SEX DIFFERENCES IN OSMOTIC STIMULATION OF VASOPRESSIN RELEASE IN RATS CHRONICALLY EXPOSED TO ALCOHOL

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHYSIOLOGY

May 2011

By

January May Andaya

Thesis Committee:

Catherine Uyehara, Chairperson
Suwit Jack Somponpun
Scott Lozanoff
ABSTRACT

Whether the purported differences in alcohol effects in males and females may be due to alcohol influence on estradiol and progesterone mediation of vasopressin (VP) release was studied in Sprague-Dawley male and female rats exposed to control, moderate alcohol (1.2% ethanol) or high alcohol (6.4-6.7% ethanol) liquid diet for 4-6 weeks. VP response to an osmotic stimulus (5% NaCl i.v. infusion) and pituitary VP stores in relationship to circulating VP were examined.

In males, alcohol did not appear to affect VP osmotic stimulation. In females, however, alcohol affected VP osmotic stimulation sensitivity depending on estrous cycle phase, and appeared to alter circulating estradiol and progesterone relationships with VP stores and release. Results thus indicate that females may be more susceptible to alcohol-induced changes in VP regulation of water balance.
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INTRODUCTION

Effects of Alcohol on Vasopressin Regulation

Based on a number of studies, it is well known that alcohol causes diuresis and influences water balance. Some studies have indicated that alcohol affects release or action of vasopressin (VP), the chief hormone that maintains water balance in the body.

According to Gulya, Dave and Hoffman (1991), chronic ethanol ingestion for 7 days resulted in a significant decrease in VP mRNA in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus in mice. Rats, chronically exposed to alcohol, and later withdrawn from alcohol showed an increase in pituitary VP content and VP release, but a decrease in VP synthesis, thus suggesting that the relationship between VP synthesis and release was disrupted (Hoffman and Dave, 1991). Moreover, Silva, Marques, and Madeira (2009) have reported a correlation between decreased VP neurons in the medial parvocellular division of the PVN after chronic alcohol use. Perhaps, alcohol may also decrease magnocellular VP neurons in the SON and PVN, thereby decreasing VP release. Despite the number of studies in this area, whether alcohol inhibits or stimulates VP release in response to a physiological stimulus remains to be elucidated.

Regulation of Vasopressin

Oliver and Schafer (1895) originally demonstrated that when extracts from the pituitary gland were intravenously injected into mammals, blood pressure would be raised to significantly high levels and would be sustained considerably longer than with maximal doses of adrenal gland extracts. This action was later attributed to VP, and other important physiological actions, such as its antidiuretic activity, were subsequently
discovered in addition to its vascular actions. What followed was years of research from examining the physiological regulation of the endogenous release of VP into the circulation.

Current understanding of the regulation of VP synthesis and release is that these different actions of VP response to blood pressure or osmotic changes are controlled by different central pathways. In a later study by Clark and Silva (1967), it was shown that VP release after hemorrhage is mediated by afferents in the vagi and sinus nerves after observing a reduction in, or no VP release, after hemorrhage when the afferents were divided. VP release stimulated by hemorrhage requires a large amount of blood loss to occur.

VP stimulation or suppression also occurs when there are changes in plasma osmolality (pOsm). The concept of osmoreceptors was introduced by Verney (1947) in which the permeability of the cell membrane of an osmoreceptor (which he believed to be a part of the brain and to monitor blood osmolality) to an osmotic agent is related to how well that agent can stimulate VP secretion. Jewell and Verney (1957) concluded that the location of osmoreceptors is in the anterior hypothalamus and preoptic region. In a later study by Mason (1980), results were consistent with Jewell and Verney’s idea on the location of osmoreceptors in the anterior hypothalamus, and his results indicated that the SON neurons are osmoreceptive. A mechanism was also defined by Bourque et al (1994) in which hypotonicity results in water movement into the VP neuron, which then stretches the membrane and inactivates the cation channel that then leads to hyperpolarization of the neuron, reduced sensitivity of VP neurons and a decrease in
excitatory transmitter release from the organum vasculosum lamina terminalis (OVLT) osmoreceptors.

VP release stimulated by an increase in pOsm does not require huge changes in pOsm. Rather, VP secretion can occur in response to minute increases in pOsm, which frequently occurs on a day-to-day basis. Alcohol is known to be a diuretic, which would influence pOsm. Therefore, in this study, I wanted to assess how alcohol influences VP response to fluctuations in pOsm. Thus, a 5% salt load infusion was used as an osmotic stimulus for studying VP secretion.

**Effects of Sex Hormones on VP Release**

Sex hormones, such as estrogen and progesterone, and their influence on VP release have been investigated. Under normal conditions, Stachenfeld et al (1998) showed that 17β estradiol treatment increases osmotic stimulation of VP secretion, thus suggesting that estradiol may contribute to water retention in females. Furthermore, estrogen treatment in post-menopausal women (Steinwell et al, 1998), and ovariectomized rats (Barron et al, 1986), enhanced VP secretion in response to an osmotic stimulus. Also, Peysner and Forsling (1990) reported that VP release is estrogen-dose dependent, with a low dose of estrogen treatment increasing VP response and a high dose of estrogen inhibiting VP response.

In an *in vitro* study by Somponpun and Sladek (2002), it is suggested that estradiol via ERβ mediation inhibits N-methyl-D-aspartate (NMDA)-induced VP release from the posterior pituitary. Furthermore, ERβ mRNA expression was found to be downregulated under chronic hyperosmolality conditions and increased in chronic hypoosmolality conditions in rats (Somponpun and Sladek, 2003). Since increases in
pOsm results in a decrease in ERβ mRNA expression, this suggests that estradiol via ERβ mediation inhibits VP synthesis and release (Somponpun and Sladek, 2003).

The effects of progesterone alone or in combination with estrogen treatment have also been examined. For example, progesterone treatment has been shown to cause a decrease in circulating VP levels in ovariectomized rats (Crofton et al, 1985) and also a decrease in circulating VP levels when combined with estrogen treatment in post-menopausal women (Steinwell et al, 1998) and ovariectomized female rats (Crofton et al, 1985). On the other hand, plasma vasopressin (pVP) was not affected in castrated male rats when given the combined estradiol and progesterone treatment (Crofton et al, 1985). Therefore, the sex differences we see in alcohol effects on water balance may be due to influence of alcohol on sex hormone mediation of VP secretion.

**Alcohol Effects on Sex Hormone Mediation of VP Regulation**

A study by Sarkola et al (1999) aimed at studying alcohol’s effects on menstrual irregularities showed that pre-menopausal women using oral contraceptives who participated in a drinking event (0.5 g/kg) followed by 28 days of no drinking exhibited a decrease in progesterone levels, but an increase in estradiol levels. This suggests that alcohol affects circulating levels of sex hormones. It is well known that alcohol affects males and females differently, and it is possible that this is due to alcohol either directly or indirectly affecting sex hormone levels. Thus, if sex hormones have an influence on VP regulation, alcohol effects on sex hormones, which in turn mediate VP responsiveness to an osmotic stimulation, would result in male and female differences in alcohol influence of water balance.
Future Relevance

The levels of sex hormones in the different phases of the estrous cycle may affect VP regulation, which may make women more susceptible to dehydration or “bloating” at certain phases of the menstrual cycle. Alcohol usage may affect females more than males in being able to maintain water balance. If alcohol is exerting its effects on sex hormone modulation of VP, it is important for researchers and clinicians to know sex-specific treatments in situations in which individuals may have difficulty in maintaining euhydration.
HYPOTHESES

Based on the literature and previous studies conducted in our laboratory, I hypothesized that:

1. **VP regulation may be different in different phases of the estrous cycle.**

   If we believe that estradiol and progesterone modulate VP release, then it would make sense that we would see a difference in VP regulation in the different phases of the estrous cycle. Thus, endogenous estradiol and progesterone concentration in plasma will be determined in each of the estrous cycle phases, and linear regression analysis will be utilized to identify relationships between progesterone or estradiol and pVP, pOsm or pituitary VP. Furthermore, relationships between pOsm and pVP or pituitary VP in males and females will be compared to examine possible sex differences in osmotic stimulation of VP release into the circulation.

2. **The purported alcohol differences in males and females may be due to alcohol effects on estradiol and progesterone influence on VP release.**

   Alcohol appears to affect males and females differently. To date, there is no study utilizing a conscious rat model to assess VP response to changes in pOsm in the different phases of the estrous cycle under the effects of alcohol. In this study, Sprague Dawley male and female rats will be fed a moderate dose of alcohol (containing 1.2% ethanol) and a high dose of alcohol (containing 6.4-6.7% ethanol) to determine how VP response to a 5% salt load is affected.
MATERIALS AND METHODS

Research Protocol Approval

All procedures were reviewed and approved by the Scientific Review Committee (SRC) and Institutional Animal Care and Use Committee (IACUC) at Tripler Army Medical Center.

