

## Angiotensin-(1-7) serves as an aquaretic by increasing water intake and diuresis in association with downregulation of aquaporin-1 during pregnancy in rats

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**Joyner J, Neves LAA, Stovall K, Ferrario CM, Brosnihan KB.** Angiotensin-(1-7) serves as an aquaretic by increasing water intake and diuresis in association with downregulation of aquaporin-1 during pregnancy in rats. *Am J Physiol Regul Integr Comp Physiol* 294: R1073–R1080, 2008. First published January 9, 2007; doi:10.1152/ajpregu.00572.2007.—We previously demonstrated that kidney and urine levels of angiotensin-(1-7) [ANG-(1-7)] were increased in pregnancy. To explore the role of ANG-(1-7) on fluid and electrolyte homeostasis during pregnancy, we evaluated the effect of the ANG-(1-7) antagonist D-alanine-[ANG-(1-7)] (A-779) on kidney function. Virgin and pregnant rats received infusion of vehicle or A-779 ( $48 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) for 8 days by osmotic minipumps. Metabolic studies were done on treatment day 7–8. Virgin and pregnant rats at day 15 and 19 were killed, and blood and kidneys were collected. Kidneys were prepared for Western blot analysis for aquaporin-1 (AQP1) and aquaporin-2. In virgin female rats, A-779 increased urine volume and decreased urinary osmolality and AQP1 with no change in water intake. In 19-day pregnant rats, A-779 significantly decreased water intake and urine volume and increased urinary osmolality and kidney AQP1 expression. Only in late gestation did A-779 treatment decrease the difference between intake and output (balance). A-779 treatment increased plasma vasopressin in late gestation but did not change vasopressin in virgins. In virgin and pregnant animals, A-779 administration had no effect on blood pressure, plasma volume, blood volume, or urinary electrolytes. These results suggest that ANG-(1-7) produces antidiuresis associated with upregulation of AQP1 in virgin rats, whereas ANG-(1-7) produces diuresis in late gestation with downregulation of AQP1. ANG-(1-7) contributes to the enhanced water intake during pregnancy, allowing maintenance of the normal volume-expanded state despite diuresis produced in part by decreased AVP and AQP1.

renin-angiotensin system; pregnancy; angiotensin-(1-7); D-alanine-[angiotensin-(1-7)]; D-alanine; aquaporin; vasopressin

NORMAL PREGNANCY IS A physiological condition characterized by hemodynamic change, including increased cardiac output and hypervolemia, decreased total peripheral resistance, and decreased or normal blood pressure (2). The renin angiotensin system (RAS), which is involved in blood pressure regulation and salt and fluid homeostasis, is markedly increased during pregnancy, as evidenced by increased circulating concentration of angiotensinogen, renin activity, and angiotensin II (ANG II; see Refs. 2, 5, and 20). Also, increased during normal pregnancy is angiotensin-(1-7) [ANG-(1-7)], the active vasodilator of the RAS. Urinary excretion of ANG-(1-7) rises progressively during human pregnancy to levels that are 20-fold higher

by the end of gestation compared with the normal menstrual cycle (35). Urinary ANG-(1-7) concentration is also increased with gestation in rats (22). ANG-(1-7), along with its forming enzyme angiotensin converting enzyme 2, is colocalized in the inner cortex/outer medulla region of kidneys from Sprague-Dawley rats, being present specifically in proximal and distal tubular cells and not in glomeruli. Renal concentration of ANG-(1-7) increases at mid (15 day)- and late (19 day) gestation in rats (17). Currently, the actions of ANG-(1-7) in the kidney of virgin female rats and during pregnancy are not known. However, studies in male animals showed contrasting effects of ANG-(1-7) on the kidney. A natriuretic effect was associated with an increase in urinary volume in kidneys perfused in situ with ANG-(1-7) (8), whereas intrarenal injection of ANG-(1-7) caused increased Na and water excretion in normal volemic male rats with denervated kidneys (14). In water-expanded male rats, Santos et al. (31) showed that ANG-(1-7) produces a potent vasopressin (AVP)-independent antidiuretic effect (30, 31) that can be accounted for by potent actions on proximal tubules (13) and inner medullary collecting ducts (31). The latter actions of ANG-(1-7) were confirmed in normal and hypertensive male rats when chronic blockade of ANG-(1-7) resulted in diuresis and natriuresis (34). Because these animals were normovolemic, it appeared that ANG-(1-7) chronically exerts renal antidiuretic effects regardless of the volume status of the animal. Based on these studies, we hypothesize that ANG-(1-7) will have an antidiuretic effect in virgin and pregnant animals. Therefore, the objective of the current study is to determine the role of ANG-(1-7) on fluid and electrolyte homeostasis by evaluating the effect of chronic administration of the ANG-(1-7) antagonist D-alanine-[ANG-(1-7)] (A-779) on kidney function in virgin and pregnant (15 and 19 day) Sprague-Dawley rats.

### METHODS

**Animals.** Timed-pregnant and virgin female Sprague-Dawley rats between 12 and 14 wk of age were obtained from Harlan Laboratories (Indianapolis, IN) and housed individually under a 12:12-h light-dark cycle in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Day 0 of pregnancy was designated as the day when sperm were found in the vaginal smear. All protocols were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine and are in compliance with National Institutes of Health guidelines.

