Sex Differences in the Regulation of Vasopressin and Oxytocin Secretion in Bile Duct-Ligated Rats

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Abstract

Introduction: Hyponatremia due to elevated arginine vasopressin (AVP) secretion increases mortality in liver failure patients. No previous studies have addressed sex differences in hyponatremia in liver failure animal models. **Objective:** This study addressed this gap in our understanding of the potential sex differences in hyponatremia associated with increased AVP secretion. **Methods:** This study tested the role of sex in the development of hyponatremia using adult male, female, and ovariectomized (OVX) female bile duct-ligated (BDL) rats. **Results:** All BDL rats had significantly increased liver to body weight ratios compared to sham controls. Male BDL rats had hyponatremia with significant increases in plasma copeptin and FosB expression in supraoptic AVP neurons compared to male shams (all $p < 0.05$; 5–7). Female BDL rats did not become hyponatremic or demonstrate increased supraoptic AVP neuron activation and copeptin secretion compared to female shams. Plasma oxytocin was significantly

higher in female BDL rats compared to female sham ($p < 0.05$; 6–10). This increase was not observed in male BDL rats. Ovariectomy significantly decreased plasma estradiol in sham rats compared to intact female sham ($p < 0.05$; 6–10). However, circulating estradiol was significantly elevated in OVX BDL rats compared to the OVX and female shams ($p < 0.05$; 6–10). Adrenal estradiol, testosterone, and dehydroepiandrosterone (DHEA) were measured to identify a possible source of circulating estradiol in OVX BDL rats. The OVX BDL rats had significantly increased adrenal estradiol along with significantly decreased adrenal testosterone and DHEA compared to OVX shams (all $p < 0.05$; 6–7). Plasma osmolality, hematocrit, copeptin, and AVP neuron activation were not significantly different between OVX BDL and OVX shams. Plasma oxytocin was significantly higher in OVX BDL rats compared to OVX sham. **Conclusions:** Our results show that unlike male BDL rats, female and OVX BDL rats did not develop hyponatremia, supraoptic AVP neuron activation, or increased copeptin secretion compared to female shams. Adrenal estradiol might have compensated for the lack of ovarian estrogens in OVX BDL rats.

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Introduction

Hyponatremia is a frequent complication observed in liver failure patients [1–3]. Hyponatremia is currently defined as a serum sodium level of less than 135 mEq/L [1]. The primary cause of hyponatremia during liver failure or heart failure is inappropriate arginine vasopressin (AVP) secretion [1, 4]. Portal hypertension and the associated systemic vasodilation lead to activation of neurohumoral mechanisms in order to maintain effective circulating volume, which include increased AVP secretion [5–8]. However, sustained AVP secretion is maintained despite decreased plasma osmolality [4, 9, 10]. Increased AVP secretion contributes to ascites, seizures, pulmonary, and cerebral edema resulting in increased mortality of liver failure patients [1, 4, 11–14]. The management of hyponatremia is challenging as conventional therapy including fluid restriction and loop diuretics are frequently ineffective [2, 4, 8, 13–15]. In addition to our limited understanding of the mechanisms underlying inappropriate AVP secretion, sex differences in the development of hyponatremia during liver failure have not been extensively investigated.

The prevalence of liver disease is higher in men and increases in women after menopause, and one study indicates that estrogen may reduce carbon tetrachloride-induced hepatic fibrosis in female rats [16–18]. This suggests protective effects of estrogen but the role of ovarian hormones in the progression of liver failure, but the regulation of AVP release in this disease modality remains to be understood [19–21]. Systemic oxytocin (OXT) and AVP are primarily produced by magnocellular neurosecretory cells (MNCs) located in the supraoptic nucleus (SON) and the paraventricular nucleus of hypothalamus [22–25]. Several studies have established that AVP MNCs express ER β while the circumventricular organs express ER α and project efferent fibers to the PVN and SON [19, 20, 26–28], indicating the potential for the regulation of MNCs by estrogen. Previous studies have shown that estrogen can inhibit AVP release in vitro [18, 21, 26] and the same studies show that estrogen inhibits OXT release in vitro as well.

In addition to sexual dimorphism in AVP secretion [29], previous studies have shown that the pituitary levels of vasopressin were maximal in proestrus, declined during estrus, and were lowest during metestrus in cycling female rats [30] and the concentration of AVP in PVN and SON was significantly lower in animals during estrus [31]. No previous studies report sex differences in development of hyponatremia and AVP secretion during liver cirrhosis. A better understanding of mechanisms under-

lying sex difference in AVP secretion associated with liver cirrhosis may open new avenues of treatment for hyponatremia and a better understanding of sex differences related to body fluid balance. The aim of this study is to investigate potential sex differences in dilutional hyponatremia and the effect of ovarian hormones on AVP secretion. Chronic bile duct ligation (BDL), a model of liver cirrhosis that develops hyponatremia, was used in this study [10, 32, 33]. This is the first study to address sex differences in hyponatremia in this liver disease model.

