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Title Page

Tolvaptan delays the onset of end-stage renal disease in a polycystic kidney disease model by suppressing the increases in kidney volume and renal injury

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Tolvaptan delays the onset of ESRD in PKD animal model

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Abbreviations:

ADPKD, autosomal dominant polycystic kidney disease ; AQP2. aquaporin 2; AVP, arginine vasopressin ; BW, body weight ; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; ERK, extracellular signal-regulated kinase; ESRD, end-stage renal disease; LKV, left kidney volume; NGAL, neutrophil gelatinase-associated lipocalin ; MRI , magnetic resonance imaging ; PCNA, proliferating cell nuclear antigen;

PKD, polycystic kidney disease; Tolvaptan, *N*-{4-[(5*RS*)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine-1-carbonyl]-3-methylphenyl}-2-methylbenzamide ; TEMPO, Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Outcome ; TKV, total kidney volume; V_{1a}, vasopressin V_{1a} receptor subtype; V_2, vasopressin V_2 receptor subtype;

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Abstract

Tolvaptan, a selective vasopressin V_2 receptor antagonist, slows the increase in total kidney volume and the decline in kidney function in patients with the results of the Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Outcome (TEMPO) 3:4 trial. However, it was unclear which dose of tolvaptan was optimal or whether tolvaptan was able to delay progression to end-stage renal disease (ESRD). Here we examined the relationship with aquaresis and the inhibitory effect on cyst development in short-term and mortality as an index of ESRD in long-term treatment of tolvaptan using DBA/2FG-*pcy* mice, an animal model of nephronophthisis. With short-term treatment from 5 to 15weeks of age, tolvaptan (0.01-0.3% via diet) dose-dependently enhanced aquaresis and prevented increases in kidney weight and cyst volume, and was associated with significant reductions in kidney cyclic AMP levels and ERK activity. Maximal effects of tolvaptan on the aquaresis and the prevention of development of polycystic kidney disease (PKD) were obtained at 0.1%. Interestingly, tolvaptan also dose-dependently reduced urinary neutrophil gelatinase-associated lipocalin levels in correlation with the kidney volume. With long-term treatment from 5 to 29 weeks of age, tolvaptan significantly attenuated the increase in kidney volume by up to 50% and reduced urinary albumin excretion. Furthermore, tolvaptan significantly reduced the mortality rate to 20% compared with 60% in the control. These data indicate that tolvaptan may delay the onset of ESRD in PKD by suppressing the increases in kidney volume and renal injury, providing a promising treatment for PKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in *PKD1* or *PKD2* genes, which encode polycystin-1 and polycystin-2 protein, respectively (Lu et al., 1999; Mochizuki et al., 1996; Torres and Harris, 2007a), resulting in the progressive development of kidney cysts, urinary concentration defects, hypertension, and ultimately end-stage renal disease (ESRD) (Grantham, 2008; Torres et al., 2007b). Total kidney volume (TKV) associates with the subsequent change in the glomerular filtration rate (GFR) and development of ESRD, and TKV forecasts the later development of renal insufficiency (Grantham et al., 2006; Chapman et al., 2012). Moreover, the increase in TKV significantly affects patients' quality of life (Rizk et al., 2009). However, there are no therapies which can slow increase in TKV and the deterioration of renal function in ADPKD. The disease affects the kidney structure and function through proliferation and growth of numerous fluid-filled cysts. Intracellular cAMP has been reported to have a central role in cyst growth by stimulating epithelial cell proliferation and trans-epithelial fluid secretion (Wallace, 2011). cAMP stimulates B-Raf, mitogen-associated/extracellular-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK) in ADPKD cells but not normal cells, and this phenotypic switch in the cellular response to cAMP effect on proliferation is due to a function of low intracellular calcium (Yamaguchi et al., 2003 and 2004).

Arginine vasopressin (AVP) is a neuropeptide hormone released from the posterior pituitary that plays a major role in body fluid regulation via V_2-receptors in the renal collecting duct. Therefore, vasopressin V_2-receptor antagonists are used for various disorders of body fluid retention, such as hyponatremia (Schrier et al., 2006) and volume overload with heart failure (Matsuzaki et al., 2011) and cirrhosis (Sakaida et al., 2014).

In the case of patients of ADPKD, AVP stimulates cAMP production in the distal nephron and collecting ducts by acting on vasopressin V_2 receptors and directly promotes cystogenesis (Wang et al., 2008). In the recent experimental and clinical studies, effectiveness of vasopressin V_2-receptor antagonists for treating polycystic kidney disease (PKD) have been reported (Chang and Ong, 2012; Higashihara et al., 2011).

Tolvaptan is a potent, highly selective, and orally effective nonpeptide V_2 receptor antagonist (Kondo et al., 1999) and inhibits vasopressin-mediated water reabsorption in the kidney by competitively blocking the binding of vasopressin to V_2 receptors, resulting in aquaresis without changing total electrolyte excretion (Hirano et al., 2000; Yamamura et al., 1998). In the Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Outcome (TEMPO) 3:4 trial (Torres et al., 2012), treatment with tolvaptan for 36 months slowed the increase in TKV (the primary endpoint) and the decline in kidney function and reduced associated symptoms (the composite secondary endpoint) in patients with ADPKD. However, it is still unclear whether tolvaptan could delay disease progression to ESRD, because the trial was limited to patients with relatively early stage ADPKD (estimated GFR: >60 mL/min/1.73m^2). Efficacy in delaying progression to ESRD could be ascertained by further studies with appropriate patient population, duration and endpoints.

As for the experimental studies, tolvaptan and/or OPC-31260 (Yamamura et al., 1992), a vasopressin V_2-receptor antagonist, have been effective in three animal models orthologous to human autosomal recessive PKD; PCK rats and ADPKD; *Pkd2*$^{ws25/-}$ mice and human adolescent nephronophthisis; CD-1/*pcy* mice (Gattone et al., 2003; Torres et al., 2004; Wang et al., 2005). These studies demonstrated that these V_2 receptor

antagonists had significant effects in reducing cyst formation, but it was unclear whether they could delay progression to ESRD because of their evaluation terms were relatively short. To solve this issue, we conducted the long-term study (29 weeks of age) to investigate the time-course of the effects of tolvaptan on kidney enlargement, measured by magnetic resonance imaging (MRI), and the mortality as an index of ESRD using DBA/2FG-*pcy* (*pcy*) mice which exhibit autosomal recessive cystic kidney disease caused by a missense mutation in *NPHP3,* which is responsible for adolescent nephronophthisis (Nagao et al., 1991; Olbrich et al., 2003; Takahashi et al., 1991). In this *pcy* mice, cysts are derived from distal tubules, and whole nephron segments become diffusely occupied by cysts accompanying disease progression by 30 weeks of age, often with the occurrence of ESRD (Nagao et al., 2012).