**Surgical procedures.** Pregnant (day 7 and 11 of gestation) and virgin rats were anesthetized with isoflurane. A 2-ml Alzet ( $5 \mu\text{l/h}$ )

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osmotic pump (Alzet Osmotic Pumps, Cupertino CA) connected to the jugular vein via PE-60 tubing was placed in the subcutaneous tissue, allowing continuous intravenous infusion of vehicle or the ANG-(1-7) antagonist A-779 (Bachem, Torrance CA) at a dose of  $48 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  for 8 days. Animals were individually placed in metabolic cages. The dose was selected based on previous publications (29, 34) that demonstrated that A-779 was a specific antagonist of ANG-(1-7) (32). Twenty-four-hour metabolic studies and water and chow consumption were measured on day 7–8 of treatment in all groups. Virgin, 15-day pregnant, and 19-day pregnant animals were killed by decapitation after 8 days of treatment. Trunk blood was collected in tubes at room temperature containing no inhibitors for measurement of serum creatinine and electrolytes and in chilled tubes containing EDTA for measurement of plasma vasopressin. Kidneys were quickly removed and placed in isolation solution (IS; 10 mM triethanolamine and 250 mM sucrose, pH, 7.6) containing protease inhibitors (100 mM phenylmethylsulfonyl fluoride and 1 mM leupeptin). Half of one kidney was minced into small pieces ( $<5$  mm) and homogenized using a Polytron homogenizer in 4 ml of IS. Samples were placed in  $2\times$  Laemmli buffer and warmed to  $60^\circ\text{C}$  for 5 min to denature protein. Kidney samples were frozen at  $-80^\circ\text{C}$ . Fetuses were counted, and their lengths and weights were measured.

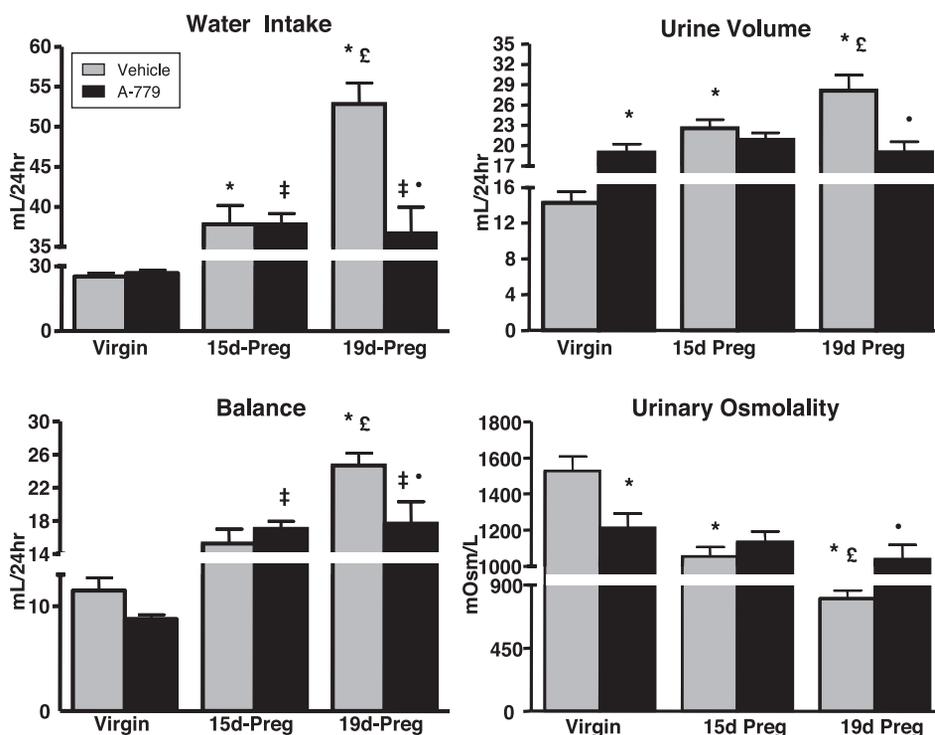
A second set of virgin and 11-day pregnant animals was instrumented with an osmotic pump as previously described. On day 7 of treatment, virgin and 18-day pregnant animals were anesthetized with isoflurane, and an incision was made for insertion of a V3 cannula in the carotid artery and a PE-10 cannula in the femoral vein. Animals were allowed to recover overnight. The next day, virgin and 19-day pregnant animals were anesthetized with isoflurane, and blood pressure was measured continuously for 30 min using the BioPac Acquisition System (BIOPAC System, Goleta, CA). Every 5 min, a 30-s mean arterial pressure was obtained, and results were averaged. Evans blue dye (0.001 g/ml diluted in saline) at a volume of 0.3 and 0.6 ml for virgin and 19-day pregnant rats, respectively, was injected in the femoral vein, and the cannula was flushed with 0.2 ml saline. The exact quantity of dye injected was determined by differential weight of the syringe. After injection (10 min), blood from anesthetized animals was obtained by heart puncture and placed in chilled tubes

containing heparin. An aliquot of blood from each animal was used for hematocrit determination. Plasma was obtained from the remainder of the blood by centrifugation and diluted 1:10 with normal saline. As previously recommended (23), samples were read at 620 and 740 nm in a spectrophotometer, and the 740-nm reading was subtracted from the 620-nm reading to control for variability in optical density caused by plasma turbidity. Blood volume was calculated as plasma volume/(1 – hematocrit).

**Biochemical measurements.** Urinary and serum Na and K concentrations were measured using the Nova Biomedical automated electrolyte analyzer (Nova Biomedical). Freezing-point depression (Precision Systems) was used to determine urinary and serum osmolality. Plasma and urine AVP was measured by radioimmunoassay (ALPCO Diagnostic Kit, Windham, NH). With the use of a colorimetric assay (Sigma Diagnostics, St. Louis, MO), urinary and serum creatinine were assessed. All clearance was calculated as the (urine concentration<sub>x</sub>)  $\times$  urine volume/(serum concentration<sub>x</sub>). Free water clearance was measured as the {urine volume – [(urine osmolality  $\times$  urine volume)/serum osmolality]}.