Feeding

Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were housed under standard lighting (12:12 hr light/dark) with lights on at 0630 hours. At approximately 8-9 weeks old, rats were started on their liquid diet. They were fed an equal caloric Bio-Serv (Frenchtown, NJ) liquid diet of either no alcohol (control) or containing one of two doses of alcohol: moderate dose (1.2% ethanol) or high dose (6.4-6.7% ethanol). Rats were allowed to feed ad lib up to 80 ml per day for 4-6 weeks, and the liquid diet provided all caloric and water requirements.

To allow ethanol-fed rats to slowly adjust to the full dose of alcohol, rats gradually received increasing levels of ethanol over a 7-day acclimatization period. For the first three days, alcohol fed-rats were given a liquid diet containing 1/3 ethanol liquid diet mixed with 2/3 control liquid diet. On the next three days, the rats were given 2/3 ethanol liquid diet mixed with 1/3 control liquid diet. Starting on the 7th day, the rats were given the full ethanol liquid diet.

Amount of liquid diet consumed and body weight were recorded every day. Based on previous studies in our laboratory, all alcohol-exposed rats consumed most, if not all, of the liquid diet. Figure 1 shows the amount of liquid diet (ml) consumed per
100 g of body weight; and Figure 2 shows body weight over 30 days from the start of the liquid diet.
Figure 1. Liquid diet consumed (ml) per 100 g body weight over 30 days. For the first 15 days, there appears to be a difference between moderate alcohol-fed males and all other groups in the amount of liquid diet consumed. However, from day 16, all rats, regardless of sex and treatment, appear to be adjusted to the liquid diet in that they seem to be consuming similar amounts. In addition, corresponding males and females appear to be consuming the same amount of liquid diet. Control males (n=15), moderate alcohol males (n=9), high alcohol males (n=7), control females (n=20), moderate alcohol females (n=19), high alcohol females (n=16). (Values represent means ± s.e.m.)
Figure 2. Daily body weight of rats after start of either the control (no alcohol) or alcohol liquid diet. Moderate alcohol-fed females (n=19) and high alcohol-fed females (n=16) appear to show normal growth similar to the control no alcohol-fed female rats (n=20). However, high alcohol-fed male rats (n=7) seem to show lower body weights than control no alcohol-fed male rats (n=15) and moderate alcohol-fed male rats (n=9). (Values represent means ± s.e.m.)
Figure 1 shows that for the first 15 days, there seems to be a difference between the moderate alcohol-fed males and all other groups in the amount of liquid diet consumed. This is possibly due to a period of adjustment to the liquid diet. However, starting on day 16, all rats seem to be adjusted to eating the liquid diet in that they appear to consume similar amounts. Corresponding treated male and female rats appear to consume similar amount of liquid diet per 100 g body weight.

In Figure 2, it appears that the high alcohol-fed males had a lower body weight than control and moderate alcohol-fed males that could be due to alcohol-induced diuresis as observed from a higher degree of excessive bed wetting when compared to control no alcohol and moderate alcohol-fed males. Their lower weights could be due to water loss that could not be compensated by extra drinking since all water and food was only provided by the liquid diet. As for females, the alcohol-fed groups appear to show normal body weight gain similar to the control no alcohol-fed group.

**Female Rat Cycle Phase Determination**

Starting at 10-11 weeks old (approximately 1 week before surgical implantation of catheters), daily vaginal smears were performed on the female rats to determine the phase of the estrous cycle. Ten to twenty microliters of 0.9% normal saline was carefully pipetted into the vaginal opening and aspirated 2-3 times. The fluid-containing cells was transferred to a slide and allowed to air dry. After air drying, the slides were stained according to the following: 95% ethanol bath for 10 seconds and allowed to air dry followed by immersion in toluidine blue solution (0.23 g/L) for 30-45 seconds, and finally rinsed by dipping in a deionized water bath and allowed to air dry.
The slides were then analyzed under a light microscope to determine cycle phase as described in previous studies (Marcondes et al., 2002). The criteria for determining phases were:

- **Anestrus** – predominately leukocytes with few nucleated epithelial cells and/or large empty space
- **Proestrus** – predominately nucleated epithelial cells
- **Estrus** – predominately cornified or flaky epithelial cells
- **Metestrus** – a mixture of leukocytes, nucleated epithelial cells and cornified or flaky epithelial cells

**Catheter Implantation**

Medical grade tygon tubing (S-54-HL, 0.030 inch O.D., Norton Co., Akron, Ohio) was used to make the vascular catheters, which were threaded through 2 small flared polyethylene (PE) 60 tubing (Sparks, MD) “dumbbells.” The “dumbbells” were used for tying catheters to vessels and to prevent kinking.

All rats underwent surgery for permanent implantation of arterial and venous catheters after 4-5 weeks on the liquid diet. Catheterization of the femoral artery and femoral vein were performed as implemented by Uyehara and Gellai (1993) and Gellai and Valtin (1979) to allow collection of arterial blood and intravenous infusion of fluids, respectively. Rats were given isoflurane anesthesia and lidocaine (topically at the incision site as needed) during surgery.

With the rat in the supine position, a 1-2 cm incision was made in the skin over the femoral artery and vein. The vessels were freed and exposed from surrounding tissue by blunt dissection; and 4-0 silk ties were used to lift and occlude the vessel. A
perforation was then made into the vessel and the catheter was carefully introduced and advanced cranially to the level of the abdominal aorta or caudal vena cava. 4-0 silk ties were then tied around the vessels to maintain hemostasis and to anchor the catheters.

The catheters were routed subcutaneously to the back of the neck and threaded through dumbbells to anchor each catheter. Vascular catheters were flushed with 0.9% normal saline infusion to check patency and function. The lines were then finally filled with a heparin-dextrose solution and plugged with stainless steel pins.

Rats were administered children’s Tylenol in water post-operatively (up to 24 hours) for post-operative analgesia. Rats were also given additional paper towels to shred as nesting material which kept them busy and less likely to play with or chew on their catheters and/or sutures. No protruding objects that have the potential of snagging the catheter lines were allowed in the cages after implantation of catheters.

To prevent infection and ensure patency and function of the lines, arterial and venous catheters were regularly flushed every 3-4 days with a heparin-saline solution (containing 10% heparin) and filled with a heparin-dextrose solution.

Rats were monitored daily and closely observed to detect any signs of irritation and/or infection at the incision sites or for any systemic side effects. No irritation or infection was found in rats used in this study.

**Animal Training**

Rats were trained a week before experiments to become accustomed to rest quietly in their acrylic experimental chambers for the duration of an experiment (up to 3 hours). The chambers allowed for easy access to arterial and venous catheters. Each rat
was acclimatized to their chamber by receiving 1-3 training sessions in which they were placed in their chambers for 1-3 hours.

Previous studies in our laboratory have shown that training was adequate in ensuring that rats were not subjected to stress when placed in their chambers (as indicated by normal mean arterial pressure, heart rate and stress hormone levels).

5% Salt Load Experiment

All rats were given at least 3 to 7 days to recover after surgical implantation of catheters before undergoing salt load experiments. Since alcohol in the blood may confound the data or interfere with measurements, the diet for the high and moderate alcohol dose groups were replaced with control liquid diet at least 12-18 hours before the salt load experiment (Figure 3) to ensure that blood alcohol levels were back to zero at the time of the blood draws.

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**Figure 3. 5% salt load experiment timeline.** All rats were given at least 60 minutes to equilibrate with normal saline followed by a baseline sample before the 5% NaCl i.v. infusion. Only 2 additional blood samples (P1 and P3 or P2 and P4) were drawn according to the timeline.
On the day of the experiment, the rat was weighed and placed in their experimental chamber. Lines were then checked for patency and function using a 10% heparinized saline solution. The arterial catheter was then connected to a Gould pressure transducer Model 5900 (Valley View, Ohio) and Gould Recorder System Model 3800 (Valley View, Ohio) for monitoring both mean arterial pressure (MAP) and heart rate (HR) to ensure that an increase in VP was not due to hemorrhage and that the rat was not in distress at any time throughout the experiment.