Materials and Methods

Animals

Adult male (450–500 g) and female (250–300 g) Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were used in the experiments for this study. Experimental protocols involving animals were approved by the UNT Health Science Center IACUC and conducted in accordance to the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. All animals were housed in a temperature (24–26 °C) and humidity (40–60%) controlled environment with ad libitum access to food (Teklad LM-485 Rat Sterilizable Diet with 0.3% Sodium; Envigo, Somerset, NJ, USA) and water unless otherwise indicated. Rats were individually housed due to the use of survival surgery and individual fluid intake measurements in the protocol. Survival surgeries were conducted using aseptic techniques. All rats were given procaine penicillin G (30,000 U, s.c.) and non-steroidal anti-inflammatory drug, carprofen (Rimadyl, 2 mg p.o.), was given before and after surgery for pain management.

Bilateral Ovariectomy

Prior to ovariectomy, female rats were anaesthetized with isoflurane (2–3%). The left side of the abdomen was shaved, cleaned, and incision was created. Ovary and uterine horn were identified and exposed. The ovary was cut by gently pulling it out by grabbing the surrounding adipose tissue to prevent detachment of a small piece of ovary, which may fall into the abdominal cavity where it may be replanted to carry on its normal function [34]. The fallopian tube was cauterized, and the remaining tissue was put back into the abdominal cavity. The incision was closed with absorbable antibiotic sutures. The procedure was repeated on the right side to remove the other ovary [35]. Two weeks later, the rats received either BDL or sham ligation surgery.

BDL Surgery

The rats were anaesthetized with isoflurane (2–3%). The abdomen was shaved and cleaned. A midline abdominal incision was performed, and the common bile duct was isolated and cauterized between two ligatures as previously described [32, 36]. Visual inspection of ascetic fluid accumulation in the peritoneal cavity was performed daily after surgery. Any rat showing morbidity or ascites that appeared to be greater than 10% of the body weight was euthanized. Sham rats received the same surgical procedure except their bile duct was not cauterized. Liver to body weight ratio was determined for every rat at the end of the study to verify the development of liver cirrhosis.

Metabolic Cage Study

After 2 weeks of recovery from BDL or sham ligation surgery, the rats were moved into metabolic cages (Lab Products, Seaford, DE, USA) to measure food intake, fluid intake, and urine excretion every day. The rats were provided with ad libitum access to food and water for 14 days to record various parameters as previously described [25]. Food intake was measured by filling the food containers up to a predetermined weight of ground chow (Teklad Diets, Madison, WI, USA) in grams. After 24 h, the food was reweighed, and the amount consumed was calculated by the difference. Fluid intake was measured by filling the bottles with a known amount of water in grams and subtracting the remaining weight 24 h later. Mineral oil was added to the urine collection vials to prevent evaporation of water from urine. Urine excretion volume was recorded, and urine samples were collected for measuring daily sodium excretion.

Estrous Cycling in Female Rats

Evaluation of vaginal cytology in the female rats on the day of decapitation was performed based on the relative abundance of each cell type in the smear as previously described [37]. Vaginal smears were collected with micropipette by inserting the tip of the pipette into the vaginal orifice with relatively shallow penetration. Distilled water (0.2–0.25 mL) was used for flushing without markedly distorting the cells to impair identification. The fluid was collected and placed on a glass slide. Smears were evaluated immediately, fresh and unstained. A standard laboratory light microscope (Olympus Corporation, Center Valley, PA, USA) was used to evaluate the sample at a total magnification of $\times 40$. Changes in vaginal epithelial cell structure were documented to establish the stage of the estrous cycle for each female rat.

Plasma Measurements

Rats were anesthetized with inactin (thiobutabarbital sodium salt hydrate; 100 mg/kg i.p.) and trunk blood was collected following decapitation. A 5–6 mL sample of blood was collected in Vacutainer tubes containing the anticoagulant, EDTA (12 mg) and the proteinase inhibitor, aprotinin (0.6 TIU/mL of blood; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). The samples were centrifuged at 1,600 g for 15 min at 4 °C. Plasma was collected from each sample for measuring copeptin (CPP), OXT, and estradiol concentrations. CPP is widely used as biomarker for AVP secretion [38]. CPP and estradiol concentrations were measured in plasma samples by using specific ELISAs according to the manufacturer's instructions (CPP: MBS724037, MyBioSource Inc., San Diego, CA, USA; estradiol: RTC009R, BioVendor Research and Diagnostic Products, Asheville, NC, USA). For OXT measurements, an aliquot of plasma was used to extract peptides by solid-phase extraction using C-18 SEP-Column (Phenomenex, Torrance, CA, USA). The elutes from the column were subjected to vacuum centrifugal concentration and were used to measure circulating OXT with specific ELISA (EK-051-01; Phoenix Pharmaceuticals). Four-parameter logistic analysis (4-PL) was performed to quantify the concentrations of CPP, OXT, and estradiol.