In the present investigation, we initially conducted a short-term dose-response study (15 weeks of age) to determine the optimal dose of tolvaptan, especially focusing on the relationship between aquaretic effect and its potency for reduction of cyst enlargement, then we conducted a long-term study to determine whether tolvaptan could delay disease progression to ESRD in *pcy* mice.

Materials and Methods

Animals and treatments

Male *pcy* mice were obtained from a breeding colony maintained at the Laboratory Animal Center, Fujita Health University, and supplied by Kyudo Co. Ltd. Age-matched wild-type DBA/2JJcl mice were from Japan Clea (Tokyo, Japan). The *pcy* mice were allocated to the control and tolvaptan groups with stratification for initial left kidney volume (LKV) measured by MRI and body weight (BW) at 4 weeks of age. Mice were maintained under a 12-hour light/dark cycle and housed at a constant temperature (22 ± 2°C) and humidity (60 ± 10%) in beta-chip-lined plastic cages (W329 ×D379 ×H175 mm), 5 mice per cage, with free access to chow and water. In the short-term study, mice received a standard chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) without (control and DBA mice) or with 0.01, 0.03, 0.1, or 0.3% tolvaptan from 5 to 15 weeks of age. In the long-term study, mice received the standard chow without or with 0.1% tolvaptan from 5 to 29 weeks of age. All surviving mice in these studies were anesthetized by inhalation of 2% isoflurane at the point of necropsy. After the blood sampling all animals were euthanized by exsanguination under isoflurane anesthesia. These studies were conducted in accordance with the Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd.

MRI

MRI was conducted using a 7.0T BioSpec 70/20 USR system (AVANCE III) with a 35-mm diameter quadrature volume coil (Bruker BioSpin MRI GmbH, Ettlingen, Germany). Mice were anesthetized by inhalation of 2-2.5% isoflurane in O_2 and N_2 gas mixture

through a face mask. Isoflurane concentration was adjusted depending on the animal's physiological condition by monitoring the respiratory rate using a small animal monitoring and gating system (SA Instruments, Inc. NY, USA). The fraction of inspired oxygen (FiO_2) used as 30%. Axial T2-weighted scout images were initially obtained using the RARE sequence with the following parameters: TR = 3300 ms, RARE factor = 8, effective TE = 44 ms, matrix size= 256 × 128, slice thickness = 1 mm, number of scans = 1, field of view = 4.0 cm × 4.0 cm. Coronal T2-weighted images were then obtained to measure kidney volume. Coronal T2-weighted images were obtained using the RARE sequence with the following parameters: TR = 2500 ms, RARE factor = 8, effective TE = 44 ms, matrix size = 256 × 256, slice thickness = 0.5 mm, number of scans = 2, field of view = 3.5 cm × 3.5 cm. Coronal T2-weighted images were acquired using a respiratory gating system (SA Instruments, New York, USA) to reduce the artifacts from respiratory motion. LKV was determined by multiplying the appropriate area from coronal images by the thickness of each slice.

Blood parameters

Blood urea nitrogen (BUN) was measured using tail vein blood samples obtained at six times in the long-term treatment study. At necropsy, blood was centrifuged at 2,150 × *g* for 10 min at 4°C to measure serum parameters and serum tolvaptan concentrations. Serum creatinine and BUN concentrations were measured using an auto-analyzer (BiOLis24i; Tokyo Boeki Medisys Inc., Tokyo, Japan). Tolvaptan concentrations were determined by high-performance liquid chromatographic-electrospray ionization tandem mass spectrometry, as previously described (Furukawa et al., 2011).

Urine parameters

Urine was collected using metabolic cages (Sugiyama-Gen Iriki, Tokyo, Japan) and centrifuged at 2,150 × *g* for 10 min at 4°C. Urine osmolality was measured using an osmometer (Model 3900; Advanced Instruments, MA, USA) as the freezing-point depression. Urine albumin and neutrophil gelatinase-associated lipocalin (NGAL) levels were measured using a Mouse Albumin ELISA Kit (Akral-121; Shibayagi, Gunma, Japan) and Mouse Lipocalin-2/NGAL Immunoassay kit (R&D Systems, Minneapolis, MN, USA), respectively. Urine albumin and NGAL were adjusted for urine creatinine concentrations, which were measured using an auto-analyzer.

Kidney cAMP levels

The kidneys were excised, immediately frozen in liquid nitrogen, and homogenized in 0.1 M HCl. After centrifuging the lysates at 2,150 × *g* for 10 min at 4°C, the supernatant was stored at −80°C until use. Kidney cAMP levels were determined using cAMP Complete ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA) and were normalized for the protein concentration measured using a Quick Start protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Kidney mRNA expression

Frozen kidney samples were homogenized and total RNA was extracted using a RNeasy mini kit (Qiagen, Hilden, Germany). Purified total RNA (2 μg) was reverse-transcribed using a High-capacity RNA-to-cDNA kit (Applied Biosystems, Tokyo, Japan). For relative mRNA expression analyses of mouse $V_{1a}R$, mouse V_2R and mouse aquaporin-2,

TaqMan® Real-Time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems) in duplicate and analyzed using Sequence Detection Software version 1.4 (Applied Biosystems). The expression levels of $V_{1a}R$, mouse V_2R and mouse aquaporin-2 were normalized for GAPDH expression.

Immunoblotting

Frozen kidney samples were homogenized in lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1 mM phenylmethanesulfonylfluoride. Homogenates were centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine albumin in Tris-buffered saline plus Tween (TBS-T) overnight at 4°C, incubated for 1 h at room temperature with anti-ERK1/2 antibody (#4695; 1:1000; Cell Signaling Technology) or anti-pERK1/2 antibody (#9101; 1:1000; Cell Signaling Technology) followed by the horseradish peroxidase-conjugated secondary antibodies in TBS-T. Protein bands were visualized using a chemiluminescence system reagent (#34075; Thermo Scientific, Waltham, MA, USA) and quantified using ImageQuant LAS4000 (GE Healthcare, Tokyo, Japan).

Immunohistochemistry

Kidneys were fixed in 10% neutral buffered formalin overnight, embedded in paraffin wax, sectioned (3–4 μm thick), and stained with hematoxylin-eosin, Masson trichrome, and proliferating cell nuclear antigen (PCNA). Cyst area (hematoxylin-eosin stained slides) and the fibrosis area (Masson trichrome stained slides) were measured using WinROOF software (Mitani, Tokyo, Japan). Cyst volume (% body weight) and fibrosis

volume (% body weight) were calculated using the following formula: cyst volume (% body weight) = cyst area / kidney cross-section area × kidney weight / body weight × 100. Fibrosis volume (% body weight) = fibrosis area / kidney cross-section area × kidney weight / body weight × 100. All PCNA-positive cells were counted in the tissue cross-sections.