The venous catheter was then connected to a Harvard Infusion Pump Model 22 (Holliston, MA) for infusion of normal saline for equilibration and 5% salt solution for the salt load test. Both solutions were at a rate of 10 μl/min/100 g of body weight. For approximately 60-90 minutes, a 0.9% normal saline infusion was provided to allow the rat to physiologically equilibrate and maintain euhydration up to the start of salt load infusion.

After physiologic equilibration, the baseline blood P0 was taken. The amount of whole blood taken was 1.4 ml [1.2 ml whole blood for pVP quantification (pg/ml) and 0.2 ml for pOsm (mOsm/kg water)].

Whole blood for VP was centrifuged for approximately 2-3 minutes using the Allegra™ 6R Centrifuge (Fullerton, CA) for VP; and blood for pOsm analysis was centrifuged for approximately 2-3 minutes using the Brinkman Eppendorf Centrifuge 5415C (Westbury, NY).

For each blood sample, the blood cells were reconstituted to the original whole blood volume with 10% heparinized saline solution and returned to the rat via the arterial catheter to prevent excessive blood loss. Once baseline sample was obtained and blood
cells returned, the 5% NaCl ramp was started in the venous line at a rate of 10 μl/100g of body weight/min. Two additional blood samples (P1 and P3 or P2 and P4) were obtained after the start of the salt load. During each draw, 0.8 ml whole blood was taken for pVP quantification and 0.2 ml for pOsm determination. Only 2 additional blood samples were taken to minimize blood collected per experiment.

The separated plasma was then collected, and one normal of HCl was added to the plasma for VP assessment and stored at -20°C if not assayed immediately. The plasma for osmolality measurement was stored as a separate aliquot at 4°C to be measured before the end of the day.

**Tissue Harvest**

Tissue harvest took place at least 2 days after the final salt load experiment to allow the animal to recover from the experiment. The alcohol-fed rats were also fed the control liquid diet at least 12-18 hours before tissue harvest to ensure blood alcohol levels were back to zero.

Before euthanization, a baseline P0 blood sample was taken for pVP and pOsm determination. The red blood cells were then resuspended in a 10% heparinized saline solution and returned to the animal if the additional 2.0-3.0 ml blood draw for clinical status check of liver function was not taken immediately.

To check the clinical status of liver function, a serum sample was obtained to perform laboratory analyses on liver enzymes, specifically alkaline phosphatase (ALKP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). This helped verify whether the models of chronic alcohol exposure had or had not caused liver damage in that liver damage can affect electrolyte and water balance, thus VP response.
Whole blood (2.0-3.0 ml) was drawn and plasma separated using the Beckman Coulter AllegraTM 6R Centrifuge (Fullerton, CA) or Brinkman Eppendorf Centrifuge 5415C (Westbury, NY). Quantification of liver enzymes was performed as standard blood chemistry analysis using the JJ 5600 at the Pathology Lab at Tripler Army Medical Center.

Table 1. Liver enzyme profile for male and female rats utilized in this study. There were no differences in AST, ALT and ALKP between control and alcohol-treated male or female rats. (Values represent means ± s.e.m., n=number of rats)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Moderate Alcohol</th>
<th>High Alcohol</th>
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<tbody>
<tr>
<td></td>
<td>Males (n=6)</td>
<td>Females (n=12)</td>
<td>Males (n=8)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>70.7±10.0</td>
<td>81.5±9.5</td>
<td>93.5±11.1</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>34.3±1.3</td>
<td>43.1±2.8</td>
<td>41.6±2.8</td>
</tr>
<tr>
<td>ALKP (U/L)</td>
<td>150.2±23.6</td>
<td>176.5±16.0</td>
<td>224.4±21.1</td>
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An additional 0.5 ml whole blood was taken from all female rats along with the 2-3 ml blood draw for liver enzymes to assess plasma estradiol (p[Estradiol]) and plasma progesterone (p[Progesterone]) levels. After obtaining all blood samples, the rat was euthanized by administering 1 ml of pentobarbital in the venous line.

The posterior pituitary was then collected and placed at -70°C until ready for VP extraction and radioimmunoassay.

Analysis

pOsm

pOsm was analyzed on the same day after the experiment using The Advanced Micro-Osmometer model 3MO plus (Norwood, Massachusetts) to assess change in osmolality caused by the salt load ramp.

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**pVP Extraction**

pVP was extracted utilizing the J.T. Baker VP extraction apparatus (Phillipsburg, NJ). Sep-Pak® Classic C18 Cartridges (Milford, MA) and 10-milliliter syringes were assembled on to the Baker apparatus. The assembled apparatus was then primed with methanol; followed by acetic acid; and finally deionized water. Filtration using a vacuum pump occurred after each addition of solution.

The plasma sample to be assayed for VP was then applied to the Baker apparatus via a plastic applicator. Normal saline was then used to wash any remaining plasma in the eppendorf tube and recovered to be applied to the Baker. The plasma-normal saline solution was then filtered with the goal of the VP remaining in the Sep-Pak cartridge. Acetic acid was then applied to the apparatus and was filtered.

Next, elution solution (95% ethanol, acetic acid brought up with deionized water) was made, and 3 ml of the solution was then applied to each sample and filtered. The filtered solution, containing VP, was captured in glass tubes coated with phosphate-sodium-bovine serum albumin (PNB) buffer. The solutions were then centrifuged and resuspended with PNB buffer ready to undergo radioimmunoassay.

**Posterior Pituitary VP Extraction**

After the posterior pituitary was thawed, it was placed in 10 ml of 0.25% acetic acid and homogenized for 30 seconds. The homogenate was then placed in boiling water for 5 minutes and centrifuged at 2500 rpm for 15 minutes at 4°C. Dilutions were then made to obtain a final dilution of 1:2500, which was used for the radioimmunoassay.
Radioimmunoassay for VP Measurement

Radioimmunoassay involves the competition between labeled VP and VP in the standards and samples to be analyzed for a fixed number of antibody binding sites. Antibody 96 (produced at the Department of Clinical Investigation at Tripler Army Medical Center) and the VP labeled with the $^{125}$I isotope (purchased from PerkinElmer, Massachusetts) were utilized for the assay.

After incubation with the antibody and the labeled VP, the amount of vasopressin was measured, with unlabeled VP in sample having an inverse relationship with the labeled VP bound to the antibody.

$p$[Estradiol] and $p$[Progesterone]

$p$[Estradiol] was analyzed using the Cayman Estradiol Enzyme Immunoassay (EIA) kit (Catalog No. 582251, Ann Harbor, MI) and $p$[Progesterone] using the Cayman Progesterone EIA kit (Catalog No. 582601, Ann Harbor, MI). Plates were read using the SpectraMax M2 Analyzer Microplate Reader (Sunnyvale, California).

Alcohol Detection

To ensure that alcohol levels were back to zero before a salt load infusion experiment and harvest, the baseline plasma sample was analyzed via an Analox Instruments AM1 Alcohol Analyzer (Lunenburg, Massachusetts). Furthermore, additional blood samples were taken from both severe and moderate alcohol-fed rats after drinking the liquid diet to determine alcohol levels in their blood.

Blood Alcohol Level Analysis

In past studies, our laboratory has found that within 30 minutes after intragastric injection of an alcohol dose of 15% ethanol (1 ml/100 g body weight), blood alcohol
levels were 100 to 150 mg/dL. This alcohol level returned to zero by 2 hours after the last drink.

In this study, I was able to take a blood sample 10-15 minutes after a high alcohol dose-fed rat took a drink, consuming approximately 30 ml of the liquid diet. However, blood alcohol levels were lower than what was expected. Thus, it is most likely that a longer wait time was needed in that previous alcohol readings in our laboratory peaked at around 30 minutes after injection of alcohol.

Alcohol levels in the blood were negative in all alcohol-exposed rats on the day of an experiment and harvest, thus eliminating any effects of blood alcohol in circulation that may influence VP response.

Statistical Analysis

Statistical analysis was performed using the JMP® 8.0.2 Analysis Program (Cary, North Carolina). Analyses of variance (ANOVA) and Student’s t-tests were carried out. In addition, the Statistical Analysis Software (SAS) Program was utilized in which a general linear model was used where VP response versus pOsm, alcohol treatment and sex was modeled. Interaction effects were then looked at; and a stepwise routine was used to remove non-significant interactions. A p-value less than 0.05 was statistically significant.
RESULTS

Sex Differences in pOsm and VP

Table 2 shows baseline mean arterial pressure, heart rate and hematocrit (Hct) in male vs. female rats. Each rat had two 5% salt load infusion experiments. If a rat was in the same phase for both experiments, the values for those days were averaged. Table 2 shows that there were no overall differences in MAP, HR and Hct between males and females. Therefore, I was studying a calm-baseline rat, and the VP response was not due to other factors.