**Western blot analysis.** Kidney homogenate samples were applied to a 10% Tris-HCl resolving gel (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose polyvinylidene difluoride immunoblot membrane (Bio-Rad). Nonspecific staining was blocked for 1 h at room temperature using milk-TBS-Tween before addition of primary rabbit polyclonal aquaporin (AQP) 1 antibody (provided by Dr. M. Knepper, NIH) (0.01  $\mu\text{g}/\text{ml}$ ) or rabbit polyclonal AQP2 antibody (Chemicon, Temecula, CA) (1:65,000) to the membrane overnight. Secondary anti-rabbit antibody (1:3,000) was applied for 1 h at room temperature. Supersignal West-Pico chemiluminescent substrate (Pierce, Rockford, IL) was added to the membrane for 5 min before exposure to Hyperfilm. Kidney homogenate from a Sprague-Dawley rat that received no treatment was run on each gel to serve as a control for gel-to-gel variability. Background levels were controlled by including a negative control lane (Laemmli buffer alone). Results were analyzed by measurement of band intensity for AQP1 (29 kDa) and AQP2 (28 kDa) using a light box apparatus. Background intensity was subtracted from AQP and  $\beta$ -actin intensities. Results were expressed as a ratio of band intensity to  $\beta$ -actin/control sample to  $\beta$ -actin. In preliminary

Fig. 1. Water intake, urine volume (UV), balance, and urinary osmolality in virgin, 15-day pregnant, and 19-day pregnant Sprague-Dawley rats treated with vehicle (gray bars) or D-alanine-[ANG-(1-7)] (A-779, black bars). During gestation, water intake, UV, and balance were increased, whereas urinary osmolality decreased. In virgin rats, A-779 increased UV and decreased urinary osmolality but did not alter water intake or balance. In 19-day pregnant rats, A-779 decreased water intake, UV, and balance and increased urinary osmolality. Values are expressed as means  $\pm$  SE. Differences between the means were evaluated by 1-way ANOVA with the Newman-Keul's post hoc test throughout gestation in each treatment group. Differences between groups at each time point during pregnancy were compared using an unpaired Student's *t*-test;  $n = 6$ –12 rats/group.  $P < 0.05$  vs. virgin vehicle (\*), vs. 15-day pregnant vehicle (£), vs. virgin A-779 (‡), and vs. 19-day pregnant vehicle (●).



studies, AQP1 and AQP2 were also observed in glycosylated forms (35–50 kDa), but these forms were not visible at the dilutions used in the study where the conditions for the nonglycosylated forms were optimized.

**Statistical analysis.** Comparisons throughout pregnancy in vehicle-treated rats only and A-779-treated rats only during pregnancy were analyzed by one-way ANOVA followed by the Newman Keul's post hoc test. Comparisons at each time point (virgin, 15-day pregnant, and 19-day pregnant) between vehicle and A-779 groups were completed by unpaired Student's *t*-test. A probability of  $<0.05$  was considered statistically significant. All values were expressed as means  $\pm$  SE. The calculation of percent during pregnancy was calculated by assigning the vehicle-treated group (either virgin or appropriate time comparison) as 100% and determining the appropriate percent increase or decrease of the value during pregnancy.

## RESULTS

Table 1 shows the baseline values for maternal body weight and fetal characteristics with and without A-779. Maternal body weight and fetal weight increased throughout gestation. There was no effect of A-779 on maternal or fetal body weight or fetal length or number. A-779 had no effect on weight gain in the virgin animals.

**Water balance.** Pregnant rats in both vehicle- and A-779-treated groups demonstrated increased water consumption at mid- and late gestation (Fig. 1), reaching levels that were 209% and 137% above virgins treated with vehicle and A-779, respectively, at late gestation. Urine volume (Fig. 1) increased throughout gestation ( $y = 0.68x + 13.97$ , urine volume over time) in the vehicle-treated rats, but the magnitude of increase of urine volume was slightly less than water intake, reaching levels that were 198% above virgins at late gestation. The difference between intake and output reflecting balance was increased at the 19th day of normal pregnancy (Fig. 1). A-779 had no effect on water intake in virgins but attenuated the amount of water intake at late gestation. In addition, A-779 treatment significantly increased urine volume in virgin animals and showed no further change in urine volume with pregnancy ( $y = 0.05x + 19.1$ , urine volume over time) (Fig. 1). Urine volume was markedly reduced at the 19th day of gestation by A-779 administration. The difference between intake and output was not altered significantly in virgin animals, whereas it was decreased significantly by A-779 treatment at late gestation (Fig. 1).

**Osmolality.** Urinary osmolality showed a similar yet inverse pattern to urine volume throughout gestation, with decreased urinary osmolality ( $y = -34.95x + 1,535$ , osmolality over time) in vehicle-treated rats and no change in urinary osmolality ( $y = 2.10x + 1,053$ , osmolality over time) in A-779-treated rats (Fig. 1). A-779 treatment decreased urinary osmolality in virgin rats and increased urinary osmolality in 19-day pregnant rats (Fig. 1). Serum osmolality decreased at mid- and late gestation in vehicle-treated animals despite no change in serum osmolality throughout gestation in A-779-treated rats (Fig. 2). Osmolar clearance increased at mid- and late gestation, whereas free water clearance decreased only at midgestation in vehicle-treated rats (Fig. 2). With A-779 administration, osmolar clearance tended to increase, and free water clearance tended to decrease at midgestation, although these values were not statistically significant (Fig. 2). Free water clearance was negative throughout gestation in both groups, demonstrating a state of water retention in both virgin and pregnant animals,