A separate 1- to 2-mL sample of blood was collected in a 2-mL microcentrifuge tube and prepared for measuring plasma osmolality and hematocrit as previously described [25, 39]. Two heparinized capillary tubes (Fisher Scientific, Hampton, NH, USA) were filled with whole blood for measuring hematocrit using Micro-Hematocrit capillary tube reader (Lancer, St. Louis, MO, USA).

The remaining blood sample was centrifuged at 1,600 g for 5–10 min, and plasma was collected to measure plasma osmolality using vapor pressure osmometer (Wescor, Logan, UT, USA).

Immunohistochemistry

Four weeks after BDL or sham surgery, rats were deeply anesthetized with inactin (thiobutabarbital sodium salt hydrate; 100 mg/kg, i.p.), perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. After the perfusion, brains were extracted from the skull and placed in 30% sucrose until dehydrated. The brains were serial sectioned at a thickness of 40 μ m using a cryostat. Sections were processed for FosB and AVP using goat polyclonal FosB (Santa Cruz Biotechnology, Cat# sc-48-G, Santa Cruz, CA, USA; 1:1,000), guinea pig polyclonal Anti-(Arg⁸)-VP (1:1,000; Cat# T-5048, Peninsula Labs, San Carlos, CA, USA). Sections were incubated in primary antibodies for 2 days at 4 °C. Following primary antibody incubation, sections were rinsed with PBS, followed by sequential incubation in respective secondary antibodies against host species. Biotinylated anti-goat secondary (1:1,000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used for FosB to visualize using 3,3'-diaminobenzidine hydrochlorides. The Cy3 fluorophores (Cy3-conjugated anti-guinea pig IgG; 1:10,000; Cat# 706-165-148, Jackson ImmunoResearch Laboratories) was used for AVP. Sections were rinsed again in PBS, mounted on gelatin-coated slides, and coverslipped with Vectashield (Vecta Labs, Burlingame, CA, USA) mounting media. Sections were examined for co-localization of the FosB with AVP immunofluorescence to determine the activation of AVP neurons.

Adrenal Gland Steroid Measurements

Four weeks after BDL or sham surgery, the adrenal glands from ovariectomized (OVX) female rats were collected and flash frozen using dry ice. The frozen (–80 °C) adrenal tissue was thawed, weighed, and homogenized using acetonitrile. The steroids were extracted from tissue samples by organic phase extraction using acetonitrile to solubilize the steroid and hexane to remove fat and lipid that may be present in the sample. The steroid separated in acetonitrile phase was evaporated to dryness using vacuum centrifugal concentrator [40]. The dried extracted samples were diluted in ethanol: assay buffer mixture (1:10). The reconstituted samples were used to measure dehydroepiandrosterone (DHEA), testosterone, and estradiol concentration using specific ELISAs according to the manufacturer's instructions (DHEA: Enzo Life Sciences Inc., Farmingdale, NY, USA; testosterone: BioVendor Research and Diagnostic Products). Four-parameter logistic analysis (4-PL) was performed to quantify the concentrations of DHEA, testosterone, and estradiol.

Experimental Groups and Statistical Analysis

The male, female, and OVX female rats were subdivided into two groups in these experiments: (1) sham ligation and (2) BDL. The female rats used in the study were in different stages of the estrous cycle, which contributed to some of the variability in certain endpoint measurements obtained from the female groups but did not affect the overall outcome of the analyses. The adrenal steroid concentration data from OVX sham and BDL rats was analyzed using Student's *t* test. All other data were analyzed using two-way ANOVA with treatment (sham vs. BDL) as first factor and sex (male vs. female vs. OVX) as second factor followed by Bonferroni post hoc comparisons using Sigma Plot 12.0. Figures

Fig. 1. Effect of BDL in male, female, and OVX rats on liver to body weight ratio (a), plasma osmolality (b), and hematocrit values (c). Data are mean $\pm$ SEM. * $p < 0.05$ vs. respective sham. + $p < 0.05$ vs. respective male group. Groups: male sham ($n = 6$); male BDL ($n = 6$); female sham ($n = 6$); female BDL ($n = 6$); OVX sham ($n = 8$); and OVX BDL ($n = 10-11$).

were assembled using the “magick” package in RStudio. The group sizes were determined by power analysis and effect size calculated from our previously published work [2, 39, 41] and preliminary data using Sigma plot 12.0. The minimal number per group for each experiment was decided to have an appropriately powered study with the effect size of approximately 0.7 (70%).

