<tr>
<th>Time min</th>
<th>HYPER</th>
<th>ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-120</td>
<td>0</td>
</tr>
<tr>
<td>Triglycerides mmol·L⁻¹</td>
<td>1.25±0.5</td>
<td>1.13±0.4</td>
</tr>
<tr>
<td>Free Fatty Acids mg·L⁻¹</td>
<td>190±70</td>
<td>150±40†</td>
</tr>
</tbody>
</table>

* significant difference between conditions at given timepoint, † significant difference from baseline of the same trial (-120 min).
FIGURE LEGENDS

Supplemental Figure 2.1.
CONSORT 2010 Flow Diagram. Chart reporting total number of participants involved during enrollment, allocation and analysis.

Supplemental Figure 2.2.
Mean plots for glycemic responses during both conditions for glucose (Panel A) and insulin (Panel B) corrected for plasma volume expansion. Error bars depict standard error of the mean. * denotes statistically significant difference between conditions for the same timepoint (P<0.05).
CONSORT 2010 Flow Diagram

Enrollment

Assessed for eligibility (n=77)

Excluded (n=17)
- Not meeting inclusion criteria (n=12)
- Declined to participate (n=5)

Randomized (n=60)

Allocation

Allocated to isotonic as the 1st trial (n=30)
- Received assigned intervention (n=30)

Allocated to hypertonic as the 1st trial (n=30)
- Received assigned intervention (n=30)

Analysis

Analysed (n=30)
- Excluded from analysis (give reasons) (n/a)

Analysed (n=30)
- Excluded from analysis (give reasons) (n/a)

Supplemental Figure 2.1.
Supplemental Figure 2.2.