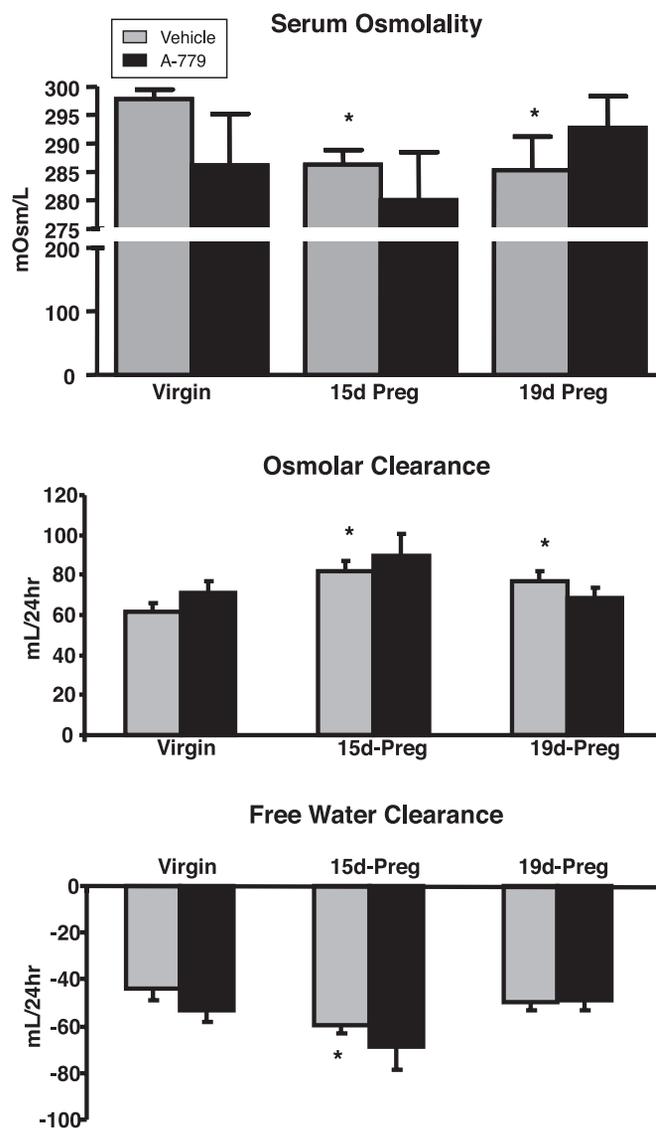


Fig. 2. Serum osmolality, osmolar clearance, and free water clearance in virgin, 15-day pregnant, and 19-day pregnant Sprague-Dawley rats treated with vehicle (gray bars) or A-779 (black bars). Serum osmolality was decreased, and osmolar clearance was increased in mid- and late gestation in vehicle-treated rats. Free water clearance decreased at midgestation in vehicle-treated rats. A-779 administration had no effect in virgin or pregnant rats on serum osmolality, osmolar clearance, or free water clearance. Values are expressed as means  $\pm$  SE. Differences between the means were evaluated as previously described in Fig. 1;  $n = 7$ –15/group. \*  $P < 0.05$  vs. virgin vehicle.

with greater retention in the 15-day pregnant compared with virgin (Fig. 2) rats. Paralleling the loss of the urine volume and urinary osmolality effects with treatment, A-779 administration also resulted in a loss of effects observed in pregnant animals on serum osmolality, osmolar clearance, and free water clearance (Fig. 2).

**Chow.** Vehicle- and A-779-treated rats consumed increased quantities of chow at mid- and late gestation compared with nonpregnant controls (Table 1). A-779 treatment had no effect on chow consumption in virgin animals or pregnant animals (Table 1).

**Electrolytes.** Urinary Na and K and serum K did not change in either vehicle- or A-779-treated animals throughout preg-

Table 1. Maternal body weight, chow consumption, and fetus characteristics in Sprague-Dawley rats treated with vehicle and A-779

	Virgin		15-Day Pregnant		19-Day Pregnant	
	Vehicle	A-779	Vehicle	A-779	Vehicle	A-779
Maternal body wt, g	232±3	229±4	287±6*	290±4‡	328±5*†	313±7‡§
Chow, g/24 h	16.7±0.8	16.1±0.5	20.2±1.2*	20.7±0.5‡	23.3±1.1*†	21.4±1.1‡
Pup no.			13±1	13±1	13±1	13±1
Pup wt, g			0.202±0.001	0.210±0.001	2.335±0.100†	2.332±0.100§
Pup length, cm					2.951±0.010	2.955±0.010

Values are expressed as means ± SE;  $n = 10-12$  pups/group. A-779, D-alanine-[ANG-(1-7)]. A one-way ANOVA with Newman-Keul's post hoc test was completed for maternal body weight. An unpaired Student's  $t$ -test was completed for pup characteristics between 15-day and 19-day pregnant groups. Differences between groups at each time point during pregnancy were compared using an unpaired Student's  $t$ -test.  $P < 0.05$  vs. virgin vehicle (\*), vs. 15-day pregnant vehicle (†), vs. virgin A-779 (‡), and vs. 15-day pregnant A-779 (§).

nancy (Table 2). Serum Na decreased at mid- and late gestation in vehicle-treated rats but remained unchanged throughout gestation with A-779 treatment (Table 2), paralleling the finding with serum osmolality. Na clearance and K clearance were increased at 19 days gestation in vehicle treated rats but did not change throughout gestation in rats treated with A-779 (Table 2). A-779 treatment did not alter urinary and serum Na and K or Na and K clearances in virgin and pregnant rats compared with vehicle-treated controls (Table 2).