Results

Liver to Body Weight Ratio

The liver to body weight ratio was measured 4 weeks after BDL/sham ligation surgery as an index of liver cirrhosis [10, 32]. The liver to body weight ratio was significantly increased in male, female, and OVX BDL rats compared to their respective sham-ligated groups. Two-way ANOVA indicated significant difference among the groups ($F(2,45) = 3.36$, $p < 0.05$). Bonferroni post hoc analysis indicates that the ratios were significantly higher in male BDL rats compared to the male sham rats (Bonferroni $t = 5.703$; $p < 0.001$; Fig. 1a). Similar increase was observed in female and OVX BDL rats (female: Bonferroni $t = 11.517$, $p < 0.001$; OVX: Bonferroni $t = 9.995$, $p < 0.001$; Fig. 1a) compared to their respective sham rats. The liver to body weight ratios were not significantly different between male, female, and OVX BDL groups. This data shows that there was no sex difference in the development of liver disease with the BDL.

Plasma Osmolality and Hematocrit Measurements

Plasma osmolality and hematocrit were measured 4 weeks after BDL/sham ligation surgery. The decrease in

plasma osmolality and hematocrit values in BDL rats indicates development of hyponatremia. The statistical analysis using two-way ANOVA showed that the BDL was associated with significant differences between some of the groups (osmolality: $F(1,44) = 8.671$, $p < 0.05$; hematocrit: $F(1,46) = 16.754$, $p < 0.05$). The sex did not significantly affect basal osmolality and hematocrit.

The male BDL rats had significantly lower plasma osmolality and hematocrit values as compared to male sham rats (osmolality: Bonferroni $t = 2.890$; $p < 0.05$; hematocrit: Bonferroni $t = 3.096$; $p < 0.05$; Fig. 1b, c). Similar decreases were not observed in intact female BDL and OVX BDL rats compared to their respective sham groups (Fig. 1b, c). Unlike male BDL rats, female and OVX BDL rats did not develop hyponatremia with liver cirrhosis.

Fluid Intake, Urine Excretion, Food Intake, and Body Weight

Fluid intake, urine excretion, and food intake were measured daily for 14 days beginning 2 weeks after BDL or sham ligation surgery. Two-way ANOVA revealed a significant interaction indicating that fluid intake was affected by BDL and sex (treatment $\times$ sex: $F(39,351) = 18.44$, $p < 0.001$; Fig. 2a). Post hoc comparisons between the factors revealed that BDL significantly increased fluid intake in all the BDL rats compared to their respective sham (male: Bonferroni $t = 4.942$, $p < 0.05$; female: Bonferroni $t = 5.271$, $p < 0.05$; and OVX: Bonferroni $t = 4.942$, $p < 0.05$). Also, fluid intake was significantly higher in both female BDL and OVX BDL rats compared to the male BDL group (female: Bonferroni $t = 5.271$, $p < 0.05$; OVX:

Fig. 2. Effects of BDL in male, female, and OVX rats on average daily fluid intake (**a**) and average daily urine excretion (**b**). Data are mean $\pm$ SEM. * $p < 0.05$ vs. respective sham. + $p < 0.05$ vs. respective male group. Groups: male sham ($n = 6$); male BDL ($n = 6$); female sham ($n = 6$); female BDL ($n = 6$); OVX sham ($n = 6$); and OVX BDL ($n = 10$).

Fig. 3. Immunohistochemistry in the SON. **a** AVP staining in male BDL rats. **b** FosB staining in the same SON sections. **c** Double labelling of FosB and AVP obtained from the merge of **a** and **b**. **d** AVP staining in female BDL rats. **e** FosB staining in female BDL rats. **f** Double labelling of FosB and AVP obtained from the merge of **d** and **e**. **g** AVP staining in OVX BDL rats. **h** FosB staining in

OVX BDL rats. **i** Double labelling of FosB and AVP obtained from the merge of **g** and **h**. Merged images (**c**, **f**, and **i**): pseudocolored yellow indicates AVP and pseudocolored green indicates FosB. Asterisks indicate co-localization of FosB and AVP; arrows indicate non-colocalized neurons.