Statistical analysis

All results are expressed as mean ± standard error. For dose-dependent studies, statistical comparisons between groups were initially analyzed by linear regression, and statistical significance was determined using the upper or lower Williams' test or two-tailed Dunnett's test. For long-term studies, variables were compared at each time using repeated-measures analysis of variance followed by two-tailed *t* tests. Survival rates were compared using the log-rank test. Differences of $P<0.05$ were considered statistically significant.

Results

Short-term Treatment Study

Dose-Dependent Effect of Tolvaptan on Aquaresis and Kidney Cyst Enlargement in pcy mice

To confirm the dose-dependent aquaretic effect, we treated *pcy* mice with tolvaptan at 0.01, 0.03, 0.1, and 0.3% via diet from 5 to 15 weeks of age. Serum tolvaptan concentrations at 15 weeks of age ranged from 7.2 ng/mL (0.01%) to 297.9 ng/mL (0.3%), within clinical ranges (Shoaf et al., 2007) (Table 1). Urine volume at 14 weeks of age was 2-fold higher and urine osmolality was substantially lower in control *pcy* mice than in DBA mice ($P<0.01$; Fig. 1). Tolvaptan significantly increased urine volume ($P<0.01$) with a maximal effect at 0.1%. Tolvaptan significantly lowered urine osmolality from 1117 ± 64 (at control) to 609 ± 11 mOsm/kg (at 0.3%; $p<0.01$) in a dose-dependent manner, with a plateau at doses ≥0.1%.

In the short-term study, only 0.3% tolvaptan caused slight decreases in BW (Table 1). The kidney weight/BW ratio (KW/BW) was significantly greater in the control *pcy* mice than in DBA mice (6.60 ± 0.49% *vs*. 1.51 ± 0.04%). Tolvaptan significantly and dose-dependently reduced the KW/BW compared with the control group (Fig. 2A). Fig. 2C–H shows representative kidney sections from control, tolvaptan-treated *pcy*, and normal DBA mice. Kidneys in the control *pcy* mice had numerous cysts and exhibited kidney expansion, accompanying disease progression. In sections from the tolvaptan-treated groups, kidney expansion, cyst enlargement, and the loss of normal renal parenchyma were markedly inhibited. Tolvaptan significantly reduced cyst volume (Fig. 2B), fibrosis volume, and the number of PCNA-positive cells (Table 1). These

histopathological analyses clearly indicated that tolvaptan was effective in halting the progression of cyst enlargement and fibrotic and proliferative deterioration in the kidney.

In the short-term study, there were no significant differences in serum creatinine or BUN at 15 weeks of age (Table 1).

Dose-dependent Effect of Tolvaptan on Kidney cAMP Pathway in pcy mice

Because cAMP levels is thought to be involved in cyst growth in PKD via ERK signaling (Wallace et al., 2011; Wang et al., 2008; Yamaguchi et al., 2003), we measured kidney cAMP content and ERK1/2 activity in normal DBA and *pcy* mice. Kidney cAMP levels and ERK1/2 activity were significantly greater in control *pcy* mice than in normal DBA mice (data not shown), and were significantly reduced by tolvaptan in a dose-dependent manner ($P<0.01$) (Fig. 3A–C). Aquaporin-2 mRNA expression was also greater in control *pcy* mice than in DBA mice, and tolvaptan reduced its expression accompanying the aquaretic effect (Fig. 3D). Although $V_{1a}R$ and V_2R mRNA expression levels were slightly increased in control *pcy* mice, their levels were not affected by tolvaptan (Fig. 3E and F).

Effect of Tolvaptan on Urine Neutrophil Gelatinase-associated Lipocalin Levels in pcy mice

To search for biomarkers associated with cyst progression in the early stage of the disease, we tested urine NGAL and urine albumin excretion collected at 14 weeks of age. Urine NGAL levels were about 70 times higher in control *pcy* mice than in DBA mice ($P<0.01$), and were significantly reduced by tolvaptan (Fig. 4A), and the urine NGAL levels in each mouse were correlated well with KW/BW measured at 15 weeks of age

(Fig. 4B). There were no significant differences in urine albumin among the study groups (Table 1).

Long-term Treatment Study

Effect of Tolvaptan on Left Kidney Volume Enlargement and Survival in pcy mice

In the long-term study, 0.1% tolvaptan was selected as an optimal dose because it showed submaximal effects and because the higher dose (0.3%) caused a slight reduction in BW. Long-term treatment with 0.1% tolvaptan did not affect BW in *pcy* mice (data not shown).

To determine the time-course of change in kidney volume, LKV was monitored by MRI from 4 to 28 weeks of age (Fig. 5). In control *pcy* mice, LKV increased linearly up to 16 weeks of age, reaching maximum of 1113 ± 54 mm^3, which was about three times greater than that of DBA mice. LKV remained constant beyond 16 weeks of age in *pcy* mice. As shown in Fig. 5, tolvaptan suppressed LKV enlargement soon after starting treatment, with an inhibition rate as high as 50% compared with the control *pcy* mice ($P<0.01$).

For the long-term treatment study, we evaluated the effects of tolvaptan on survival rates in *pcy* mice. Nine out of 15 control *pcy* mice died between 20 and 29 weeks of age, corresponding to a mortality rate of 60% at 29 weeks of age (Fig. 6). By contrast, only 3/15 tolvaptan-treated mice died, corresponding to a mortality rate of 20% at 29 weeks of age, which was significantly lower than that in the control group ($P=0.0418$).

Effect of Tolvaptan on Renal Function in pcy mice

In the long-term study, the urine volume gradually increased and urine osmolality decreased with age, showing urinary concentration defects in the control *pcy* mice. Tolvaptan significantly increased urine volume and decreased urine osmolality at each time point compared with the control mice (Fig. 7A and B). Urine albumin excretion also increased gradually and significantly in control *pcy* mice compared with DBA mice ($P<0.01$) and reached a peak excretion rate of 2 mg/20 h. Tolvaptan significantly inhibited the increase in urine albumin excretion at 19and 23weeks of age ($P<0.05$ and $P<0.01$, respectively) (Fig. 7C). BUN increased gradually in *pcy* mice, accompanying the progression of kidney disease. Tolvaptan slightly but not significantly decreased BUN at 27 weeks of age (Fig. 7D).

Discussion

ADPKD lead to progressive destruction of normal kidney structure leading to ESRD, which is defined as the requirement of dialysis or transplantation. An estimated 45% to 70% of ADPKD patients progress to ESRD by age 65 (Mitcheson et al., 1977), and ADPKD shows high phenotypic variability and ESRD occurs 20 years earlier in patients with *PKD1* than those with *PKD2* (Hateboer et al., 1999; Cornec-Le et al., 2013). Substantial clinical reports showed that higher rates of kidney enlargement were associated with a more rapid decrease in renal function. These indicate that suppression of the increase in kidney volume may delay the onset of ESRD.