**Creatinine.** Serum creatinine decreased throughout gestation in both vehicle- and A-779-treated animals, whereas urinary creatinine decreased only in vehicle-treated rats during pregnancy (Table 2). Creatinine clearance increased at 15 days gestation compared with virgin animals and decreased at day 19 of gestation compared with 15-day pregnant animals (Table 2). There was no difference in serum or urinary creatinine or creatinine clearance in virgin rats treated with A-779 compared with vehicle-treated controls (Table 2). At day 19 of gestation, creatinine clearance was not different, whereas serum creatinine decreased by 126% and urinary creatinine increased by 160% with A-779 treatment compared with 19-day pregnant vehicle controls (Table 2).

**Vasopressin.** Plasma AVP (Fig. 3) was increased at mid- and late gestation in both vehicle- and A-779-treated animals compared with their virgin counterparts. Vehicle-treated animals showed increased urinary AVP at 15 days of pregnancy that

was not maintained during late gestation (Fig. 3). A-779-treated rats demonstrated no difference in urinary AVP throughout pregnancy. In virgin animals, plasma (Fig. 3) and urinary AVP (Fig. 3) were not changed by A-779 treatment. Plasma AVP was increased further in 19-day pregnant rats treated with A-779 above the level of vehicle-treated 19-day pregnant rats, suggesting it as a potential mechanism contributing to decreased urine volume in 19-day pregnant rats treated with A-779. A-779 treatment compared with vehicle treatment did not alter urinary AVP at 19 days of gestation.

**Aquaporin.** Kidney AQP1 was increased at day 19 of gestation in rats treated with A-779 but did not change during pregnancy in vehicle controls (Fig. 4). Kidney AQP2 expression, although demonstrating a tendency to increase at late gestation with both treatments, did not change statistically throughout pregnancy with vehicle or A-779 treatment (Fig. 4). AQP1 was downregulated in virgin animals treated with A-779 compared with controls, whereas AQP2 was not changed (Fig. 4).

**Blood pressure/blood and plasma volume.** During pregnancy, blood pressure decreased, whereas plasma volume and blood volume increased at day 19 of gestation in both vehicle- and A-779-treated animals (Fig. 5). Hematocrit decreased at day 19 of gestation in vehicle-treated rats and showed a similar trend for A-779-treated animals (Fig. 5). A-779 treatment did not alter blood pressure, plasma vol-

Table 2. Urinary and serum electrolytes and creatinine and electrolyte and creatinine clearances in virgin and pregnant (day 15 and 19) rats treated with vehicle or A-779

	Virgin		15-Day Pregnant		19-Day Pregnant	
	Vehicle	A-779	Vehicle	A-779	Vehicle	A-779
Urinary Na, mmol/24 h	1.33±0.06	1.33±0.10	1.41±0.09	1.20±0.10	1.29±0.10	1.15±0.1
Urinary K, mmol/24 h	3.39±0.23	3.23±0.16	3.98±0.40	3.50±0.10	3.74±0.20	3.17±0.20
Urinary creatinine, mg/dl	79±8	63±8	54±7*	58±6	26±3*	42±3§
Serum Na, mmol/l	141±3	135±2	126±2*	141±2.7	123±4*	132±3
Serum K, mmol/l	6.26±0.23	5.64±0.36	5.60±0.19	5.28±0.15	5.59±0.15	5.86±0.19
Serum creatinine, mg/dl	0.56±0.03	0.50±0.03	0.41±0.04*	0.39±0.02‡	0.42±0.04*	0.31±0.03†‡§
Na clearance, $\mu$ l/min	5.44±0.51	6.23±0.68	6.63±0.90	6.55±0.60	8.85±0.76*	7.33±1.32
K clearance, $\mu$ l/min	287±33	378±31	470±44*	437±20	447±37*	402±56
Creatinine clearance, ml/min	1.41±0.21	1.53±0.22	2.09±0.27*	2.22±0.25	1.20±0.11†	1.59±0.26

Values are expressed as mean ± SE. Differences between the means were evaluated by a two-way ANOVA with Bonferroni post hoc test. Differences between the means were evaluated by a one-way ANOVA with Newman-Keuls posthoc test throughout gestation in each treatment group. Differences between groups at each time point during pregnancy were compared using an unpaired student's  $t$ -test.  $n = 5-14$ /group.  $P < 0.05$  vs. virgin vehicle (\*), vs. 15-day pregnant vehicle (†), vs. virgin A-779 (‡), and vs. 19-day pregnant vehicle (§).

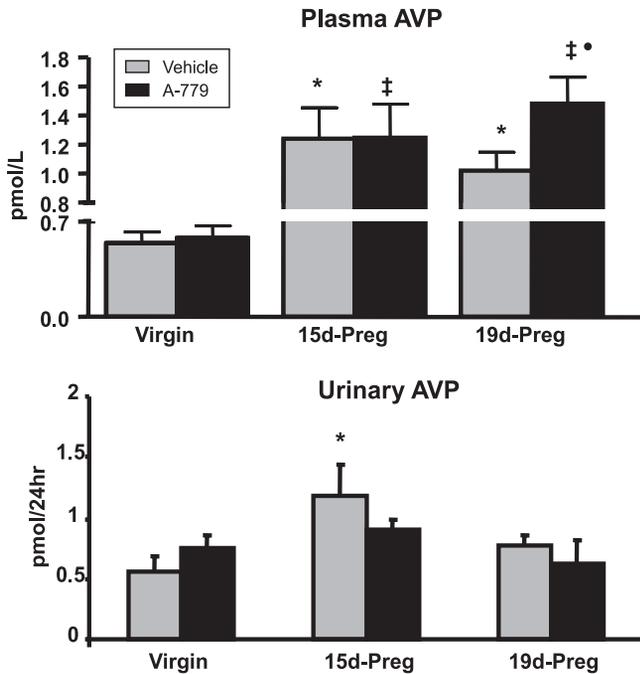


Fig. 3. Plasma AVP and urinary AVP in virgin, 15-day pregnant, and 19-day pregnant Sprague-Dawley rats treated with vehicle (gray bars) or A-779 (black bars). Plasma AVP increased during pregnancy in both vehicle- and A-779-treated rats. Urinary AVP increased in 15-day pregnant vehicle controls and tended to decrease at 19 days gestation, whereas, in A-779-treated rats, AVP did not change throughout gestation. A-779 treatment increased plasma AVP in 19-day pregnant animals. Values are expressed as means  $\pm$  SE. Differences between the means were evaluated as previously described in Fig. 1;  $n = 6-12$ /group.  $P < 0.05$  vs. virgin vehicle (\*), vs. virgin A-779 (‡), and vs. 19-day pregnant vehicle (•).