Fig. 4. Quantification of the average number of neurons positive for FosB and FosB+AVP in male sham, male BDL, female sham, female BDL, OVX sham, and OVX BDL. Data are mean $\pm$ SEM. * $p < 0.05$ vs. respective sham. + $p < 0.05$ vs. respective male group. Groups: male sham ($n = 6$); male BDL ($n = 6$); female sham ($n = 6$); female BDL ($n = 9$); OVX sham ($n = 6$); and OVX BDL ($n = 7$).

Bonferroni $t = 4.942$, $p < 0.05$). A significant interaction between the factors were observed in the volume of urine excreted (treatment $\times$ sex $F(39,351) = 24.419$, $p < 0.05$; Fig. 2b). The average daily urine volume significantly increased in all the BDL groups compared to their respective sham rats (Bonferroni t tests, $p < 0.05$). Post hoc comparisons show that the urine excretion was significantly higher in female and OVX BDL rats compared to the male BDL group. The food intake and body weight were not significantly different among the groups, and increase was not significantly different among the four groups.

FosB and AVP Expression in SON Neurons

Staining for FosB was measured in the SON of male, female, and OVX rats after sham and BDL surgeries (Fig. 3b, e, h). FosB in the SON contributes to changes in gene expression that promotes development of hyponatremia in this animal model [32]. The neurons were double labeled with AVP (Fig. 3a, d, g) to determine if FosB expression was observed in AVP neurons (Fig. 3c, f, i). The numbers of FosB-positive neurons in SON was increased in male, female, and OVX BDL rats compared to their respective shams. In male BDL rats, 61% of the FosB-positive cells were vasopressinergic compared with 14% in sham-ligated controls. FosB staining was evident in AVP neurons in male BDL rats, whereas in female and

OVX BDL rats the FosB was not co-localized in AVP neurons. The 24% of the FosB-positive neurons were also AVP positive in female BDL rats compared with 19% in female sham rats. In OVX BDL rats, 29% of the FosB-positive neurons were colocalized with AVP compared with 18% in female sham rats. The number of FosB- and AVP-positive neurons in female and OVX BDL rats was not significantly higher as compared to the female shams (Fig. 4).

Plasma CPP Concentration

Plasma CPP concentration was measured 4 weeks after BDL or sham ligation surgery. The statistical analysis using two-way ANOVA showed a significant effect of BDL among the groups ($F(1,32) = 13.219$, $p < 0.05$). The sex of the animal significantly affected circulating CPP ($F(2, 32) = 40.489$; $p < 0.05$). The plasma CPP concentration was significantly higher in both female groups compared to the male groups (Bonferroni t tests, all $p < 0.05$). The concentration of CPP in male BDL rats was significantly greater compared to the male sham rats (Bonferroni $t = 3.096$; $p < 0.05$; Fig. 5a). In contrast to the males, BDL was not associated with significantly higher circulating plasma CPP concentrations in female BDL rats compared to the female shams (Fig. 5a).

Circulating CPP was significantly different in OVX sham rats compared to male and female sham rats. Also, the CPP concentration in OVX BDL rats was significantly different compared to female rats but not to male rats. Similar to intact female rats, the OVX BDL rats did not have significantly greater plasma CPP concentration as compared to the OVX sham rats (Bonferroni t tests, all $p < 0.05$; Fig. 5a). These data indicate sex differences in basal circulating CPP concentration along with the difference in their response to BDL.

Plasma OXT Concentration

Plasma samples collected 4 weeks after BDL/sham ligation surgery were used for OXT measurements. The two-way ANOVA analysis shows that plasma OXT was significantly influenced by BDL and the sex of the animal (surgical treatment ($F(1,31) = 44.448$, $p < 0.05$; sex ($F(2, 31) = 3.628$, $p < 0.05$). Plasma OXT concentration was not significantly higher in male BDL rats compared to sham-ligated males, whereas female BDL rats had significantly higher circulating OXT compared to sham-ligated females (Bonferroni $t = 5.242$; $p < 0.001$; Fig. 5b). Also, plasma OXT concentration was significantly higher in female BDL rats compared to the male BDL group (Bonferroni $t = 3.306$; $p < 0.05$).

Fig. 5. Effect of BDL in male, female, and OVX rats on plasma CPP concentration (a), plasma OXT concentration (b), and plasma estradiol (E2) concentration (c). Data are mean $\pm$ SEM. * $p < 0.05$ vs. respective sham. + $p < 0.05$ vs. respective male group. ϕ $p < 0.05$ vs. respective female group. Groups: male sham ($n = 6-7$); male BDL ($n = 6$); female sham ($n = 6-7$); female BDL ($n = 9$); OVX sham ($n = 6-10$); and OVX BDL ($n = 6-10$).