In a recent studies, there have been reported that upregulation of cAMP by vasopressin play a central role in the pathophysiology of ADPKD and the inhibition of cystogenesis by V_2 receptor antagonists attenuate the disease progression in three different PKD animal models. However, these studies were conducted in the early stage of disease, when the renal function was not deteriorated yet, therefore the association of the progression of PKD and mortality has remained unknown. Because of their models showing slow progressive renal cystic disease, their life span are over 1 year (Nagao et al., 2012; Doctor et al., 2010).

In the present study, we tested the efficacy of tolvaptan in DBA/2FG-*pcy* mice, a spontaneously occurring PKD model. The PKD mutation first occurred in the KK mouse strain and the mutant locus was transferred to the DBA/2J strain (Takahashi et al., 1991). Enlarged renal tubules and growing cysts are observed from 3 weeks of age, and histological findings in advanced ADPKD by 30 weeks of age, often with the occurrence of ESRD. The life span of *pcy* mice is approximately 35 weeks of age.

The TEMPO trial was a pivotal trial and provided clear evidence that tolvaptan delayed the progression of ADPKD (Torres et al., 2012). However, the study had some limitations because of its study design and the difficulty in monitoring the efficacy of tolvaptan in this slowly progressive disease. First, there was no dose-related evidence of tolvaptan against TKV and aquaretic action because a titration regimen consisting of split doses (45/15 mg to 90/30 mg) was used in the TEMPO trial and 55% of participants tolerated the maximum dose (90/30 mg) for 3 years. Therefore, the relationship between the TKV inhibition and aquaresis was not fully elucidated, although polyuria was the most common adverse event in the TEMPO trial. Second, the clinical trial was performed in a patient population with relatively early stage and preserved kidney function. Therefore, it is not clear whether tolvaptan can prevent the progression to ESRD in ADPKD patients.

In the present study, we examined (1) whether the aquaretic effects and inhibitory effects on cyst development are dose-dependent, and (2) whether tolvaptan can prevent kidney dysfunction and improve mortality at the end stage of PKD in a rapidly progressive animal model.

To evaluate the relationship between aquaresis and inhibitory effects on cyst development, we treated *pcy* mice with four doses of tolvaptan in a short-term study. As expected, the serum tolvaptan concentrations increased in a dose-dependent manner, and the concentrations at 15 weeks of age were comparable with those in the TEMPO study. These results confirmed that the mice were exposed to adequate concentrations of tolvaptan and allowed us to evaluate its dose-dependent effects. In fact, tolvaptan dose-dependently increased urine volume and decreased urine osmolality, indicating that the doses were reasonable to observe the *in vivo* V_2R antagonistic effects of tolvaptan in *pcy*

mice. The aquaretic effects of tolvaptan were saturated at the dose of 0.1% in *pcy* mice. Tolvaptan also dose-dependently inhibited the development of PKD, decreasing KW and kidney cyst volume. The maximum inhibitory effect on KW/BW and the maximum aquaretic effect were observed at 0.1%. Elevated kidney cAMP levels were reported in animal models of PKD, including *pcy* mice, *jck* mice, PCK rats, and *Pkd2*$^{WS25/-}$ mice (Masyuk et al., 2013; Smith et al., 2006; Yamaguchi et al., 1997). Wallace *et al*. reported that AVP stimulation increased intracellular cAMP and activated ERK signaling, and that tolvaptan inhibited AVP-stimulated *in vitro* cyst growth and transepithelial fluid chloride secretion in ADPKD cells (Reif et al., 2011). Tolvaptan dose-dependently reduced kidney cAMP levels and reduced ERK activation, and completely blocked at 0.1%, which achieved a plasma tolvaptan concentration similar to those used in prior *in vitro* studies. The dose-dependent decrease in cAMP was correlated with aquaporin-2 expression and the aquaretic effects of tolvaptan. Treatment with tolvaptan for 10 weeks also decreased KW in a dose-dependent manner. Because the maximum inhibitory effect of tolvaptan on KW/BW and aquaretic effect were observed at 0.1%, we considered that tolvaptan exerted both effects by suppressing cAMP signaling via V_2R antagonism, and support the idea that blocking AVP may delay ADPKD progression. The most common adverse events in the tolvaptan group in the TEMPO trial were predominantly from polyuria. We confirmed suppression of cyst volume and KW/BW increases by tolvaptan treatment, even at lower doses in which its aquaretic effects were not maximal. Therefore, finding the optimal tolvaptan dose balanced with efficacy and polyuria in PKD treatment is important for improving the quality of life of PKD patients.

NGAL is an established biomarker of acute kidney injury (Koyner et al., 2012; Mori et al., 2007) and is increased in human ADPKD-derived cell lines and tissue

(Husson et al., 2004), and in rodent models (Mrug et al., 2008; Riera et al., 2006). It was reported that NGAL levels reflect damage in glomeruli, proximal tubules, and distal nephrons (Kuwabara et al., 2009). In the short-term study, urinary NGAL levels increased significantly at 14 weeks of age in control *pcy* mice, and were reduced by tolvaptan in a dose-dependent manner. Interestingly, the urinary NGAL levels in each mouse were correlated with KW. It was also reported that NGAL is abundant in the cyst fluid of ADPKD patients (Parikh et al., 2012; Park et al., 2012). Therefore, elevated NGAL might be a common feature of PKD in human patients and rodent models. These data also suggest that NGAL might be a sensitive biomarker for changes in KV in ADPKD, and could be used to evaluate drug efficacy.

Recent studies have demonstrated the potential of using MRI to monitor the development of kidney cysts in animal models of cystic diseases (Reichardt et al., 2009). The surrounding parenchyma and cysts can be clearly distinguished by the differences in signal intensities between parenchyma and cysts in T2-weighted images. In the long-term study, we used 0.1% of tolvaptan which exerted maximal inhibitory effects on PKD in a short-term study and evaluated the time-course of changes in LKV and the effects of tolvaptan on LKV. We found that LKV and cystic area (data not shown) increased in a linear manner up to 16 weeks of age in control *pcy* mice. Tolvaptan significantly suppressed this increase soon after starting its administration, and the effectiveness continued thereafter. In the 3-year TEMPO trial, TKV increased by 2.8% per year in the tolvaptan group versus 5.5% in the placebo group. Therefore, tolvaptan reduced the increase in KV by almost 50%. The reduced increase in KV in the tolvaptan group was apparent in the first year of treatment and was sustained for 3 years. Consistent results were obtained in the present study as 0.1% tolvaptan reduced the increase in KV during

the first 12 weeks of treatment, which was maintained for the remainder of the study. Tolvaptan also significantly improved the survival rate as an index of ESRD. These results suggest that the sustained inhibition of kidney cyst enlargement by tolvaptan not only suppress the progression of PKD but also delay ESRD.

