Effects of hydration in rats and mice with polycystic kidney disease

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Running Title: Effects of hydration on PKD

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K.H., X.W., and V.E.T. co-designed the study and co-wrote/co-illustrated the manuscript. P.C.H. assisted in experimental design. K.H. performed the experimental trial in the Pkd1 R3277C mouse model while X.W. performed the PCK rat studies. H.Y. performed the Pkd2\textsuperscript{WS25\textsuperscript{L}} DDAVP study and aided in animal maintenance/dissection. M.V.I.
assisted in histological evaluation. All authors edited the manuscript and approved the final version.

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Abstract

Vasopressin and V2 receptor signaling promote polycystic kidney disease (PKD) progression, raising the question whether suppression of vasopressin release through enhanced hydration can delay disease advancement. Enhanced hydration by adding 5% glucose to the drinking water has proven protective in a rat model orthologous to ARPKD. We wanted to exclude a glucose effect and explore the influence of enhanced hydration in a mouse model orthologous to ADPKD. PCK rats were assigned to normal (NWI) or to high water intake groups achieved by feeding a hydrated agar diet (HWI-agar) or by adding 5% glucose to the drinking water (HWI-glucose), the latter to recapitulate prior published results. Homozygous Pkd1 R3277C (Pkd1RC/RC) mice were assigned to NWI and HWI-agar groups. To evaluate the effectiveness of HWI, kidney weight and histomorphometry were assessed and urine vasopressin, renal cAMP levels, and phosphodiesterase activities measured. HWI-agar, like HWI-glucose, reduced urine vasopressin, renal cAMP levels and PKD severity in PCK rats, but not in Pkd1RC/RC mice. Compared to rat kidneys, mouse kidneys had higher phosphodiesterase activity and lower cAMP levels, and were less sensitive to the cystogenic effect of DDAVP as was previously shown for Pkd1RC/RC mice and confirmed here in Pkd2WS25/- mice. We conclude that the effect of enhanced hydration in rat and mouse models of PKD differs. More powerful suppression of V2 receptor mediated signaling than achievable by enhanced hydration alone may be necessary to affect the development of PKD in mouse models.
Keywords: Polycystic Kidney Disease, vasopressin, cyclic AMP, cyclic nucleotide phosphodiesterase, hydration.
INTRODUCTION

Polycystic kidney diseases are characterized by the development and growth of cysts arising from renal tubules and associated with enlargement of the kidneys and destruction of the renal parenchyma. Autosomal Dominant Polycystic Kidney Disease (ADPKD), the fourth leading cause of end-stage kidney disease in adults, is caused by mutations to either of two genes, *PKD1* or *PKD2*. Autosomal Recessive Polycystic Kidney Disease (ARPKD), an important cause of ESRD and mortality in infants and children, is caused by mutations to *PKHD1* (9, 23).

A large body of evidence indicates that vasopressin and V2 receptor signaling promote the progression of polycystic kidney disease via cyclic adenosine 3',5'-cyclic monophosphate (cAMP) and protein kinase A (22). Administration of the V2 receptor agonist 1-deamino-8-D-arginine vasopressin (DDAVP) aggravates the disease in orthologous models of ARPKD and PKD1 (10, 28). Genetic elimination of circulating vasopressin markedly inhibits the development of polycystic kidney disease in PCK rats, an effect that was reversed by the administration of DDAVP (28). Treatment with selective V2 receptor antagonists (mozavaptan or tolvaptan) inhibits renal cyst development in cpk mice (8) and in orthologous models of ARPKD (7, 26), PKD1 (10, 14), PKD2 (24, 25), and juvenile nephronophthisis (2, 7). A phase 3 randomized, double blind clinical trial has shown that tolvaptan administered over three years slows kidney growth and renal function decline in patients with ADPKD (20).
The effects of V2 receptor agonists and antagonists on polycystic kidney disease beg the question of whether suppressing vasopressin release through enhanced hydration can also delay disease progression. Indeed, Nagao et al showed that enhanced hydration by adding 5% glucose to the drinking water increased urine output 3.5-fold and slowed the progression of polycystic kidney disease in the PCK rat (16). Recently, the demonstration that phlorizin-induced glycosuria and osmotic diuresis is protective in Han:SPRD cy/+ rats has raised the question of whether the protective effect of 5% glucose in PCK rats could be due to glycosuria and increased urine flow rather than due to suppression of vasopressin release (29). In addition, at this stage no hydration studies have been performed in a mouse model or an orthologous ADPKD model. Therefore, the purpose of this study was to determine whether suppressing vasopressin release through enhanced hydration using a hydrated agar diet would be protective in PCK rats and \( Pkd1^{RC/RC} \) mice.