Figure 4 shows pOsm at baseline after the 60-90 minute 0.9% normal saline infusion prior to starting the salt load infusion. Depending on when the rats had their last drink before a salt load experiment, not all rats will have similar hydration status. Thus, the 0.9% normal saline infusion was given to provide similar hydration status between male or female rats so that the osmotic stimulation of VP could be assessed from a euhydration baseline.

Baseline pOsm (Figure 4) was similar between control and alcohol-fed males and between control and high alcohol-fed female groups. However, moderate alcohol-fed females showed a significant decrease in pOsm when compared to control females.

Figure 5 shows basal pOsm and Figure 7 shows basal pVP after euhydration in males versus females by estrous cycle phase. A rat may be counted twice due to different estrous cycle phases on experiment days. If a rat had the same estrous cycle for both experiment days, the pOsm or pVP values for both days were averaged. Figure 5 shows that there was a tendency for females by estrous cycle phase to have a lower pOsm than...
males, which concurs with other studies showing that males typically have a higher pOsm than females.

Figure 6 shows that there were no differences in pVP at baseline between control and alcohol-fed males or females. In addition, Figure 7 shows that VP response was similar between control and alcohol-fed females by estrous cycle phase and when compared to males. Moreover, the similar levels in circulating VP in the plasma suggest that all rats at baseline were equally hydrated prior to salt load infusion.

In Figure 8, pituitary VP stores were significantly lower in alcohol-fed females than their male counterparts. It might be expected that the posterior pituitary in females are smaller, thus accounting for the lower VP content when compared to that in males. However, only the alcohol-fed rats were significantly lower than their male counterparts whereas the controls were not. Therefore, it could be that alcohol-fed females had a compensatory increase in VP release when compared to alcohol-fed males.

Figure 9 shows that there was a tendency for alcohol-fed females by estrous cycle phase to have lower pituitary VP stores than corresponding alcohol-fed males, which may be due to the low number of n’s. Thus, it was hard to detect statistical significant differences between phases. Therefore, the relationships of pOsm, pVP and pituitary VP with direct estradiol and progesterone hormone levels were assessed.
Table 2. Baseline mean arterial pressure, heart rate and hematocrit in males versus females by estrous cycle phase. MAP, HR and Hct were all within normal values reported for male and female rats. There was no obvious influence of alcohol on male versus female baseline values. (Values represent means ± s.e.m., n=number of estrous cycle phases at baseline)

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<thead>
<tr>
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<th>MAP</th>
<th>HR</th>
<th>Hct</th>
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<td></td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>119 ± 2 (n=12)</td>
<td>412 ± 13 (n=12)</td>
<td>43 ± 1 (n=13)</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anestrus</td>
<td>121 ± 3 (n=7)</td>
<td>442 ± 8 (n=7)</td>
<td>39 ± 1 (n=8)</td>
</tr>
<tr>
<td>Proestrus</td>
<td>114 ± 5 (n=2)</td>
<td>423 ± 39 (n=2)</td>
<td>43 ± 2 (n=3)</td>
</tr>
<tr>
<td>Estrus</td>
<td>122 ± 2 (n=5)</td>
<td>426 ± 19 (n=3)</td>
<td>42 ± 0 (n=5)</td>
</tr>
<tr>
<td>Metestrus</td>
<td>118 ± 4 (n=5)</td>
<td>477 ± 33 (n=4)</td>
<td>41 ± 1 (n=5)</td>
</tr>
<tr>
<td><strong>Moderate Alcohol Dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>122 ± 4 (n=9)</td>
<td>446 ± 14 (n=9)</td>
<td>44 ± 1 (n=9)</td>
</tr>
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<td><strong>Females</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>114 ± 3 (n=8)</td>
<td>425 ± 15 (n=8)</td>
<td>42 ± 1 (n=8)</td>
</tr>
<tr>
<td>Proestrus</td>
<td>116 ± 3 (n=4)</td>
<td>414 ± 9 (n=4)</td>
<td>42 ± 1 (n=4)</td>
</tr>
<tr>
<td>Estrus</td>
<td>113 ± 2 (n=12)</td>
<td>419 ± 9 (n=13)</td>
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<tr>
<td>Metestrus</td>
<td>119 ± 6 (n=5)</td>
<td>458 ± 18 (n=5)</td>
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<tr>
<td><strong>High Alcohol Dose</strong></td>
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<td><strong>Males</strong></td>
<td>116 ± 2 (n=7)</td>
<td>416 ± 11 (n=7)</td>
<td>42 ± 1 (n=7)</td>
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<tr>
<td><strong>Females</strong></td>
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</tr>
<tr>
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</tr>
<tr>
<td>Estrus</td>
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<td>440 ± 14 (n=3)</td>
<td>43 ± 1 (n=3)</td>
</tr>
<tr>
<td>Metestrus</td>
<td>115 ± 1 (n=4)</td>
<td>416 ± 19 (n=4)</td>
<td>42 ± 1 (n=3)</td>
</tr>
</tbody>
</table>
In general, all females had lower basal pOsm than males. Alcohol exposure did not appear to affect baseline pOsm in males and females with the exception of moderate alcohol-fed females being lower than control females. (Values represent means ± s.e.m., * = significantly different from control of same sex rats, † = significantly different from corresponding male rats, ANOVA, p<0.05)
Figure 5. Effect of alcohol on baseline plasma osmolality in male versus female rats by estrous cycle phase. There was a tendency for females by estrous cycle phase to have a lower pOsm than males. (Values represent means ± s.e.m.)
Figure 6. Effect of alcohol on baseline plasma vasopressin in male versus female rats. There were no differences in pVP circulating levels between control and alcohol-fed males or females. (Values represent means ± s.e.m.)
Figure 7. Effect of alcohol on baseline plasma vasopressin in males versus female rats by estrous cycle phase. Circulating pVP levels were similar between control and alcohol-fed females by estrous cycle phase and when compared to males. (Values represent means ± s.e.m.)
Figure 8. Posterior pituitary vasopressin content in males versus female rats at tissue harvest. Pituitary VP stores were significantly lower in the alcohol-fed females than their male counterparts. (Values represent means ± s.e.m., † = significantly different from corresponding male rats, ANOVA, p<0.05)
Figure 9. Pituitary vasopressin content in males versus females by estrous cycle phase at tissue harvest. High alcohol-fed females in metestrus had a significantly lower pituitary VP content than control females in metestrus. There was also a tendency for alcohol-fed females to have a lower pituitary VP content than corresponding alcohol-fed males. (Values represent means ± s.e.m., * = significantly different from control females in metestrus, ANOVA, p<0.05)
Influence of Estradiol and Progesterone on VP

Figures 10 and 11 show p[Estradiol] and p[Progesterone] levels, respectively. Plasma samples for estradiol analysis were obtained on non-experiment days, at baseline prior to salt load infusion, and at tissue harvest. Plasma samples for progesterone analysis were obtained only at baseline prior to salt load infusion and at tissue harvest. Both figures show that no statistical significance could be detected between phases in control and alcohol-fed groups due to the low number of n’s. Although I was not able to detect the typical peak of circulating p[Estradiol] levels in proestrus and estrus in control female rats, I was able to detect the characteristic peak of estradiol levels in proestrus in moderate alcohol-fed rats as well as the characteristic estradiol peaks in proestrus and estrus in high alcohol-fed rats. As for progesterone, I was able to detect the characteristic peak that occurs in anestrus.

A significant negative correlation between p[Estradiol] and pOsm occurs in control females (Figure 12A). However, alcohol disrupts this relationship. When examining p[Estradiol]’s relationship with pVP, no relationship was found in control or alcohol-treated female rats (Figure 12B). However, there was a significant negative relationship between p[Estradiol] and pituitary VP content in high alcohol-fed rats (Figure 12C) indicating a different response based on alcohol dose.