To elucidate whether tolvaptan improved biomarkers at the late stage of the disease, we measured urine albumin, serum creatinine, and BUN, and found that tolvaptan delayed the increases in urine albumin over 7–27 weeks of age. Vasopressin plays a critical role in regulating urine albumin excretion in normal rats, in healthy humans, and in diabetic albuminuria via V_2R (Bardoux et al., 2003a; Bardoux et al., 2003b). Tolvaptan may reduce urine albumin by slowing PKD progression and direct V_2 antagonism. Tolvaptan did not significantly improve serum creatinine (control (n=6) *vs*. Tolvaptan-treated (n=12) : 0.96 ± 0.20 *vs*. 1.08 ± 0.11) or BUN levels at 29 weeks of age, which may be due to the high mortality in control mice.

In conclusion, tolvaptan dose-dependently reduced cystogenesis in parallel with its aquaretic effect by reducing kidney cAMP signaling pathways in *pcy* mice. We also observed that long-term treatment with tolvaptan at the maximal dose was highly effective in inhibiting cystogenesis, preventing renal dysfunction, and improving mortality rates in *pcy* mice. Our data suggest that preventing cyst formation and expansion in the early stages of the disease can delay disease progression and should maintain kidney function in ADPKD, which may delay the progression to ESRD. These data also strongly indicate that vasopressin-induced cAMP elevation plays a pivotal role in the pathogenesis of PKD, and that tolvaptan ameliorates the progression by V_2R antagonism.

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Authorship Contributions

Participated in research design: Aihara, Fujiki, Nagano, and Yamamura.

Conducted experiments:

Aihara, Fujiki, Mizuguchi, Hattori, Ohmoto, and Ishikawa.

Contributed new reagents or analytic tools: Ishikawa.

Performed data analysis:

Aihara, Fujiki, Mizuguchi, Hattori, Ohmoto, and Ishikawa.

Wrote or contributed to the writing of the manuscript:

Aihara, Nagano, Fujiki, and Yamamura.

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Footnotes

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Figure Legends

Figure 1. Effects of tolvaptan on urine volume (A) and urine osmolality (B) in a short-term treatment study. DBA mice (n=5) were untreated and DBA/2FG-*pcy* mice were treated without (n=13) or with 0.01% (n=14), 0.03% (n=14), 0.1% (n=11), or 0.3% (n=12) tolvaptan from 5 to 15 weeks of age. Urine samples were collected at 14 weeks of age. Values are expressed as the mean ± SEM. $^{\#\#}P<0.01$ vs. normal DBA mice (*t* test); $^{**}P<0.01$ vs. control *pcy* mice (Dunnett's test for urine volume; Williams' test for urine osmolality).

Tolvaptan showed a dose-dependent aquaretic effect in the short-term study.

Figure 2. Effects of tolvaptan on kidney weight (A), cyst volume (B) and cyst formation (C–H). (A) Kidney weight/body weight ratio in DBA mice (n=5) and in *pcy* mice treated without (n=13) or with 0.01% (n=14), 0.03% (n=14), 0.1% (n=11), or 0.3% (n=12) tolvaptan from 5 to 15 weeks of age. Values are expressed as the mean ± SEM. $^{\#\#}P<0.01$ vs. normal DBA mice (*t* test); $^{*}P<0.05$ and $^{**}P<0.01$ vs. control *pcy* mice (Williams' test). (B) Cyst volume (% body weight) were calculated using the following formula: cyst volume (% body weight) = cyst area / kidney cross-section area × kidney weight / body weight × 100. (C–H) Cyst formation was assessed at 15 weeks of age using hematoxylin/eosin-stained kidney sections (magnification: ×20). (C) Untreated *pcy* mice. (D–G) *pcy* mice treated with 0.01, 0.03, 0.1, and 0.3% tolvaptan. (H) Untreated normal DBA mice. Tolvaptan significantly reduced the kidney enlargement in a short-term treatment study.

Figure 3. Effects of tolvaptan on kidney cAMP levels (A), ERK activity (B, C), mRNA expression levels of aquaporin-2 (D), the vasopressin V_{1a} (E) and V_2 (F) receptors in the short-term study. *pcy* mice were treated without (n=13) or with 0.01% (n=14), 0.03% (n=14), 0.1% (n=11), or 0.3% (n=12) tolvaptan from 5 to 15 weeks of age. Values are expressed as the mean ± SEM. (A) cAMP levels were measured using an immunoassay kit. **P*<0.05 and ***P*<0.01 vs. control *pcy* mice (Williams' test). (B) ERK activity expressed as a ratio of pERK1/2 to total ERK1/2. ***P*<0.01 vs. control *pcy* mice (Dunnett's test). (C) Western blots of total kidney lysates from *pcy* mice. Tolvaptan significantly and dose-dependently reduced kidney cAMP content and ERK activity in the short-term treatment study. Relative mRNA expression was normalized to GAPDH. Its expression level in normal DBA mice was set at 1.0. (D) Aquaporin-2 expression was markedly increased in control *pcy*, and was reduced by tolvaptan. ***P*<0.01 vs. control *pcy* mice (Williams' test). There were no significant differences in V_{1a}R (E) or V_2R (F) expression among any of the groups (Dunnett's test).

Figure 4. Effect of tolvaptan on urine neutrophil gelatinase-associated lipocalin (NGAL) level (A) and correlation between urinary NGAL level and the kidney weight/body weight ratio (KW/BW) (B). NGAL was measured in urine sample obtained at 14 weeks of age and KW/BW was measured at 15 weeks of age. Data are included for 64 mice. Tolvaptan significantly reduced the urine NGAL levels in the short-term treatment study.

Figure 5. Effect of tolvaptan on left kidney volume. (A) Left kidney volume measured by MRI in untreated DBA mice (n=9), control *pcy* mice (n=15), and in *pcy* mice treated with 0.1% tolvaptan (n=15) from 5 weeks of age until necropsy. Values are expressed as the mean ± SEM. The numbers of mice are shown in parentheses. Statistical significance was analyzed by repeated-measures ANOVA followed by two-tailed *t* tests. ##$P<0.01$ vs. normal DBA mice; *$P<0.05$ and **$P<0.01$ vs. control *pcy* mice. (B) Representative T2-weighted images of the left kidney in control and tolvaptan-treated *pcy* mice. Sequential images from the same mouse in each group are shown. Cystic fluid is indicated by high signal intensities in T2-weighted images. Tolvaptan suppressed left kidney volume enlargement with an inhibition rate as high as 50% compared with the control *pcy* mice in the long-term treatment study.