Fig. 6. Effect of BDL and OVX on adrenal glands. Concentrations of adrenal estradiol (a), adrenal testosterone (b), and adrenal DHEA concentration (c). Data are mean $\pm$ SEM. * $p < 0.05$ vs. OVX sham. Groups: OVX sham ($n = 7$) and OVX BDL ($n = 5$).

The circulating OXT concentration was significantly increased in OVX BDL rats compared to OVX sham rats (Bonferroni $t = 4.293$; $p < 0.001$; Fig. 5b). Similar to female rats, plasma OXT concentration in OVX BDL rats was significantly higher compared to the male BDL group (Bonferroni $t = 2.729$; $p < 0.05$). These data show existence of sex differences in OXT secretion in BDL rats but not in the basal circulating concentration.

Plasma Estradiol Concentration

Circulating estradiol was measured in plasma samples collected from rats 4 weeks after BDL/sham ligation surgery. Both the treatment and sex factors had significant effects in all BDL rats compared to the sham groups (treatment: $F(1,37) = 61.421$, $p < 0.05$; sex: $F(1,37) = 9.471$, $p < 0.05$). Plasma estradiol concentration was significant-

ly increased in both male and female BDL rats compared to their respective sham groups (male: Bonferroni $t = 3.320$, $p < 0.05$; female: Bonferroni $t = 4.084$, $p < 0.05$; Fig. 5c). Also, the estradiol concentration was significantly higher in both the female groups compared to their respective male groups (Bonferroni t tests, all $p < 0.05$).

Plasma estradiol concentration was measured in the OVX sham and BDL rats to compare the values with intact female sham and BDL rats and verify the efficiency of ovariectomy procedures. Plasma estradiol was significantly reduced in OVX sham rats compared to the intact female sham rats. This indicates the ovariectomy procedures were successful. However, in OVX rats with BDL, circulating estradiol was not reduced. Bonferroni comparisons indicate that plasma estradiol was significantly decreased with ovariectomy in sham rats compared to the

intact female shams (Bonferroni $t = 2.883$; $p < 0.05$; Fig. 5c). The OVX BDL rats showed significant increase in circulating estradiol concentration compared to the OVX sham rats (Bonferroni $t = 6.561$; $p < 0.05$; Fig. 5c).

Adrenal Steroid Concentration

Adrenal steroids were extracted to measure the concentrations of estradiol, testosterone, and DHEA. BDL was associated with a greater adrenal estradiol concentration in OVX rats compared to the sham rats. This increase in adrenal estradiol concentration might contribute to the observed increase in circulating estradiol. In contrast, the concentration of estradiol precursors, testosterone and DHEA, were lower in the adrenal glands of OVX BDL rats compared to the OVX sham rats. The lower concentration of the substrates of estradiol synthesis with the greater concentrations of estrogen suggests the possibility of increased enzymatic activity.

Adrenal estradiol concentration in OVX BDL rats was significantly higher than the OVX sham rats ($t = -5.632$; d.f. = 11; $p < 0.001$; Fig. 6a). Adrenal testosterone and DHEA were significantly lower in the OVX BDL rats compared to the OVX sham rats (testosterone: $t = 4.685$, d.f. = 11, $p < 0.001$, Fig. 6b; DHEA: $t = 2.783$, d.f. = 11, $p < 0.05$, Fig. 6c). These data suggest increased aromatase transcription or activity in the adrenal glands.

Discussion/Conclusion

It has been suggested that cirrhosis and other types of liver disease causes fibrosis, which blocks portal blood flow due to intrahepatic vasoconstriction leading to portal hypertension. Once portal hypertension develops, it influences extrahepatic vascular beds in the splanchnic and systemic circulations resulting in arterial vasodilation [42]. Peripheral vasodilation causes hypovolemia [43]. The decrease in effective blood volume is reported to cause baroreceptor-mediated non-osmotic stimulation of AVP [1, 6, 11, 15, 23]. In patients with liver cirrhosis, increased AVP secretion leads to hyponatremia, resulting in life-threatening complications such as cerebral and pulmonary edema [2, 14, 44]. Although decreased effective blood volume is the most common explanation for inappropriate AVP release associated with cirrhosis, the mechanism is not known. Other factors such as systemic inflammation could contribute to the changes in body fluid balance related to BDL. Furthermore, no previous studies have reported sex differences in the development of hyponatremia during liver failure.