**MATERIALS AND METHODS**

**Animal models.** PCK rats (30) and C57BL/6 \( Pkd1^{RC/RC} \) (10, 11), \( Pkd2^{WS25/WS25} \) and \( Pkd2^{+/−} \) (33) mice were maintained in the Animal Facilities of the Department of Veterinary Medicine of the Mayo Clinic, Rochester, MN. \( Pkd2^{+/−} \) mice and \( Pkd2^{WS25/WS25} \) mice were crossed to generate double heterozygote \( Pkd2^{WS25/−} \) mice. The Institutional Animal Care and Utilization Committee approved all experimental protocols for the work described within this manuscript.
Increased hydration protocols. PCK rats were randomly assigned at 4 weeks of age to control normal water intake (NWI) or to one of two high water intake (HWI) groups. High water intake was achieved by adding 5% glucose to the drinking water (HWI-glucose) or by feeding a hydrated agar diet (HWI-agar) containing 5 g of powdered food (5053 regrind LabDiet), 50 ml of water and 0.5 g of agar (Sigma-Aldrich) per 100 g of body weight. Rats in the NWI or HWI-glucose groups received the same amounts of powdered food and agar without added water. All the groups had ad lib access to water in bottles. At 6, 8 and 10 weeks of age the rats were placed in metabolic cages to measure urine volume. They were sacrificed at 10 weeks of age for blood and tissue harvest.

Pkd1^{RC/RC} mice were randomly assigned at 4 weeks of age to NWI or HWI-agar groups. Mice in the HWI-agar group were fed 5 g of powdered food (5053 regrind LabDiet) and 0.25g of agar (Sigma-Aldrich) in 25 ml of water per mouse. NWI mice received the same amounts of powered food and agar. Both groups had ad lib access to water and throughout the trial period bottled water intake was measured weekly and the mice were placed in metabolic cages every four weeks to measure urine output. NWI mice drank 4.08 ml/mouse/day whereas HWI mice drank only 0.75 ml/mouse/day from the bottles. HWI animals ate >90% of food/agar mixture daily, providing a ~5.7-fold increase in daily water intake compared to NWI mice. All animals were sacrificed at 24 weeks of age for blood and tissue harvest.
Administration of DDAVP. DDAVP or saline vehicle was administered subcutaneously via osmotic minipumps (Alzet 2004 and 2ML4 for rats and Alzet 1004 for mice, replaced every three weeks) to PCK rats (10 ng/100 g/hour) between 3 and 10 weeks of age (28) and to Pkd1<sup>RC/RC</sup> and Pkd2<sup>WS25/-</sup> mice (30 ng/100 g/hour) between 4 and 12 weeks and between 4 and 16 weeks of age, respectively (10).

Urine vasopressin measurement. Urine vasopressin was measured in PCK rats and Pkd1<sup>RC/RC</sup> mice by using an arg8-Vasopressin EIA kit (ADI900-017, Enzo Life Science) according to manufacturer’s protocol. Urines from HWI-glucose and HWI-agar groups were concentrated using Centrifugal Filter Units (Millipore) before measurement.

Tissue and blood harvest/analysis. At sacrifice, the animals were weighed and anesthetized with ketamine 60 mg/Kg and xylazine 10 mg/Kg, IP (rats) or euthanized by CO<sub>2</sub> exposure (mice). Blood was obtained by cardiac puncture for determination of serum creatinine and BUN levels. The right kidney and part of the liver were placed into pre-weighed vials containing 10% formaldehyde in phosphate buffer (pH 7.4). These tissues were embedded in paraffin for histological studies. The left kidney was immediately frozen in liquid nitrogen for determination of cAMP levels and phosphodiesterase activities.