Figure 6. Effect of tolvaptan on survival rates. Survival rates are plotted for 5–29 weeks of age in untreated DBA mice (n=9), control *pcy* mice (n=15), and in *pcy* mice treated with 0.1% tolvaptan (n=15) from 5 weeks of age until necropsy. Statistical significance was analyzed using the Log-rank test. Tolvaptan significantly improved survival rates in the long-term treatment study.

Figure 7. Effects of tolvaptan on urine volume (A), urine osmolality (B), urine albumin (C) and blood urea nitrogen (D) in the long-term treatment study. DBA mice (n=9) were untreated and *pcy* mice were treated without (n=15) or with 0.1% tolvaptan (n=15) from 5 to 29 weeks of age. All urine parameters were measured at 7, 11, 15, 19, 23, and 27 weeks of age. Values are expressed as the mean ± SEM. The numbers of mice are shown in parenthesis. Statistical significance was analyzed by repeated measures ANOVA

followed by two-tailed *t* tests. NS: not significant; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. normal DBA mice; $*P<0.05$ and $**P<0.01$ vs. control *pcy* mice.

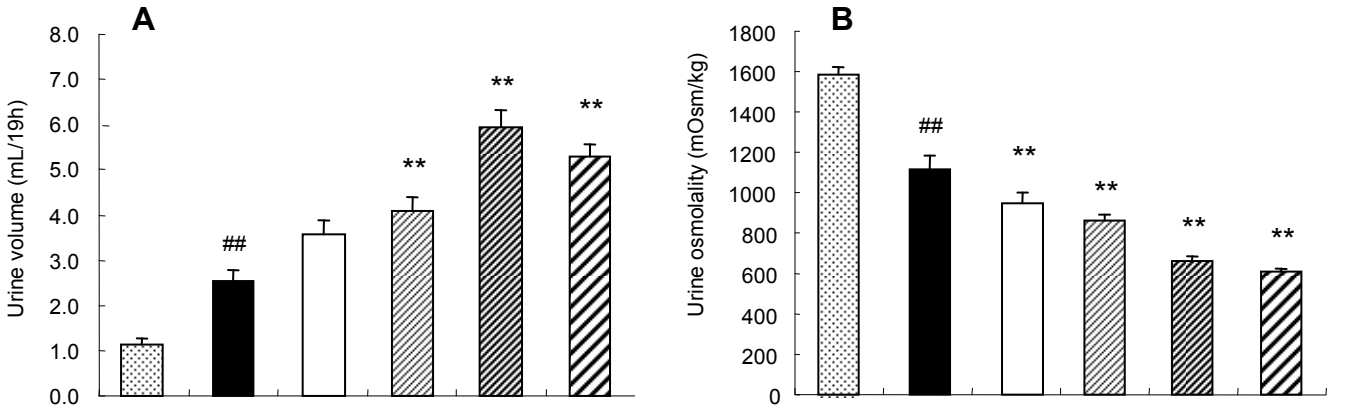
Tolvaptan showed sustained aquaretic effect and inhibited increases in urinary albumin excretion at 23 and 27 weeks of age, and slightly decreased BUN at 27 weeks of age.

Table 1. Effects of tolvaptan from 5 to 15 weeks of age on the progression of polycystic kidney disease.

	DBA normal mice	**Control *pcy* mice**	**Tolvaptan-treated *pcy* mice**			
			0.01%	0.03%	0.1%	0.3%
n	5	13	14	14	11	12
BW (g)	33.7 ± 0.6	28.0 ± 0.6$^{\#\#}$	27.4 ± 0.4	26.8 ± 0.6	27.6 ± 0.5	25.7 ± 0.3**
Kidney fibrosis volume (%BW)	—	0.93 ± 0.1	0.90 ± 0.09	0.77 ± 0.07	0.72 ± 0.08	0.64 ± 0.06**
PCNA-positive cells ($\times 10^4$ cells/kidney)	63.1 ± 6.2	113.0 ± 17.9$^{\#}$	123.4 ± 17.7	99.2 ± 8.2	107.5 ± 8.22	62.5 ± 8.4**
BUN (mg/dL)	36.1 ± 0.8	51.2 ± 2.4$^{\#\#}$	52.0 ± 3.3	56.4 ± 2.4	51.2 ± 3.9	51.9 ± 2.8
Serum Creatinine (mg/dL)	0.13 ± 0.01	0.17 ± 0.01$^{\#\#}$	0.18 ± 0.01	0.21 ± 0.02	0.19 ± 0.01	0.18 ± 0.01
Serum tolvaptan (ng/mL)	—	—	7.2 ± 1.8	35.5 ± 3.1	146.0 ± 18.1	297.9 ± 58.8
Urine albumin (μg/mg Cr)	0.27 ± 0.07	0.34 ± 0.08	0.43 ± 0.11	0.62 ± 0.27	0.14 ± 0.02	0.26 ± 0.12

Values are expressed as mean ± SEM.

$^{\#}p<0.05$ and $^{\#\#}P<0.01$ *vs.* normal DBA mice (*t* test); $^{**}P<0.01$ *vs.* control *pcy* mice (Williams' test)