I also looked to see if there was a correlation between p[Progesterone] and pOsm, pVP or pituitary VP (Figure 13A-C). While there was a tendency for progesterone to have a positive relationship with pOsm in control female rats, there was no relationship in the alcohol-fed groups (Figure 13A). As for pVP and pituitary VP (Figure 13B), there was no relationship with p[Progesterone] in control and moderate alcohol-fed rats.
However, female rats exposed to the high alcohol dose showed a significant positive relationship between p[Progesterone] and pVP and a significant negative relationship with pituitary VP, indicating a different response based on alcohol dose.
Figure 10. **Plasma estradiol by estrous cycle phase.** Samples were collected at baseline prior to salt load infusion, at tissue harvest, or on non-experiment days. No statistical significant differences could be detected between phases in control and alcohol-fed groups. Interestingly, in the high alcohol-fed groups, it seems that the typical peaks of estradiol in proestrus and estrus were observed whereas in the controls they were not. (Values represent means ± s.e.m.)
Figure 11. Plasma progesterone by estrous cycle phase. Samples were collected at baseline prior to salt load infusion, at tissue harvest, or on non-experiment days. No statistical significance was found in plasma progesterone levels between phases in control, moderate alcohol and high alcohol-fed rats. (Values represent means ± s.e.m.)
A. p[Estradiol] vs. pOsm

Control (n=10):
\[ y = -1.64 \times 10^{-1} x + 303.79, \quad r=0.84, \quad p=0.002 \]

Moderate Alcohol (n=15):
\[ y = 5.44 \times 10^{-2} x + 291.85, \quad r=0.22, \quad p=0.42 \]

High Alcohol (n=7):
\[ y = 5.92 \times 10^{-2} x + 288.22, \quad r=0.60, \quad p=0.15 \]

B. p[Estradiol] vs. pVP

Control (n=7):
\[ y = 2.3 \times 10^{-3} x + 2.15, \quad r=0.05, \quad p=0.91 \]

Moderate Alcohol (n=11):
\[ y = -0.02 x + 2.71, \quad r=0.33, \quad p=0.32 \]

High Alcohol (n=7):
\[ y = 1.24 \times 10^{-2} x + 0.48, \quad r=0.65, \quad p=0.11 \]

C. p[Estradiol] vs. Pituitary VP

Control (n=6):
\[ y = 1.19 \times 10^{-1} x + 492.36, \quad r=0.03, \quad p=0.95 \]

Moderate Alcohol (n=15):
\[ y = -6.13 \times 10^{-1} x + 503.727, \quad r=0.18, \quad p=0.52 \]

High Alcohol (n=7):
\[ y = -3.03 x + 659.73, \quad r=0.84, \quad p=0.02 \]

Figure 12. Relationship between plasma estradiol and (A) plasma osmolality, (B) plasma vasopressin, and (C) pituitary vasopressin at time of tissue harvest. (A) Whereas a significant negative correlation between p[Estradiol] and pOsm exists in control rats, alcohol disrupts this relationship. (B) No relationship between p[Estradiol] and pVP occurs in control and alcohol-fed groups. (C) A negative relationship between p[Estradiol] and pituitary VP only occurs in high alcohol-fed rats. (Linear regression analysis, p<0.05)
A. p[Progesterone] vs. \textit{pOsm}

Control (n=8):
\[ y = 1.27 \times 10^{-1}x + 285.52, r=0.68, p=0.06 \]

Moderate Alcohol (n=15):
\[ y = 5.27 \times 10^{-2}x + 289.73, r=0.28, p=0.33 \]

High Alcohol (n=7):
\[ y = 7.97 \times 10^{-2}x + 287.38, r=0.45, p=0.31 \]

B. p[Progesterone] vs. pVP

Control (n=6):
\[ y = 1.10 \times 10^{-2}x + 1.29, r=0.30, p=0.56 \]

Moderate Alcohol (n=11):
\[ y = -7.16 \times 10^{-3}x + 2.79, r=0.18, p=0.60 \]

High Alcohol (n=7):
\[ y = 2.96 \times 10^{-2}x - 0.53, r=0.83, p=0.01 \]

C. p[Progesterone] vs. Pituitary VP

Control (n=6):
\[ y = -1.38x + 600.37, r=0.18, p=0.73 \]

Moderate Alcohol (n=14):
\[ y = 7.21 \times 10^{-1}x + 410.37, r=0.28, p=0.33 \]

High Alcohol (n=7):
\[ y = -6.12x + 835.91, r=0.94, p<0.01 \]

Figure 13. Relationship between plasma progesterone and (A) plasma osmolality, (B) plasma vasopressin, and (C) pituitary vasopressin at time of tissue harvest. (A) There was a tendency for pOsm to be positively correlated with p[Progesterone] in control rats. (B) A significant positive relationship was observed between p[Progesterone] and pVP, and (C) a significant negative relationship occurred between p[Progesterone] and pituitary VP in only high alcohol-fed females. (Linear regression analysis, p<0.05)
Sex Differences in the Effect of Alcohol on VP Stimulation

All salt load figures indeed show an osmotic stimulation in that when pOsm increases, pVP also increases. Figure 14A shows that there were no differences in VP response to a 5% salt load between control, moderate alcohol, and high alcohol treated-males. However, Figure 14B shows that moderate alcohol-fed females had a more sensitive VP response than control and high alcohol-fed females.

Figure 15A shows that in control males, we see a typical VP response in that once a certain pOsm threshold is reached, there is an exponential release of VP into the circulation. However, in control females, the pOsm threshold is not as obvious when grouping the females together regardless of estrous cycle phase.

Nevertheless, alcohol did not seem to obliterate VP release to an osmotic stimulus in both males and females in that we still had an increase in pOsm with a resultant increase in VP release. Interestingly, in the moderate alcohol group (Figure 15B), females seemed to have a lower threshold and had a more sensitive VP response than males for equivalent osmolalities.

Males versus females in each of the estrous cycle phases by treatment (Figure 16A-D) were analyzed. No significant differences could be detected between males and females in metestrus (Figure 16A) or females in anestrus (Figure 16B). In addition, the slope for the VP response curve in females in anestrus was lower than that in males (Figure 16B). VP response was also similar between control males and females in proestrus and estrus (Figure 16C-D), but moderate alcohol-treated females had a lower threshold and a more sensitive VP response than moderate alcohol-treated males.
Nevertheless, when exposed to high alcohol, males and females in proestrus and estrus had a similar VP response.

Figure 17 shows the effect of alcohol in each of the different cycle phases more clearly. In proestrus and estrus, moderate alcohol increases VP sensitivity to an osmotic stimulus based on a significant shift of the VP response curve to the left. During metestrus, exposure to high alcohol shifted the VP response curve to the left in females in metestrus. These results indicate that alcohol effects are different depending on estrous cycle phase.
A. Males

Control (n=13):
\[ y = 45.77x - 259.51 \]
\[ r = 0.66 \]

Moderate Alcohol (n=9):
\[ y = 50.06x - 284.02 \]
\[ r = 0.64 \]

High Alcohol (n=7):
\[ y = 36.32x - 205.32 \]
\[ r = 0.66 \]

B. Females

Control (n=13):
\[ y = 27.15x - 153.25 \]
\[ r = 0.59 \]

Moderate Alcohol (n=18):
\[ y = 31.16x - 175.64 \]
\[ r = 0.57 \]

High Alcohol (n=11):
\[ y = 33.92x - 191.68 \]
\[ r = 0.72 \]

Figure 14A-B. Vasopressin response to a 5% salt load in (A) males and (B) females. There was a similar VP response between control and alcohol-fed males. However, moderate alcohol-fed females showed a more sensitive VP response than control (p<0.01) and high alcohol-fed (p=0.02) females. (Multiple Regression Analysis)
A. Control

Males (n=13):
y = 45.77x – 259.51
r = 0.66

Females (n=13):
y = 27.15x – 153.25
r = 0.59

B. Moderate Alcohol

Males (n=9):
y = 50.06x -284.02
r = 0.64

Females (n=19):
y = 31.16x – 175.64
r = 0.57

C. High Alcohol

Males (n=7):
y = 36.32x – 205.32
r = 0.66

Females (n=11):
y = 33.92x – 191.68
r = 0.72

Figure 15A-C. Vasopressin response to a 5% salt load in (A) control, (B) moderate alcohol, and (C) high alcohol-treated male and female rats. There were no differences in VP response between control or high alcohol-fed males and females. Moderate alcohol-fed females had a more sensitive VP response than moderate alcohol-fed males. (Multiple regression analysis, p<0.01)
A. Metestrus vs. Males

i. Control

Males (n=13):
y = 45.77x – 259.51  
r = 0.66

Females (n=4):
y = 27.99x-157.96  
r = 0.58

ii. Moderate Alcohol

Males (n=9):
y = 50.06x -284.02  
r = 0.64

Females (n=6):
y = 23.98x – 135.13  
r = 0.54

iii. High Alcohol

Males (n=7):
y = 36.32x – 205.32  
r = 0.66

Females (n=4):
y = 33.44x – 188.38  
r = 0.84

Figure 16A. Vasopressin response to a 5% salt load in male versus female rats by estrous cycle phase. No significant differences could be detected between males and females in metestrus.
B. Anestrus vs. Males

i. Control

Males (n=13):
y = 45.77x – 259.51
r = 0.66

Females (n=10):
y = 24.97x – 140.76
r = 0.56

ii. Moderate Alcohol

Males (n=9):
y = 50.06x -284.02
r = 0.64

Females (n=8):
y = 25.60x – 144.07
r = 0.56

iii. High Alcohol

Males (n=7):
y = 36.32x – 205.32
r = 0.66

Females (n=5):
y = 42.19x – 238.98
r = 0.80

Figure 16B. Vasopressin response to a 5% salt load in male versus female rats by estrous cycle phase. The slope for the VP osmotic stimulation curves in females in anestrus was lower than that in males pass a certain threshold, indicating that the females’ VP response was not as sensitive to changes in pOsm. In high alcohol-fed groups, there was no difference between males and females. (Multiple regression analysis, p=0.04).
### C. Proestrus vs. Males