This study addresses sex differences in development of hyponatremia and AVP secretion in an experimental model of liver cirrhosis. Chronic bile duct-ligated rats were used as an animal model in this study. Cirrhosis was induced in BDL rats by cauterizing the bile duct and obstructing the flow of bile from liver to duodenum. The BDL rats develop jaundice and cirrhosis due to necrosis of hepatocytes caused by bile accumulation in liver. The yellowish pigmentation of skin and plasma, along with an increase in liver to body weight ratio, were used as gross indices of disease progression in this study and previous studies from these groups [10, 36, 39, 45]. Although these indices are related to disease progression in this model, they are not clinically reliable indicators such as bilirubin [33, 46]. Based on these results, it would be worth examining not only bilirubin and possibly other clinical markers of hepatic function.

Plasma CPP measurements were used as an indicator for circulating AVP in our experiments. Plasma CPP is often used as a surrogate marker for AVP as CPP is secreted at equal molar concentration to AVP and has longer half-life compared to AVP. Plasma CPP is known to correlate with AVP measurements at various plasma osmolalities [24, 38, 47].

The results from this study show a distinct sex difference in the development of hyponatremia during liver cirrhosis using the BDL rat model. The stage of the estrous cycle was recorded for intact female rat at the time of sacrifice but did not appear to influence the changes in CPP or OXT measured at the end of the experiment. Unlike male rats, female BDL rats develop liver failure but not hyponatremia. Ovariectomy was performed to understand the influence of circulating estrogen in preventing AVP release in female BDL rats. Plasma estradiol analysis shows that removal of ovaries in BDL rats did not eliminate the estradiol from circulation. We found that adrenal-derived estradiol contributes to the observed increase in circulating plasma estradiol in OVX BDL rats. Similar to the intact female rats, OVX BDL rats developed cirrhosis but did not increase AVP secretion and hyponatremia. The adrenal estradiol might have compensated for the absence of gonadal estradiol preventing OVX BDL rats from becoming hyponatremic.

Plasma estradiol concentrations were higher in all BDL groups compared to their respective shams. Increases in circulating estradiol has been reported in cirrhosis patients [48, 49]. Elevated estradiol secretion might be protective, as hyponatremia did not develop with increased estradiol in our experiments. However, the underlying mechanism leading to an increase in estradiol

and its role in liver failure are not understood. This study attempted to understand the role of estradiol in AVP secretion and hyponatremia in female BDL rats by performing ovariectomy.

Ovariectomy significantly decreased plasma estradiol concentration in sham rats compared to intact female sham ligated rats. However, BDL in OVX rats increased the circulating estradiol concentration. Increased estradiol was observed in intact female and male BDL rats as well. Adrenal glands were collected at the end of protocol to determine a possible source of estradiol in OVX BDL rats. This study is the first to show that BDL increases adrenal estradiol and decreases its precursors in OVX rats compared to the sham rats. This increase in adrenal estradiol concentration might be contributing to the observed increase in circulating estradiol. The decrease in substrates of estradiol synthesis pathway, testosterone and DHEA, and the increase in estradiol suggests the possibility of increased aromatase enzyme activity. Clinical studies show that aromatase inhibitors utilized for treatment of breast cancer induce hepatotoxicity [50, 51]. Aromatase overexpression has been reported in hepatitis and hepatocellular carcinoma, but the role of aromatase in liver disorders remains unclear. Also, liver cirrhosis in BDL rats might impair estradiol metabolism and possibly contribute to the increased estradiol in circulation. At this point, we cannot address the role of estrogens in the absence of hyponatremia in female BDL rats.

The transcription factor FosB creates a heterodimer with Jun to form activator protein-1 (AP-1) complexes. AP-1 complexes bind with the target gene promoter sequence to either stimulate or inhibit transcription. Our previous study indicates that changes in gene expression associated by increased FosB in the SON may be necessary for increased vasopressin secretion [32]. In other systems, FosB has been shown to contribute to neural sensitization and adaptations in neural function [52–54]. In accordance with the previous literature, male BDL rats had greater numbers of FosB positive neurons in the SON and 61% of these were positive for AVP. Both the intact female BDL and OVX BDL rats had significantly more FosB-positive neurons in the SON as compared to sham-ligated controls, but only 24 and 19%, respectively, were AVP positive. As OXT MNCs are in the SON and PVN along with AVP neurons, this suggests that in female rats the FosB staining was in OXT neurons.

A sex difference was observed in OXT secretion in BDL rats, which could be consistent with the FosB staining. Plasma OXT concentration was higher in intact female BDL and OVX BDL rats compared to female sham.

This increase was not observed in male BDL rats. Studies have reported increases in OXT gene expression associated with increases in plasma estradiol [55, 56], but the relationship between estrogens and OXT release is not clear. While estrogen inhibits AVP release in vitro, the same studies show that it inhibits OXT release in vitro as well [18, 21, 26]. Additional studies will be required to determine the mechanisms that increase circulating OXT in female BDL rats.