Histomorphometric analysis. Four μm transverse tissue sections of the kidney, including cortex, medulla, and papilla, and of the liver were stained with hematoxylin-eosin and picrosirius red to measure cystic and fibrotic indices, respectively. Image analysis
procedures were performed with Meta-Morph software (Universal Imaging, West Chester, PA). Digital images were acquired using a light microscope with a high-resolution Nikon Digital camera (Nikon DXM 1200). The observer interactively applied techniques of enhancement for a better definition of interested structures and to exclude fields too damaged to be analyzed. A colored threshold was applied at a level that separated cysts from non-cystic tissue and picrosirius red positive material from background in order to calculate the indices of renal cysts as percentages of total tissue. Histomorphometric analyses were performed blindly, without knowledge of group assignment.

Immunohistology. Immunostaining for proliferating cell nuclear antigen (PCNA) with a monoclonal IgG2a antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to measure epithelial cell proliferation. Fields (400X) of renal medulla and cortex were randomly selected and 1,000 tubular epithelial cell nuclei per tissue section were counted. Proliferative indices were calculated as the percent of cells positive for PCNA.

Western Blot Analysis. Total ERK1/2, phosphorylated ERK1/2, and PCNA were measured by Western blot analysis using anti-ERK1/2, anti-phosphorylated ERK1/2, and anti-PCNA antibodies (Santa Cruz Biotechnology).

Phosphodiesterase activities. Kidneys were homogenized in ice-cold homogenization buffer containing 50 mM Tris pH 7.5, 0.25M sucrose, 5mM MgCl2, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT, and supplemented with a protease inhibitor tablets (Roche).
PDE activities were measured using 1μM cAMP as substrate in buffer containing 50 mM Tris (pH 7.5), 5 mM MgCl2, 4mM 2-mercaptoethanol and 0.1% BSA. 3H-cAMP was included as a tracer for quantitation. Assays were initiated by the addition of substrate and incubated for 10 min at 30°C. The reaction was stopped by incubation for 3 min in boiling-water. Crotalus atrox snake venom was then added and after 15-min incubation at 30°C, hydrolyzed nucleotide were separated using high capacity pre-activated ion exchange resin (FabGennix, Frisco, TX). Slurries were mixed thoroughly and left to stand for 15 min on ice before centrifugation at 12,000g for 3 min. The radioactivity in 150μl aliquots of the resulting supernatants was determined by liquid scintillation counting. To determine the activity in a sample due to a specific PDE, various activators or inhibitors were included in the assay. The PDE activity in aliquots incubated without calcium and with 2 mM EGTA was determined as a basal activity. Calmodulin-stimulated PDE1 activity was determined by subtracting the basal activity from the activity in the presence of 2.01 mM CaCl2 and 10μg/ml calmodulin. PDE3 and PDE4 activities were determined as cAMP-PDE inhibitable by 10μM cilostamide or rolipram, respectively. Hydrolysis of cAMP was linearly proportional to incubation time and enzyme protein. Specific activities were defined as pmoles of cAMP hydrolyzed/minute/mg protein (27).

Renal cAMP content. The kidneys were ground to fine powder under liquid nitrogen in a stainless steel mortar and homogenized in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. After centrifugation at 600g for 10 min, the supernatants were extracted with 3 volumes of water-saturated ether. After drying the aqueous extracts, the
reconstituted samples were processed without acetylation using an enzyme immunoassay kit (Sigma-Aldrich, Inc., St. Louis MO). The results were expressed in pmol/mg of protein.

Statistical analysis. Data are expressed as means ± SD. Comparisons between the groups were performed by the t test.