<table>
<thead>
<tr>
<th>Condition</th>
<th>Males (n)</th>
<th>Females (n)</th>
<th>Equation</th>
<th>Correlation Coefficient</th>
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<td><strong>i. Control</strong></td>
<td>13</td>
<td>3</td>
<td>( y = 45.77x - 259.51 )</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( r = 0.66 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( y = 39.03x - 220.89 )</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>ii. Moderate Alcohol</strong></td>
<td>9</td>
<td>4</td>
<td>( y = 50.06x -284.02 )</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( r = 0.64 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( y = 49.85x - 281.64 )</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>iii. High Alcohol</strong></td>
<td>7</td>
<td>3</td>
<td>( y = 36.32x - 205.32 )</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( r = 0.66 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( y = 44.03x - 249.69 )</td>
<td>0.89</td>
</tr>
</tbody>
</table>

**Figure 16C.** Vasopressin (VP) response to a 5% salt load in male versus female rats by estrous cycle phase. Moderate alcohol-fed females in proestrus had a more sensitive VP response to osmolality with the dose response curve shifted to the left of the males, whereas in control and high alcohol-fed rats, there was no difference between male and female VP responses. (Multiple regression analysis, \( p<0.01 \))
D. Estrus vs. Males

i. Control

*Males* (n=13):
\[ y = 45.77x - 259.51 \]
\[ r = 0.66 \]

*Females* (n=5):
\[ y = 20.01x - 112.75 \]
\[ r = 0.39 \]

ii. Moderate Alcohol

*Males* (n=9):
\[ y = 50.06x - 284.02 \]
\[ r = 0.64 \]

*Females* (n=12):
\[ y = 38.81x - 219.00 \]
\[ r = 0.65 \]

iii. High Alcohol

*Males* (n=7):
\[ y = 36.32x - 205.32 \]
\[ r = 0.66 \]

*Females* (n=3):
\[ y = 32.54x - 183.82 \]
\[ r = 0.75 \]

Figure 16D. Plasma vasopressin response to a 5% salt load in male versus female rats by estrous cycle phase. Control females in estrus had a flatter, less sensitive VP response to osmotic changes than males. Moderate alcohol shifted the VP response of females to the left of the males, showing that females had greater sensitivity to osmotic changes. There were no differences in VP response between high alcohol-fed males and females. (Multiple regression analysis, p<0.01)
A. Metestrus

Control: \( y = 27.99x - 157.96, r = 0.58 \)

MA: \( y = 23.98x - 135.13, r = 0.54 \)

HA: \( y = 33.44x - 188.38, r = 0.84 \)

B. Anestrus

Control: \( y = 24.97x - 220.89, r = 0.56 \)

MA: \( y = 25.60x - 281.64, r = 0.64 \)

HA: \( y = 42.19x - 249.69, r = 0.89 \)

C. Proestrus

Control: \( y = 39.03x - 220.89, r = 0.88 \)

MA: \( y = 49.85x - 281.64, r = 0.64 \)

HA: \( y = 44.03x - 249.69, r = 0.89 \)

D. Estrus

Control: \( y = 20.01x - 112.75, r = 0.39 \)

MA: \( y = 38.81x - 219.00, r = 0.65 \)

HA: \( y = 32.54x - 183.82, r = 0.75 \)

Figure 17A-D. Vasopressin response to a 5% salt load in control, moderate alcohol and high alcohol-fed females by estrous cycle phase. During (A) metestrus, high alcohol shifted the VP response curve to the left (p=0.02). In (C) proestrus and (D) estrus, moderate alcohol increases VP sensitivity to an osmotic stimulus (p<0.01) based on a significant shift of the VP response curve to the left. These results indicate that alcohol effects are different depending on estrous cycle phase. (Multiple regression analysis)
DISCUSSION

Sex Differences in pOsm and VP

Males had a similar pOsm at baseline between control and alcohol-treated groups. Also, in females, pOsm at baseline was similar between control and high alcohol-fed groups. However, moderate alcohol-fed females showed a significantly lower pOsm than that in control females. Previous studies in our laboratory have shown that in response to the alcohol-induced diuresis in moderate alcohol-fed males, renal V2R was upregulated (Wu et al, 2003). Thus, it is possible that when exposed to a moderate dose of alcohol, the female renal response overcompensates by an upregulation in renal V2R, that leads to an increase in water retention and a lower pOsm. Indeed, previous work in our laboratory showed that when exposed to a high dose of alcohol, females also compensate with an upregulation of V2R (Huckstep et al, 2008).

Control and alcohol-fed females also had a significantly lower pOsm than their male counterparts and there was a tendency for pOsm in all estrous cycle phases to be lower than that in males, both of which concurs with previous studies (such as Dai and Yao, 1995) showing that pOsm is typically lower in females than males. Furthermore, the significantly lower pOsm at baseline in alcohol-fed females than that in their male counterparts suggests that alcohol-fed females may have had a higher compensatory increase in VP synthesis, thus higher VP stores released into the plasma in response to an osmotic stimulus, and thus greater compensatory water retention by the kidneys. Furthermore, the significantly lower pOsm in high alcohol-fed females when compared to the pOsm in high alcohol-fed males may be a result of an increase in renal V2R in high
alcohol-fed females but not high alcohol-fed males, which was previously demonstrated in our laboratory (Huckstep et al, 2008).

On tissue harvest day, pOsm was again assessed (Appendix, Figure 18). pOsm at harvest was different from pOsm at baseline in that on tissue harvest day, rats were not given the 0.9% normal saline infusion to achieve euhydration status before blood sample collection. Nevertheless, pOsm in males tended to be higher in the alcohol-fed groups than in the control no alcohol-fed group, indicating perhaps that there was an alcohol-induced diuresis that was not as completely compensated for by an increase in VP synthesis/release. In contrast, with females, there was a tendency for alcohol-fed groups to have a lower pOsm than the control no alcohol-fed group. This again suggests that the females may have had a higher compensatory response via increased VP synthesis and/or release or an upregulation in V2R receptors to counteract the alcohol-induced diuresis.

pVP at harvest (Appendix, Figure 20) and pituitary VP content (Figure 8) on tissue harvest day tend to correlate with the harvest pOsm data for males. The data shows that severe alcohol-fed males had a significantly lower pVP than control males. In addition, there was a tendency for high alcohol-fed males to have a higher pituitary VP content than moderate alcohol and control no alcohol-fed males. Assuming that VP synthesis was not causing the higher pituitary VP content, this suggests that pituitary VP content increases in high alcohol-fed males due to suppressed VP release. Since pVP is low, renal water loss would be higher, which then corresponds with the tendency for high alcohol-fed males to have a higher pOsm at harvest than control males.

Furthermore, based on Figure 23 (Appendix), there was a significant negative correlation between pVP and pOsm in high alcohol-fed males. Thus, when pOsm is high,
pVP levels are low, which would concur with my speculation that if VP release is suppressed, pituitary VP stores will increase and pVP levels will decrease. Thus, since pVP levels are low, there is a decrease in water reabsorption and pOsm will increase.

Figure 22 (Appendix) also shows that there is a tendency for pituitary VP and pVP to be positively linked in control and moderate alcohol-fed males. However, in high alcohol-fed males, pituitary VP stores are no longer linked with pVP. This suggests that VP is still able to be released into the plasma under normal conditions; and when exposed to a moderate dose of alcohol, males may still be able to compensate by releasing VP into the plasma. However, with high alcohol exposure, the positive relationship between pituitary VP stores and circulating VP is disrupted, thus possibly accounting for the significant decrease in VP circulating levels despite a tendency for an increase in pituitary VP content when compared to control male rats.