ume, blood volume, or hematocrit in virgin or 19-day pregnant rats (Fig. 5).

DISCUSSION

The present study is the first demonstration of the effects of endogenous ANG-(1-7) by use of its antagonist A-779 on fluid,

electrolyte, and blood pressure in pregnant and virgin female rats. ANG-(1-7) elicits contrasting effects depending upon the animal's physiological state, producing diuresis associated with decreased urinary osmolality in late gestation and antidiuresis associated with an increase in urinary osmolality in virgin females, with no change in blood pressure in either condition. The studies uncover an unexpected effect of ANG-(1-7) on drinking during pregnancy that was not present in virgin animals, suggesting a central action of ANG-(1-7) during pregnancy. The increased urine volume in late gestation could be mechanistically regulated by ANG-(1-7) in part by the increased water intake, decreased plasma AVP, and downregulation of kidney AQP1. These renal effects occur in the presence of increased renal and urinary excretion of ANG-(1-7) at gestational day 19, as previously reported (22). In virgin animals, the decrease in urinary volume produced by ANG-(1-7) is paralleled by an increase in urinary osmolality. The reabsorption of water by ANG-(1-7) in virgin animals could in part be mediated by an upregulation of the AQP1 water channel.

Pregnancy in humans and rats is associated with hormonal, biochemical, hemodynamic, and renal changes. The entire nature of volume sensing and control in pregnancy appears to differ compared with that seen in the nonpregnant state. As found in this study, urine volume increases throughout gestation, reaching the highest level during late gestation. As expected, urinary osmolality shows an inverse pattern by decreasing during pregnancy. Increased urinary volume excretion appears to be an overall consequence of pregnancy, as demonstrated by metabolic studies in goats, sheep, and Sprague-Dawley and Long-Evans rats (4, 22, 26). Pregnancy shows, however, greater fluid retention, as reflected by an increase in plasma volume, paradoxically to the increased diuresis.

Previously, the actions of ANG-(1-7) on the female kidney or during pregnancy had not been evaluated. Several studies showed that ANG-(1-7) in male Sprague-Dawley rats has diuretic and natriuretic actions that are independent of renal blood flow and glomerular filtration rates (8, 14). In the proximal tubules, ANG-(1-7) inhibits  $Na^+K^+$ -ATPase activ-

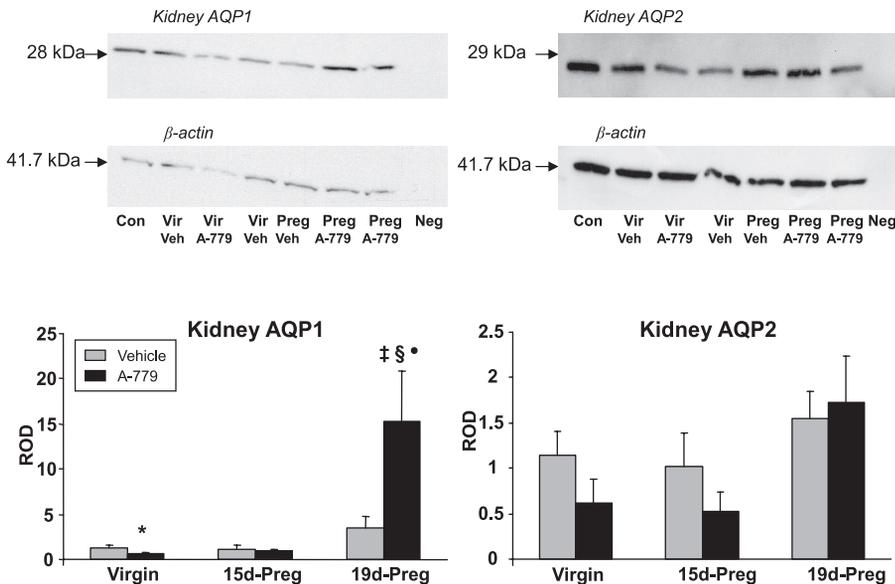


Fig. 4. Representative Western blots show kidney aquaporin (AQP) 1 and AQP2 in virgin (vir) and 19-day pregnant (preg) rats treated with vehicle (veh) or A-779. A positive control (con, kidney homogenate) and a negative control (neg) were run on each gel to control for gel-to-gel variation and background, respectively. Kidney AQP1 and AQP2 expression in virgin, 15-day pregnant, and 19-day pregnant Sprague-Dawley rats treated with vehicle (gray bars) or A-779 (black bars). Kidney AQP1 increased at day 19 of gestation in A-779-treated rats with no changes during pregnancy in AQP2 expression. A-779 treatment caused downregulation of AQP1 in virgin rats and upregulation of AQP1 in 19-day pregnant rats. Values are expressed as means  $\pm$  SE. Differences between the means were evaluated as previously described in Fig. 1;  $n = 6-12$ /group.  $P < 0.05$  vs. virgin (\*), vs. virgin A-779 (‡), vs. 15-day pregnant A-779 (§), vs. 19-day pregnant vehicle (•).