RESULTS

Effect of enhanced hydration in PCK rats. Enhanced hydration in PCK rats, achieved by adding 5% glucose to the drinking water (HWI-glucose, n= 10 male and 10 female) or by feeding a hydrated 1% agar diet (HWI-agar, n= 10 male and 10 female) between 4 and 10 weeks of age, resulted in significant and similar four-fold increases in urine output compared to rats on normal water intake (NWI, n= 10 male and 10 female). Both treatments were associated with significant overall reductions in urine vasopressin and renal levels of cAMP, with a marked protective effect on the development of polycystic kidney disease, as reflected by lower kidney weights, cystic and fibrotic indices, plasma urea, and (HWI-agar only) plasma creatinine (Table 1 and Figure 1A). Further, ERK phosphorylation and cell proliferation pathways stimulated by vasopressin were significantly downregulated in HWI rats (Figure 2A, C, E). There was no detectable effect on tail cuff blood pressures (Table 1).
Effect of HWI-agar in Pkd1\textsuperscript{RC/RC} mice. As in the PCK rats, urine flow rates were markedly increased in HWI-agar (n= 12 male and 12 female) compared to NWI (n= 12 male and 12 female) Pkd1\textsuperscript{RC/RC} mice (Table 2). This, however, was not accompanied by significant effects on urine vasopressin, renal cAMP, ERK phosphorylation, cell proliferation, kidney weight, cystic and fibrotic indices, and plasma urea or plasma creatinine levels (Table 2 and Figures 1B and 2B, D, F).

Cyclic AMP phosphodiesterase activities and cAMP levels in PCK rats versus Pkd1\textsuperscript{RC/RC} mice. In a previous study we showed that renal phosphodiesterase activities are higher in wild-type and polycystic kidney disease (Pkd2\textsuperscript{WS25/-}) mice compared to wild-type and polycystic kidney disease (PCK) rats (27). Hence, we wondered whether the inability to demonstrate a beneficial effect of enhanced hydration in Pkd1\textsuperscript{RC/RC} mice could be due to increased phosphodiesterase activity and lower basal levels of renal cAMP, which would require a more powerful suppression of V2 receptor-mediated signaling than that achievable by enhanced hydration alone. Indeed, renal phosphodiesterase activities were significantly higher and the cAMP levels were significantly lower in Pkd1\textsuperscript{RC/RC} mice compared to PCK rats (Figure 3).

Effects of DDAVP in Pkd1 and Pkd2 mouse models compared to PCK rats. If the different ability to demonstrate an effect of water hydration in Pkd1\textsuperscript{RC/RC} mice compared to PCK rats was due to higher phosphodiesterase activity, we reasoned that rat models of polycystic kidney disease would be more sensitive than mouse models to the cystogenic effects of the V2 receptor agonist DDAVP. In previous studies where we
ascertained the effect of DDAVP on the development of polycystic kidney disease, this appeared to be larger in PCK rats than in \( Pkd1^{RC/RC} \) mice (10, 28). To confirm this observation in a different mouse model of polycystic kidney disease, we treated \( Pkd2^{WS25/-} \) mice with DDAVP. The combined results show that the administration of DDAVP at 10 ng/100 g/hour increased the kidney/body weight by 256 and 301% in PCK rats, whereas DDAVP at 30 ng/100 g/hour increased kidney/body weight by only 38 and 46% in \( Pkd1^{RC/RC} \) and by 27 and 79% in \( Pkd2^{WS25/-} \) mice, male and female, respectively (Figure 4).

**DISCUSSION**

A large body of evidence indicates that the vasopressin V2 receptor and cAMP signaling play an important role in the pathogenesis of polycystic kidney disease (22). Based on this evidence a recommendation has been made that patients with ADPKD and normal renal function increase the amount of solute-free water drunk evenly throughout the day in order to decrease plasma vasopressin concentrations and mitigate the action of cAMP on renal cysts (19). Although the hypothesis that enhanced hydration may slow the progression of ADPKD has not been adequately tested in patients, Nagao et al showed that enhanced hydration induced by the addition of 5% glucose to the drinking water suppresses vasopressin/V2 receptor signaling, reduces the renal levels of cAMP, and inhibits the development of polycystic kidney disease in the PCK rat, a model orthologous to ARPKD (16). The possibility that the beneficial effect of adding 5% glucose to the drinking water was due to the induction of glycosuria
and increased urine flow rather than to the suppression of vasopressin has been raised by a recent report showing that the development of glycosuria and osmotic diuresis by phlorizin induced inhibition of sodium glucose cotransporters ameliorated polycystic kidney disease in Han:SPRD Cy/+ rats (29). Our study has shown an equally protective effect when hydration was induced by the utilization of a hydrated agar diet. This was associated with reduced urine vasopressin excretion and renal levels of cAMP, making it unlikely that the beneficial effect of adding 5% glucose to the drinking water was due to the induction of glycosuria or due to metabolic effects of the glucose load.