Influence of Estradiol and Progesterone on VP

According to Emanuele et al (2002) and Butcher et al (1974), estradiol levels peak sometime in proestrus with another peak, but at lower levels, occurring sometime in estrus. Nevertheless, my measurements of plasma estradiol in control female rats were similar across all phases. Perhaps, more samples were needed in each phase to see the purported trend of estradiol levels through the course of the cycle. Also, the similar estradiol levels between phases could be due to analyzing only 1 to 2 phases per rat instead of analyzing all 4 phases within the same rat. Furthermore, because the hormone peaks are short in duration and typically occur at night and do not last for the entire phase, I may have missed the peaks during blood sampling since I took my samples during the day.
Although no statistical significant differences could be detected between phases in the alcohol groups, interestingly, alcohol may alter the timing of the peaks in estradiol levels such that it seems I was able to catch the peaks that are typical in proestrus in both alcohol groups and in estrus in only the high alcohol-fed group.

p[Progesterone], on the other hand, peaks sometime in proestrus, and at lower levels in anestrus (Emanuele et al, 2002). Based on my own measurements of p[Progesterone] in control rats, it seems I was able to catch the peak in anestrus but not proestrus.

Although I was not able to catch the estradiol peaks typical in proestrus and estrus in the control rats as well as the progesterone peak typical in proestrus, I felt that it was important to look at the relationship between circulating levels of estradiol and progesterone in the blood with pOsm, pVP and pituitary VP regardless of estrous cycle phase.

In control rats, there was a significant negative correlation between p[Estradiol] and pOsm. This relationship was disrupted in alcohol-fed rats. As for p[Estradiol] and pVP, there was no correlation between the two in control and alcohol-fed rats. Furthermore, under normal and moderate alcohol conditions, there was no relationship between p[Estradiol] and pituitary VP stores. However, with high alcohol exposure, there was a strong negative correlation between p[Estradiol] and pituitary VP stores such that high estradiol levels are associated with low pituitary stores and low estradiol levels are associated with high VP stores. This may suggest that with high alcohol exposure, low estradiol levels are not inhibiting VP synthesis and thus VP stores are increased. Alternatively, it could mean that high alcohol exposure causes high estradiol levels to
stimulate release of VP stores, thereby decreasing pituitary VP content. On the other hand, estradiol may not affect central VP synthesis and release, but may act on the kidneys, which alters pOsm which in turn alters pVP. This would support a study by Wang et al (1993), in which it was shown that VP 2 receptors (V2R) in the papillary collecting duct (PCD) cells was lower in females than in males, thus suggesting that gonadal steroid hormones may have an effect on VP action.

As for p[Progesterone], there was no correlation with pOsm in control or alcohol-fed rats. While not evident in normal conditions, high alcohol may unmask a compensatory mechanism whereby progesterone may stimulate VP release as we see that p[Progesterone] was positively correlated with pVP. Also, there was a significant negative correlation between p[Progesterone] and pituitary VP stores in high alcohol-fed female rats, that was not seen in control and moderate alcohol-fed females. The negative correlation between p[Progesterone] and pituitary VP stores suggest that progesterone may stimulate VP release. When circulating progesterone levels are high, pituitary VP stores go down, and VP circulating levels go up.

Nevertheless, it is also possible that progesterone may have other actions which may influence VP release. According to Gambling et al (2004), progesterone may increase gamma-Epithelial Sodium Channel (γENaC) mRNA expression in the renal collecting tubules, thus increasing sodium reabsorption along with water. Despite water reabsorption, it is possible that VP release may have become more sensitized to small changes in sodium levels due to alcohol exposure. With progesterone, it is possible that there may have been an increase in renal ENaC mRNA expression, thus inducing VP release.
Sex Differences in the Effect of Alcohol on VP Stimulation

The similar VP response to an osmotic stimulation between treatment groups in males, Figure 14, further supports possible compensatory upregulation of VP synthesis/release and/or V2R receptors in the kidney in alcohol-fed males.

In control males, a typical VP response is also seen in that once a certain pOsm threshold is reached, there is an exponential release of VP into the circulation. However, in control females, the VP response seems to be blunted and not as sensitive to changes in pOsm when grouping the females together regardless of estrous cycle phase. Nevertheless, alcohol did not seem to obliterate VP release to an osmotic stimulus in that we still had an increase in pOsm with a resultant increase in VP release in females.

Interestingly, in the moderate alcohol group, the VP response in females seemed to be higher than the VP response in males for equivalent osmolalities and there was a significantly higher VP release in moderate alcohol-fed females than control and high alcohol-fed females. This suggests that with moderate alcohol exposure, there is a compensatory VP response. Likewise, high alcohol exposure also results in a compensatory VP response, but not to the same degree observed in female rats exposed to the moderate alcohol dose. Perhaps, the high dose of alcohol slightly decreases sensitivity of VP release to an osmotic stimulus.

VP response between females by estrous cycle phase and males showed differences which may be due to the different levels of estrogen and progesterone in the different phases of the estrous cycle.
In metestrus females, there was a tendency for a lower slope in VP release when compared to males, and moderate alcohol exposure did not seem to alter this pattern. However, there were no obvious differences in VP response between males and females.

For females in anestrus versus males, the slope for the VP osmotic stimulation curves seems to be steeper in males once you pass a certain threshold. Furthermore in anestrus, there seems to be a tendency for alcohol exposure to cause the female response to become similar to that of the males. This suggests that alcohol may unmask a stimulatory effect of progesterone on VP and in anestrus, progesterone levels are high. Therefore, the stimulatory effect of progesterone on VP would be higher during this phase thus accounting for females to have an increased sensitivity of VP release to pOsm.

In proestrus, both progesterone and estradiol levels supposedly peak during this phase. Unlike metestrus and anestrus, in proestrus, the male and female response curves are superimposable in control animals. This would suggest that progesterone, in the presence of estradiol, may stimulate VP sensitivity to an osmotic stimulus in proestrus females as opposed to females in anestrus. In anestrus there is also a peak in progesterone levels without the peak in estradiol. Since progesterone levels are higher than estradiol levels in proestrus, it is possible that progesterone may overcome the inhibitory effect of estradiol on VP, thus resulting in a higher VP response in control females in proestrus.

With moderate alcohol exposure (also shown in Appendix, Figure 25B), the VP response in proestrus female rats are even more enhanced with a shift of the osmotic VP curve to the left. This is likely due to the high progesterone levels that occur in proestrus and with alcohol unmasking progesterone’s stimulatory effect on VP release. With high
alcohol, stimulation may now have resulted in depletion in pituitary stores as seen earlier with the progesterone-pituitary VP graph (Figure 13C).

Estrus females seem to return to similar patterns of less sensitivity of VP osmotic stimulation than control males. This could be due to the purported inhibitory effects of estradiol on VP under normal conditions during estrus (when progesterone levels are low and not around to counteract the action of estradiol). Perhaps estradiol binds to ERβ, found predominantly in the SON and in select subnuclei of the PVN (Somponpun and Sladek, 2003), thereby inhibiting VP release, which was previously demonstrated in hypothalamic explants in vitro (Sladek & Somponpun, 2008).

However, moderate alcohol caused a shift in VP osmotic stimulation sensitivity for females in estrus. This would suggest that moderate alcohol interferes with the estradiol inhibitory effect on VP release. Alternatively, if estradiol levels are enhanced with high alcohol, perhaps VP synthesis is inhibited, thus explaining why a significant negative relationship occurred between estradiol and pituitary VP stores in our estradiol-pituitary VP graph (Figure 12C). This would also cause a dampened VP response in high alcohol-fed females in estrus.

Relevance of these Findings

Based on this study, the natural fluctuation in levels of sex hormones in the different phases of the estrous cycle seem to affect VP regulation which would have an effect on water loss or retention.

Alcohol seems to also affect the modulation of VP release by sex hormones. Thus, it is important to know sex-specific treatments in situations in which individuals
may be subjected to dehydration or water retention. An individual’s lifestyle and current health status can be affected by sex hormones and complicated by the use of alcohol.

For example, maintaining euhydration may be problematic in post-menopausal women or women who may have inadequate levels of sex hormones. Based on this study, it appears that progesterone may have a stimulatory effect on VP. Thus, if progesterone levels are insufficient, dehydration may result. Also, women athletes may also experience a disruption in sex hormones due to their training. Thus, when exercising, they may be more susceptible to dehydration than men. Therefore, a possible recommendation for females is to hydrate more before exercising depending on the phase of the cycle.