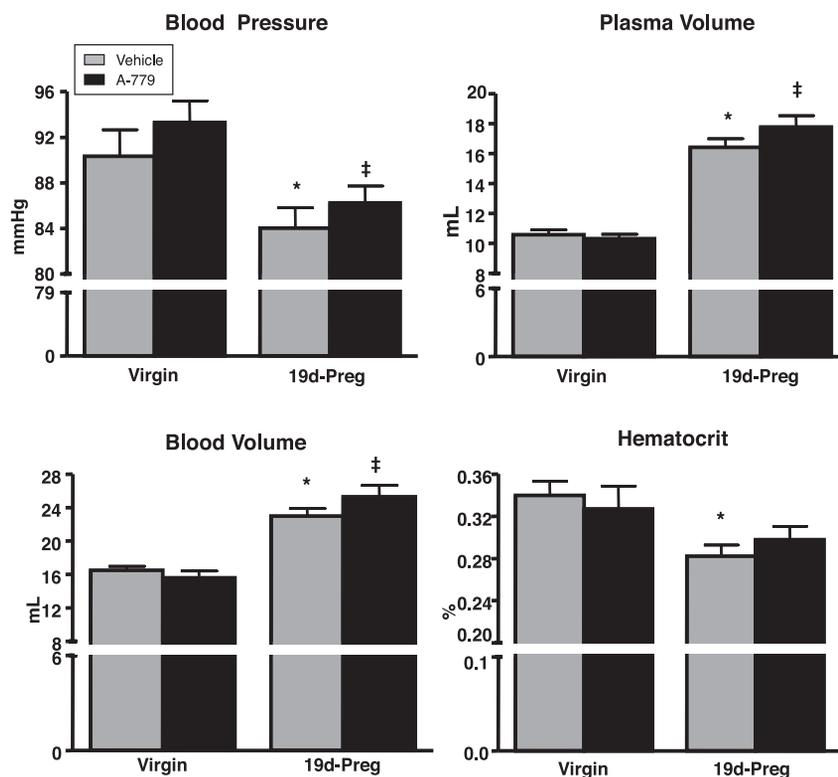


Fig. 5. Blood pressure (BP), plasma volume (PV), blood volume (BV), and hematocrit in virgin, 15-day pregnant, and 19-day pregnant Sprague-Dawley rats treated with vehicle (gray bars) or A-779 (black bars). In 19-day pregnant vehicle- and A-779-treated animals, BP decreased, whereas PV and BV increased. Hematocrit decreased at 19 days gestation in vehicle-treated animals. Drug treatment did not affect BP, PV, BV, or hematocrit at either time point. Values are expressed as means  $\pm$  SE. Differences between the means were evaluated by unpaired Student's *t*-test throughout pregnancy and at each time point;  $n = 11$ – $16$ /group.  $P < 0.05$  vs. virgin vehicle (\*) and vs. virgin A-779 (†) and (‡).

ity and causes a decrease in energy dependent transcellular  $\text{Na}^+$  transport. These diuretic actions of ANG-(1-7) contrast with the observation by Santos and Baracho (30) who demonstrated that ANG-(1-7) is a potent antidiuretic peptide in volume-expanded male rats and by Simoes e Silva et al. (34) who showed in male Wistar and spontaneously hypertensive rats that chronic treatment with A-779 resulted in diuresis and natriuresis. The specificity of the ANG-(1-7) effects was demonstrated in mas receptor knockout mice where the antidiuretic effect of ANG-(1-7) after acute water load was lost (32).

Based on the data of Simoes e Silva et al. (34) and Santos and Baracho (30), we hypothesized that ANG-(1-7) would produce antidiuresis regardless of the volume status of the animal. In agreement with Simoes e Silva et al. (34), ANG-(1-7) did produce antidiuresis in virgin female rats, suggesting that both males and females respond to ANG-(1-7) similarly. However, in pregnant rats, ANG-(1-7) produced diuresis in late gestation, in contrast to the findings in volume-expanded males. These findings indicate that the primary role of ANG-(1-7) shifts from one of fluid conservation in virgin females to fluid loss during pregnancy. Because the urine volume data in both groups were paralleled by appropriate changes in urinary osmolality with no alterations in urinary electrolyte excretion, these findings are consistent with an aquaretic role of ANG-(1-7).

As seen in the present study, gestation has generally been associated with a decrease by 8–10 mosmol/kgH<sub>2</sub>O in serum osmolality accounted for predominately by decreased Na. This reduction in serum Na with pregnancy is confirmed by our study. Also during pregnancy, there is a resetting or lowering of the osmotic threshold for AVP secretion and thirst (4, 9, 19). In pregnancy, the reduction in osmolality is reported to be accompanied by levels of circulating AVP that are unchanged

compared with virgin levels, indicating that the levels of AVP have been reset to a higher than expected level (9). The current data showing increased circulating AVP above virgin levels are consistent with the leftward shift of higher levels of AVP at every level of osmolality but differ from previous reports in that plasma AVP in our study may be associated with an even greater leftward shift in the relationship. The increased circulating AVP in the current study suggests that this antidiuretic hormone compensates for factors that increase diuresis such that normal expansion of plasma volume can occur. Thus blockade of the V<sub>2</sub> receptor antagonist during pregnancy results in further diuresis, indicating an endogenous role of AVP in pregnancy as one of the factors involved in contributing to fluid balance.