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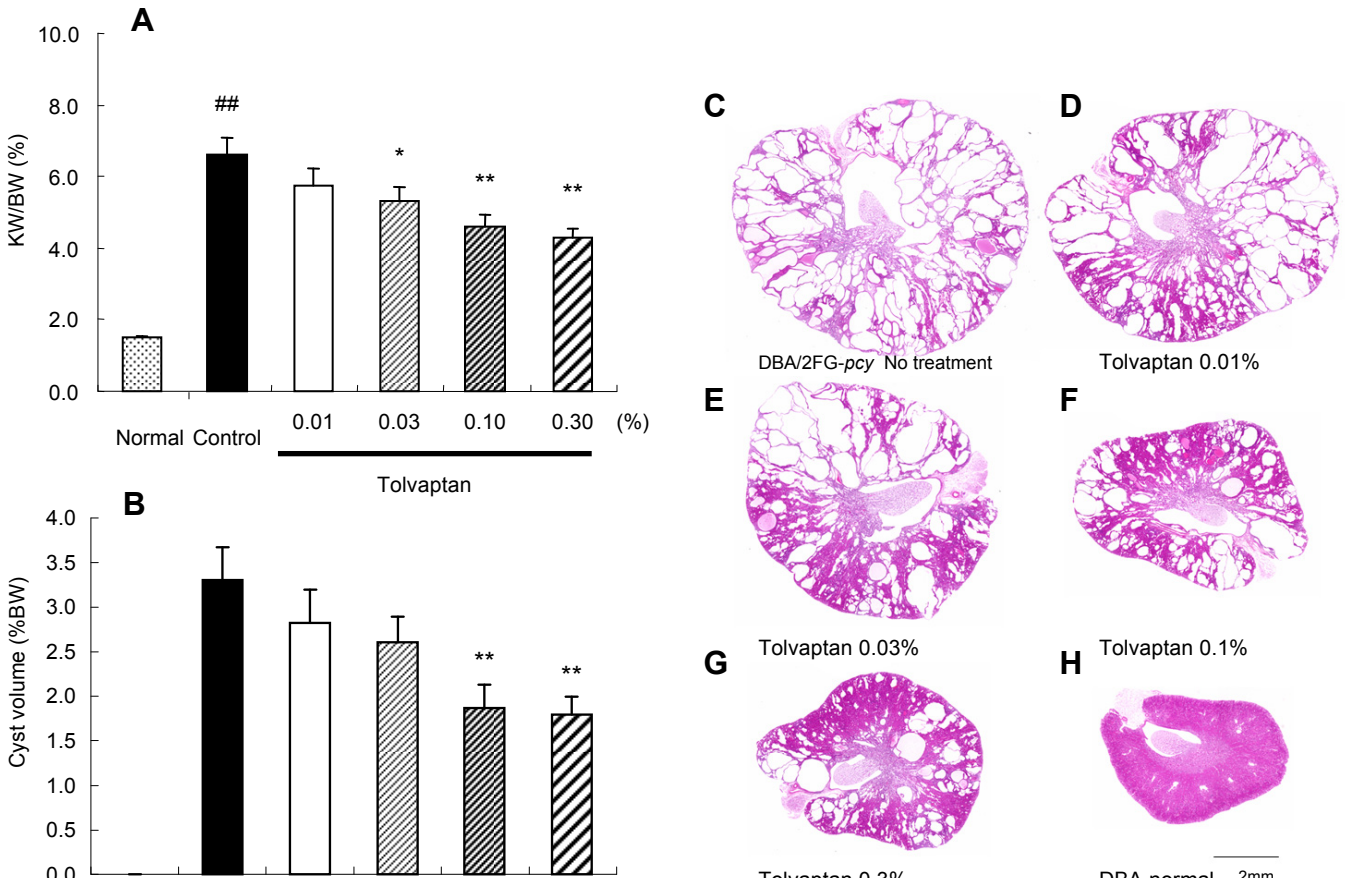

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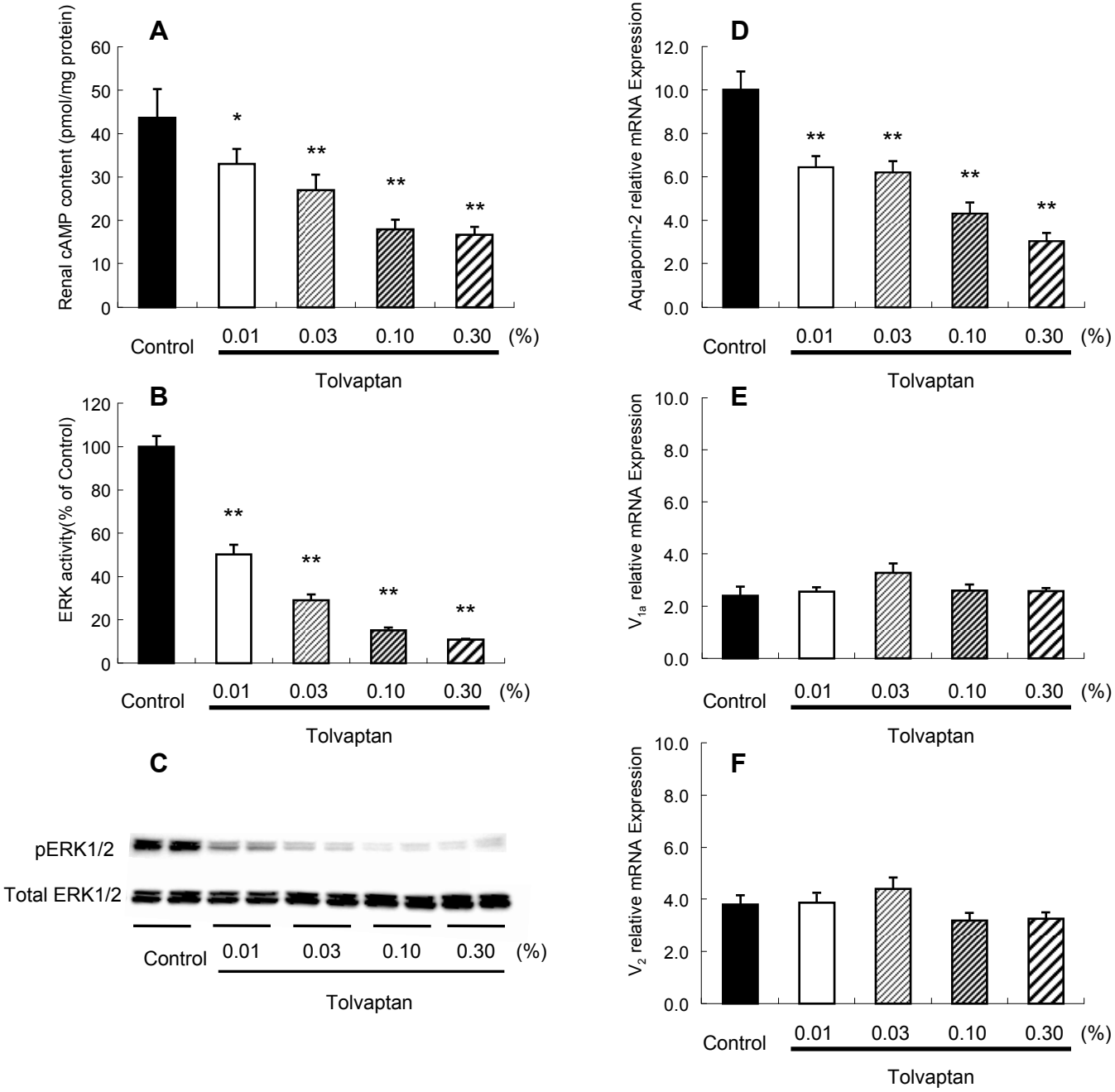