Furthermore, chronic use of a moderate or high dose of alcohol can impair the kidney’s ability to maintain proper fluid balance in the body that can lead to dehydration. Since alcohol affects males and females differently and sex hormones seem to affect VP regulation, it is important to be aware of the differences between males and females in how they regulate water. For example, if alcohol inhibits estradiol and if estradiol inhibits VP, the inhibitory effect of estradiol on VP is removed. Thus, circulating VP levels increase and will act on the kidney to retain water. As a result, individuals who may be on medications may not be able to properly excrete metabolic byproducts that may become toxic.
SUMMARY

There were indeed differences in VP regulation in the different phases of the estrous cycle. In control rats not exposed to alcohol, osmotic stimulation appears to be less sensitive in females in metestrus, anestrus and estrus when compared to males. However, in proestrus, the VP osmotic stimulation curve for females was similar to males.

In males, there was no effect of alcohol on osmotic stimulation of VP. In fact, the response between control and alcohol-fed groups were superimposable. In females however, there were differences depending on estrous cycle phase. In control females, estradiol had a significant negative relationship with pOsm. If estradiol inhibits VP release, as pOsm increases, we should expect to see a decrease in p[Estradiol] so that the negative inhibition on VP is removed.

In control females in proestrus, in which progesterone and estradiol levels are high, the higher levels of progesterone may overcome estradiol’s inhibitory effect on VP. Therefore, progesterone is able to stimulate VP release. Thus, there is a higher VP response in control females in proestrus than in control females in anestrus.

Unlike males where alcohol did not seem to alter osmotic stimulation of VP, in females, alcohol did affect VP osmotic stimulation sensitivity depending on the phase of the cycle which was related to different levels of estrogen and progesterone during those phases.

In estrus and proestrus, alcohol seems to remove the influence of estradiol on VP. Thus, the VP responsiveness to an osmotic stimulus is enhanced. Therefore, with an
increase in VP release, there is a decrease in pituitary VP stores that is eventually seen with high alcohol exposure.

Collectively, the results suggest that alcohol did not seem to affect osmotic stimulation of VP in males. However, females may be more susceptible to alcohol-induced changes in VP regulation of water balance.
FURTHER DIRECTION

The dynamic nature of estradiol and progesterone through the course of the phases of the estrous cycle is complex especially when analyzing effects of estrous cycle phase on VP response to changes in pOsm. For instance, there is only a certain time period within estrus or proestrus where estradiol levels are high. It is possible that some experiments and blood sampling may have occurred when estradiol levels are low and vice versa, which makes it difficult to assess whether or not an increase or decrease in VP response is because of estradiol, progesterone or a combination of both.

Further studies that include elucidating mechanisms of estrogen and progesterone on vasopressin synthesis should be done. To do so, hypothalamic VP, estrogen receptor $\beta/\alpha$, and VP 1 receptor mRNA expression should be analyzed.

Furthermore, a limitation of this study was that p[Estradiol] and p[Progesterone] was not measured in males. Rather, it was assumed that there were no changes in estradiol and progesterone levels. However, future studies could include these measurements along with p[Testosterone] and compare to what was found with the estrous cycle in females. Also, future studies should look at assessing possible estrogen and progesterone effects on osmotic stimulation of VP in males and hormone replacement treated-ovariectomized females. In addition, studies should take a closer look at how the different doses and duration of sex hormone changes affect VP release.
Figure 18. Plasma osmolality in male versus female rats at tissue harvest. No differences were observed in pOsm at harvest between control and alcohol-fed males or females. However, there was a tendency for alcohol-fed males to have a higher pOsm than control males. On the other hand, high alcohol dose-fed females had a significantly lower pOsm than high alcohol dose-fed males. In addition, there was a tendency for moderate alcohol-fed females to have a lower pOsm than moderate alcohol-fed males. These results suggest that alcohol-fed females have a higher compensatory response to alcohol-induced diuresis than males. (Values represent means ± s.e.m., ANOVA, p<0.05, † = significantly different from corresponding male rats)
Figure 19. Plasma osmolality harvest in males versus females by estrous cycle phase at tissue harvest. pOsm at harvest was similar in control and alcohol-fed females in anestrus, proestrus, estrus and metestrus. (Values represent means ± s.e.m.)
Figure 20. Plasma vasopressin in male versus female rats at tissue harvest. pVP at harvest was significantly decreased in high alcohol dose-fed males. However, there were no differences in pVP in females, but there was a tendency for high alcohol-fed females to have a higher pVP than high alcohol-fed males. (Values represent mean ±s.e.m., ANOVA, Student’s t-test, p<0.05, * = significantly different from control males)
Figure 21. Plasma vasopressin in males versus females by estrous cycle phase at tissue harvest. Control and alcohol-fed females in anestrus, proestrus, estrus and metestrus had similar pVP at harvest. (Values represent means ± s.e.m., ANOVA, p<0.05, * = significantly different from control males)
A. Males

**Control** (n=9):
\[ y = 170.80x + 292.69, r=0.63, p=0.07 \]

**Moderate Alcohol** (n=6):
\[ y = 168.64x + 258.09, r=0.70, p=0.12 \]

**High Alcohol** (n=5):
\[ y = 562.54x + 500.98, r=0.48, p=0.42 \]

B. Females

**Control** (n=14):
\[ y = -2.46x + 511.78, r=0.02, p=0.95 \]

**Moderate Alcohol** (n=12):
\[ y = 10.02x + 449.25, r=0.16, p=0.63 \]

**High Alcohol** (n=14):
\[ y = 5.09x + 479.10, r=0.03, p=0.91 \]

**Figure 22. Relationship between pituitary vasopressin and plasma vasopressin in (A) males and (B) females.** There was a tendency for a positive relationship to occur between pituitary VP and pVP in control and moderate alcohol dose-fed male rats (A). There was no relationship between pituitary VP and pVP in high alcohol dose-fed male rats (A) and control and alcohol-fed female rats (B).
A. Males

Control (n=10):
\[ y = 4.85e^{-2}x - 12.23, \quad r=0.25, \quad p=0.24 \]

Moderate Alcohol (n=7):
\[ y = 7.06e^{-2}x - 18.81, \quad r=0.31, \quad p=0.50 \]

High Alcohol (n=5):
\[ y = -5.45e^{-2}x + 16.80, \quad r=0.94, \quad p=0.02 \]

B. Females

Control (n=14):
\[ y = 6.61e^{-2}x - 17.62, \quad r=0.40, \quad p=0.16 \]

Moderate Alcohol (n=12):
\[ y = 4.06e^{-2}x - 9.78, \quad r=0.19, \quad p=0.54 \]

High Alcohol (n=14):
\[ y = 1.80e^{-2}x - 51.17, \quad r=0.49, \quad p=0.08 \]

Figure 23. Relationship between plasma osmolality and plasma vasopressin in (A) males and (B) females. There was no relationship between pOsm and pVP in control and moderate alcohol dose-fed males (A) or females (B). However, a significant negative relationship occurs in high alcohol dose-fed males (A) along with a tendency for a positive relationship to occur between pOsm and pVP in high alcohol dose-fed females (B). The significant negative relationship between pOsm and pVP in high alcohol-fed males suggest that VP release may be suppressed, thus a low pVP is associated with a high pOsm. (Linear regression analysis, p<0.05)
A. Males

Control (n=10):
y = -0.69x + 801.51, r=0.02, 0.96

Moderate Alcohol (n=6):
y = -15.51x + 5312.94, r=0.28, p=0.60

High Alcohol (n=6):
y = -49.33x + 15446.03, r=0.57, p=0.24

B. Females

Control (n=13):
y = 4.03x – 657.86, r=0.16, p=0.59

Moderate Alcohol (n=16):
y = -3.35x + 1461.70, r=0.25, p=0.35

High Alcohol (n=13):
y = 11.168x – 2785.44, r=0.20, p=0.51

Figure 24. Relationship between pituitary vasopressin and plasma osmolality in males (A) and females (B). There was no relationship between pituitary VP content and pOsm in control, moderate alcohol and high alcohol-treated male or female rats.
Figure 25A-C. Vasopressin response to a 5% salt load by estrous cycle phase in control, moderate alcohol and high alcohol-fed females. (A) Control female rats in metestrus, anestrus, proestrus and estrus appear to have a similar VP response to a 5% salt load. (B) Moderate alcohol-fed females in proestrus and estrus showed a more sensitive VP response than females in metestrus (p=0.02). (C) High alcohol-treated rats in metestrus had a more sensitive VP response than rats in anestrus and proestrus (p<0.01) and estrus (p=0.04). These results show that alcohol affects VP response in the different phases of the estrous cycle. (Multiple regression analysis)
LITERATURE CITED