The present study also confirms previous reports demonstrating increased water intake (1, 4, 9) and food consumption (16, 21) during gestation. In fact, a decrease in the osmotic threshold for drinking as well as for AVP secretion is necessary to maintain the new steady state of fluid homeostasis. In our study, the 6-ml increase in intake over excretion at the 19th day of gestation compared with nonpregnant controls indicates that the pregnant animals are in positive fluid balance, which agrees with the previous report characterizing water balance in pregnant rats by Atherton et al. (1). In general, ANG-(1-7) is not regarded as a dipsogen (12). In virgin animals, this is confirmed, suggesting that the ANG-(1-7) effect on water balance is restricted to the kidney. In pregnant animals, our study provides evidence consistent with ANG-(1-7) acting as a dipsogen, contributing significantly to the enhanced water intake. Whether this action of ANG-(1-7) on fluid intake has a central component during pregnancy requires further study.

Recently, water channels AQP1 and AQP2 have been shown to be involved in water retention. AQP1, the AVP-independent

water channel, is highly expressed in the descending thin limb and proximal tubule. It has not been assessed during pregnancy. AQP2, an AVP-sensitive collecting duct water channel, has been shown to increase in the kidney medullary papillae during pregnancy. This observation was made in the presence of normal levels of circulating AVP, suggesting a non-AVP dependent mechanism of action during gestation (24) or resetting of the AVP-AQP2 regulation in pregnancy. One would expect that, in the presence of increased AVP, water permeability would increase by upregulating the AQP2 channel. However, in the current study, pregnancy did not alter AQP1 or AQP2 protein expression in whole kidney homogenate despite increased circulating AVP. Our study may differ in regard to the previous study evaluating AQP2 in pregnancy, since we evaluated whole kidney homogenates and not a specific medullary region. Until regional specific evaluation of AQP1 in proximal tubules and descending thin limb and AQP2 in collecting ducts is done during pregnancy, their role cannot be eliminated.

However, in pregnant animals, ANG-(1-7) appears to mediate the diuresis seen during pregnancy by direct action on the kidney by downregulation of the AQP1 water channel. Although aquaretic changes without alterations in collecting duct permeability are not typical, AQP1 knockout mice are unable to concentrate their urine appropriately in response to water deprivation and show decreased transepithelial osmotic water permeability in isolated proximal tubules (33), suggesting that AQP1 can mediate water reabsorption in this part of the nephron. Furthermore, immortalized proximal tubules exposed to ANG-(1-7) showed decreased AQP1 mRNA expression that was blocked by A-779 treatment (18), a finding consistent with the regulation of AQP1 that we found in pregnancy. Although functionally this alteration in AQP1 during pregnancy is more likely to occur in the descending thin limb, the proximal tubular presence of both AQP1 and ANG-(1-7) and downregulation of AQP1 by ANG-(1-7) (18) within proximal tubules indicate that a role of AQP1 within this part of the nephron cannot be ruled out.

Characteristic hemodynamic alterations seen during normal pregnancy include increased cardiac output by 30–40% despite normal or decreased blood pressure and a marked increase in plasma volume by 50% occurring mainly during the last week of gestation (3). The current study demonstrates a 155% increase in plasma volume paralleled by a 79% decrease in hematocrit, a 139% increase in blood volume, and a 6 mmHg decrease in mean arterial pressure at the 19th day of gestation. Treatment with A-779 was associated with no blood pressure effect in pregnant animals, a finding consistent with the study by Sampaio et al. (29) who showed that blood pressure was not changed by ANG-(1-7), A-779, or ANG-(1-7) + A-779 administration in male Wistar rats. In the study by Sampaio et al. (29), the intravenous administration of A-779 resulted in increased total peripheral resistance and decreased cardiac output. The increase in total peripheral resistance with A-779 is predicted based on the studies that show that ANG-(1-7) serves mainly as a vasodilator producing relaxation of canine middle cerebral artery (11), feline systemic vasculature (27), and mesenteric microvessels in rats (10, 25). The vasodilator response of ANG-(1-7) is increased in mesenteric resistance vessels (22) of pregnant rats, all of which is consistent with ANG-(1-7) contributing to the decreased total peripheral

resistance and counterbalancing the actions of ANG II. Characterization of the complete systemic hemodynamic response to A-779 in pregnancy is required to understand how ANG-(1-7) influences the volume and hemodynamic status during pregnancy.

Pregnancy is also characterized by renal hemodynamic changes, including increased glomerular filtration rate (7) and increased proximal tubule length (1). Creatinine clearance, as a measure of glomerular filtration rate, increases during midgestation and falls significantly in late gestation in the present study, confirming previous findings (6). In pregnancy, ANG-(1-7) produced no effect on glomerular filtration rate, as measured by creatinine clearance. This finding agrees with the study of Handa et al. (14) who found that in vivo administration of ANG-(1-7) did not alter blood pressure or renal blood flow. However, it differs from the studies showing that ANG-(1-7) in isolated denervated kidneys can increase glomerular filtration rate (15) and dilate renal afferent arterioles through nitric oxide release (28).

#### PERSPECTIVES AND SIGNIFICANCE

Previous studies from our laboratory showed an increased renal RAS, as shown by increased urinary (22) and renal (17) levels of ANG-(1-7) during late gestation. In the setting of increased renal expression of ANG-(1-7), the current studies demonstrate that endogenous blockade of ANG-(1-7) actions produce contrasting effects on renal fluid balance depending on the whether the animal is pregnant or not. ANG-(1-7) produces antidiuresis in virgin female animals associated with upregulation of AQP1, whereas, in pregnancy, ANG-(1-7) produces diuresis associated with downregulation of AQP1. The studies also show that ANG-(1-7) contributes to the dipsogenic response observed during pregnancy. The water intake is increased to a level that is able to overcome decreased AVP and a downregulation of kidney AQP1 such that animals can maintain an expanded volume state. These studies demonstrate that ANG-(1-7) is an important factor in mediating normal fluid expansion of pregnancy.

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