In contrast to its protective effect in the PCK rat model, enhanced hydration did not slow PKD progression in Pkd1RC/RC mice and urine vasopressin levels remained unchanged in the HWI despite their ~ten-fold greater urine output. This lack of urine vasopressin suppression may be consistent with the report of inappropriate expression of vasopressin in the brain of Pkd1+/− mice despite chronic low plasma osmolality(1) or due to higher urine concentrating abilities of mice compared to rats, hence requiring greater water loading to reduce vasopressin levels(34). Further, it should be considered that urine vasopressin levels do not reliably reflect generation or plasma levels of vasopressin(4, 17, 18). For example, diuresis from a large water load may be accompanied by a transient paradoxical rise in vasopressin excretion while plasma osmolality decreases(17). Therefore, we cannot definitely conclude from our results that vasopressin release was not suppressed in the HWI Pkd1RC/RC mice and how this correlated to the lack of disease alleviation.
Additional factors might have contributed to these unexpected results. Since vasopressin V2 receptor antagonists are effective in PCK rats, $Pkd1^{RC/RC}$ (10) and $Pkd2^{WS25/-}$ (24, 25) mice, it seems unlikely that the genes mutated (PCK rat: $Pkhd1$; $Pkd1^{RC/RC}$ mouse: $Pkd1$; $Pkd2^{WS25/-}$: $Pkd2$) account for the different effects of hydration in these animal models.

Instead, differences in intrinsic susceptibility to the development of PKD between rats and mice may be a contributory factor. In this regard, it is telling that the PCK rat exhibits renal cysts at or soon after birth (13), whereas most mouse $Pkhd1$ knockouts only develop renal cysts at an advanced age or not at all (3, 5, 6, 15, 31, 32). The renal phenotype of $Pkd1$ or $Pkd2$ heterozygous knockout mice is similarly normal or very mild; unfortunately, no $Pkd1$ or $Pkd2$ rat model currently exists (21). Further, in previous studies we and others had reported higher PDE activities in kidneys from mice compared to those from rats (12, 27). Since the hydrolytic capacity of PDEs far exceeds the maximum rate of synthesis by adenylyl cyclases, cellular levels of cAMP may be more sensitive to changes in PDEs compared to those in adenylyl cyclases. Therefore, variation in cyclic nucleotide phosphodiesterase activities may determine variable susceptibility to renal cystic disease. The observations in the present study, namely the lower renal cAMP phosphodiesterase activities and higher cAMP levels, and the increased susceptibility to the cystogenic effect of DDAVP in rats compared to mice provide support to this hypothesis.

Acknowledgments
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Disclosures
None

References


Legends

Figure 1. (A) Representative hematoxylin-eosin stained kidney cross-sections from PCK rats drinking water ad lib (NWI) or on enhanced hydration induced by adding 5% glucose to the drinking water (HWI 5% Glucose) or by administration of a hydrated agar diet (HWI 1% Agar) (B) Representative hematoxylin-eosin stained kidney sections from Pkd1RC/RC mice drinking water ad lib (NWI) or on enhanced hydration induced by administration of a hydrated agar diet (HWI 1% Agar).

Figure 2. Western blots of total and phosphorylated ERK1/2, PCNA and GAPDH in whole kidney lysates: ERK1/2 phosphorylation and PCNA expression are significantly reduced in PCK HWI rats (A, E) but not in Pkd1RC/RC HWI mice, compared to their NWI controls (B, F). PCNA immunostaining: Proliferative indices were reduced in HWI PCK rats (C, E) but not in HWI Pkd1RC/RC mice (D, F) compared to NWI controls. N =10 per group (E, PCK rats), n = 12 per group (F, Pkd1RC/RC mice).

Figure 3. Total PDE, PDE1, PDE3 and PDE4 phosphodiesterase activities and cAMP levels measured in whole kidney tissue homogenates from PCK rats and Pkd1RC/RC mice. Phosphodiesterase activity: n=10 per group and model, cAMP levels: N=20 (PCK rats), n=24 (Pkd1RC/RC mice).