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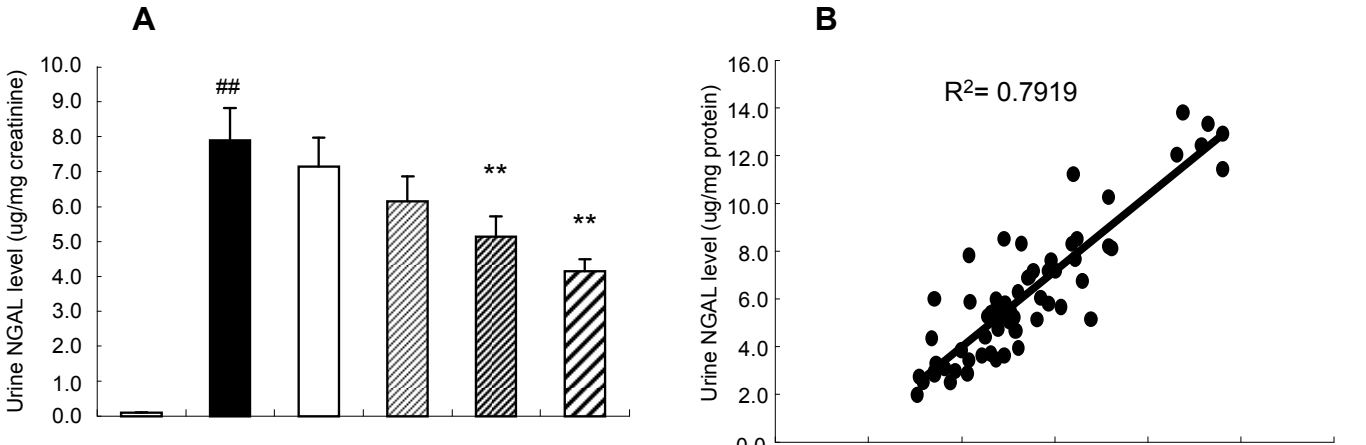

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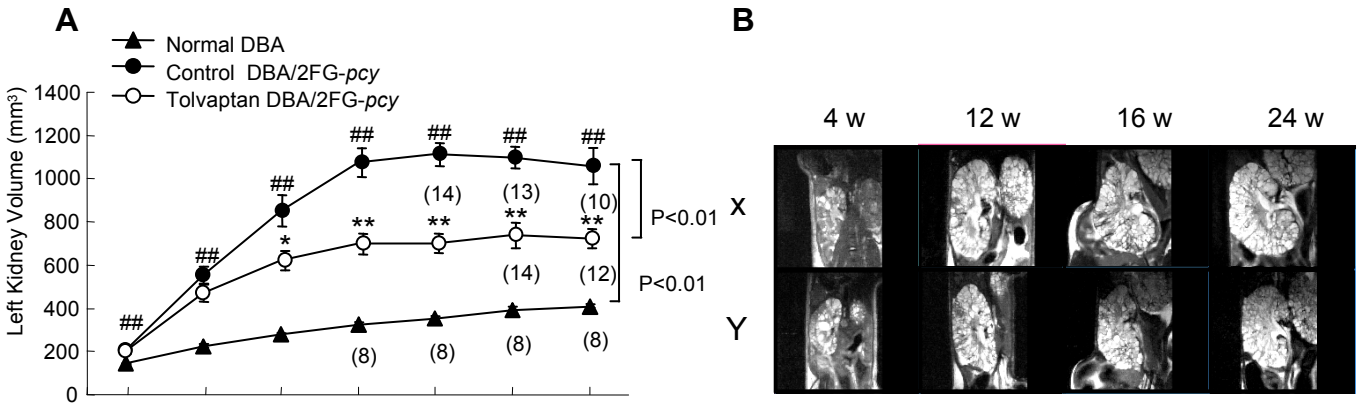

Fig. 5.

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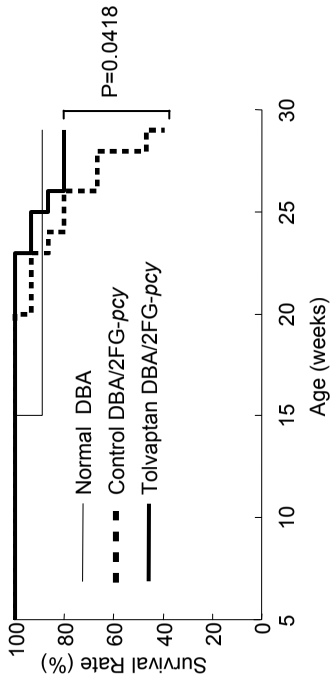

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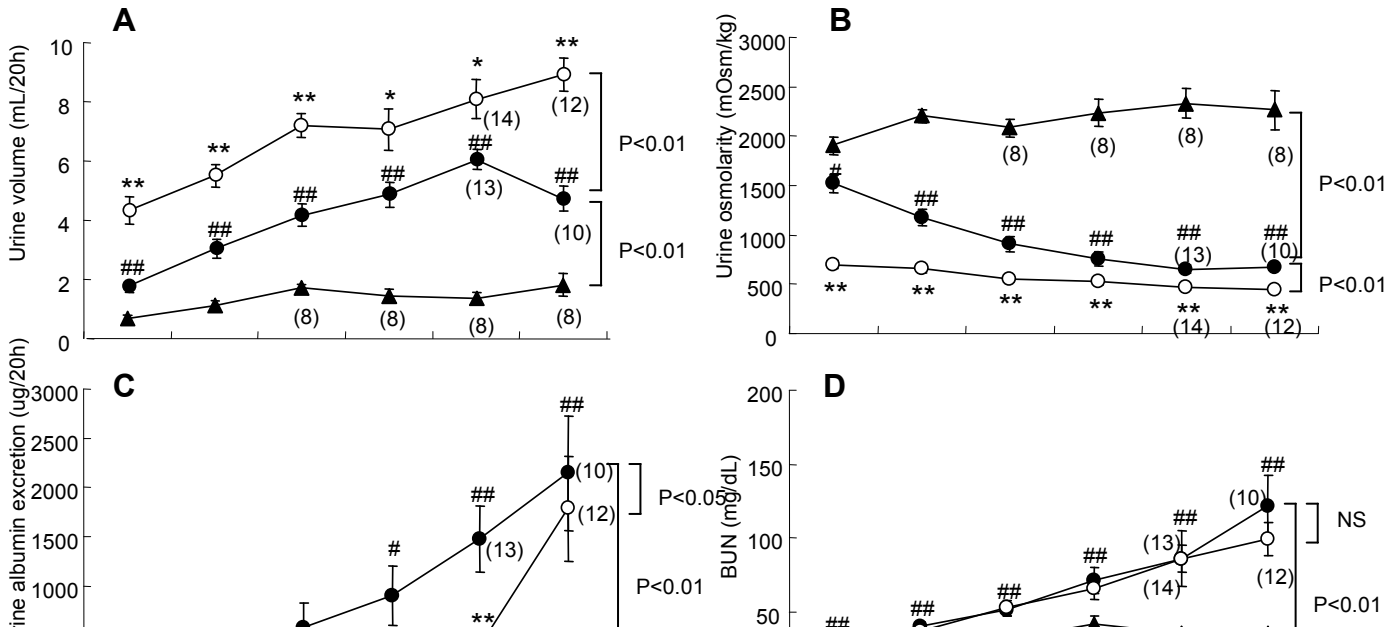

Fig. 7.