Both AVP and OXT are metabolized by the liver, and altered hepatic metabolism could contribute to the increases in these hormones that were observed in BDL rats. It was observed that AVP (in this study CPP) was increased in male BDL rats while OXT was increased in BDL female rats. It could be that decreased hepatic metabolism is contributing to increases in AVP and OXT in BDL rats. In male BDL rats, it was previously shown that increased circulating AVP is associated with increased heteronuclear RNA (hnRNA) for AVP in the SON and increased FosB expression. Together, these observations indicate that increased AVP synthesis and release contributes to water retention in male BDL rats. Similar observation about OXT hnRNA have not been made in male BDL rats. It could be that the trend of increased plasma OXT in male BDL rats is due to changes in the OXT metabolism. In female BDL rats, increased plasma OXT was associated with increased FosB staining in OXT neurons of the SON consistent with increased release. Again, in female BDL rats, there was a trend for increased circulating AVP, and this trend could be due to changes in hepatic metabolism. The mechanisms responsible for OXT and AVP secretion in the BDL model are yet to be understood.

Daily fluid intake and urine excretion were recorded for 14 days beginning after 2 weeks of recovery from BDL/sham ligation surgery. Fluid intake and urine excretion were greater in BDL rats despite their low plasma osmolalities, suggesting a possible role for non-osmotic mechanisms in drinking behavior of BDL rats. Previous studies from our laboratory have shown that angiotensin type 1 receptors in the subfornical organ and BDNF in the SON contribute to increased fluid intake in male BDL rats [10, 32, 45, 57]. Sex differences were observed in fluid intake and urine excretion of BDL rats. The intact female and OVX BDL rats appeared to increase their urine output to compensate for increased average daily water intake as compared to male rats, which might contribute to the observed absence of hyponatremia. Previous studies have shown that BDL in male rats significantly decreases sodium excretion compared to sham rats [24]. The effects of BDL on sodium excretion may be different in female

rats because OXT, which was selectively increased in females, can be natriuretic and stimulates atrial natriuretic peptide secretion [58, 59]. It could be that OXT release by the female BDL rats contributed to their ability to maintain a more normal plasma osmolality. However, changes in blood pressure and sodium excretion or the functional consequences of OXT release have not been tested in female rats and will be addressed in future studies.

Angiotensin II (Ang II) is the main hormone of the renin angiotensin system, and it influences AVP release both as a hormone and a putative neurotransmitter [60, 61]. In BDL rats, central infusions of losartan decreased water intake but did not prevent the decrease in plasma osmolality associated with BDL [10]. Blocking angiotensin type 1 receptors in the subfornical organ also reduced water intake associated with BDL but, again, had no effect on plasma osmolality or FosB staining in AVP SON neurons [62]. Based on these results, Ang II appears to be more involved in fluid intake than AVP release in male BDL rats. Estrogen influences the peripheral and central effects of Ang II [63, 64], and androgens influence gene expression in the peripheral renin angiotensin system [65]. The role of estrogen in the sex differences that we have observed in BDL rats is still not clear. It could be that testosterone, a combination of estrogen and testosterone, or a different ovarian hormone is primarily responsible for the differences in body fluid balance or neurohypophyseal hormone release or has differential effect on kidney function in BDL rats.

This study advances current understanding of sex differences in the regulation of AVP and OXT related to disorders of body fluid balance associated with cirrhosis. However, the adrenal production of estradiol appeared to confound the use of OVX to determine the role of this hormone in the failure of the female rats to develop hyponatremia during the course of this experiment. It could be that the additional estradiol produced by the adrenal was sufficient to maintain a protective effect of estrogens. Alternatively, the protective mechanism may be unrelated to estrogens such as a chromosomal or organizational effect. Additional experiments are required to understand the role of estradiol in BDL as well as the sex difference re-

lated to AVP and OXT release in this model. Expression of ER β in MNCs and its possible role in AVP release suggests ER involvement in the prevention of BDL-induced hyponatremia in female rats. Future studies combining ER antagonist in this model of hyponatremia will provide further insight into mechanisms underlying sex differences in hyponatremia related to liver failure.

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Statement of Ethics

Animal protocols were approved by the UNT Health Science Center IACUC and conducted in accordance to the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

Disclosure Statement

The authors declare that they have no competing interests.

Availability of Data and Materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

J.T.C., R.L.C., and K.B. designed experiments; K.B., J.T.L., and M.E.B. performed experiments; K.B., M.E.B., and J.T.C. analyzed data; K.B., R.L.C., and J.T.C. wrote the manuscript. All authors read and approved the final manuscript.

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