Figure 4. Effect of vehicle or DDAVP administration to PCK rats, Pkd1RC/RC and Pkd2WS25/- mice on disease severity (reflected by percent kidney/body weight). The data on the PCK rats and Pkd1RC/RC mice have been previously published(10, 28)
Table 1. Effect of enhanced hydration on the development of polycystic kidney disease in PCK rats

<table>
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<tr>
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<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td>NWI (n=10)</td>
<td>HWI-Agar (n=10)</td>
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<tr>
<td>Body wt (g)</td>
<td>398.7±19.5</td>
<td>363.6±16.7 ‡</td>
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<tr>
<td>Total Kidney wt (g)</td>
<td>5.72±0.84</td>
<td>3.63±0.39 ‡</td>
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<td>Kidney wt (%BW)</td>
<td>1.44±0.24</td>
<td>1.00±0.12 ‡</td>
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<tr>
<td>Kidney cystic index (%)</td>
<td>21.9±6.13</td>
<td>15.1±6.56 *</td>
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<tr>
<td>Kidney fibrotic index (%)</td>
<td>2.24±1.62</td>
<td>0.81±0.47 *</td>
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<tr>
<td>Renal cAMP (pmol/mg protein)</td>
<td>16.3±5.67</td>
<td>12.0±2.33 *</td>
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<td>Renal cAMP (pmol/mg protein)</td>
<td>50.7±3.65</td>
<td>42.7±2.35 ‡</td>
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<td>Plasma Urea (mg/dl)</td>
<td>0.36±0.05</td>
<td>0.31±0.04 *</td>
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<td>24 hrs urine (ml)</td>
<td>15.8±4.08</td>
<td>62.6±13.5 ‡</td>
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<tr>
<td>Urine vasopressin (pg/24 hrs)</td>
<td>830±400</td>
<td>251±61 †</td>
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<td>BP (mmHg)</td>
<td>117.5±6.77</td>
<td>119.5±5.99</td>
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Compared to NWI * p<0.05, † p<0.01, ‡ p<0.001

Table 2. Effect of enhanced hydration on the development of polycystic kidney disease in Pkd1RC/RC mice

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<tr>
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<td>NWI (n=12)</td>
<td>HWI-Agar (n=12)</td>
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<td>Body wt (g)</td>
<td>28.0±2.6</td>
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<td>Kidney cystic index (%)</td>
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<td>15.66±4.90</td>
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<td>Kidney fibrotic index (%)</td>
<td>1.50±1.35</td>
<td>1.58±1.15</td>
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<tr>
<td>Renal cAMP (pmol/mg protein)</td>
<td>6.55±3.73</td>
<td>7.07±3.29</td>
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<td>Plasma Urea (mg/dl)</td>
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<td>24 hrs urine (ml)</td>
<td>0.22±0.03</td>
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<tr>
<td>Urine vasopressin (pg/12 hrs)</td>
<td>172±66</td>
<td>196±113</td>
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Compared to NWI * p<0.05, † p<0.01, ‡ p<0.001


**PCK rat**

- NWI

**Male**

**Female**

**HWI (1% Agar)**

**Male**

**Female**

**HWI (5% Glucose)**

**Male**

**Female**

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**Pkd1^{RC/RC} mouse**

- NWI

**Male**

**Female**

**HWI (1% Agar)**

**Male**

**Female**

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Renal PDE activities

Renal cAMP

pmol cAMP/min/mg protein

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<th>PCK</th>
<th>Pkd1&lt;sup&gt;RC/RC&lt;/sup&gt;</th>
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<td>Total PDE</td>
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<td>PDE1</td>
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<td>PDE3</td>
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<td>5</td>
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<tr>
<td>PDE4</td>
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<td>10</td>
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</table>

* t-test Pkd1<sup>RC/RC</sup> vs PCK *p<0.05; †p<0.001
Male

- PCK
- Pkd2^+/^WS25: %Δ 256
- Pkd1^RC/RC: %Δ 27

Female

- PCK
- Pkd2^+/^WS25: %Δ 301
- Pkd1^RC/RC: %Δ 79

Kidney wt (%BW)

- t-test vs PCK: *p<0.05; †p<0.01; ††p<0.001