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BASIC SCIENCE ARTICLE

Hydrogen-rich water reduced oxidative stress and renal fibrosis in rats with unilateral ureteral obstruction

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BACKGROUND: Congenital obstructive nephropathy (CKD) is commonly implicated in the pathophysiology of chronic kidney disease occurring in the pediatric and adolescent age groups and the release of reactive oxygen species contribute to the worsening of renal fibrosis. Molecular hydrogen (H_2) protects against tissue injury by reducing oxidative stress. We evaluated the efficacy of oral H_2-rich water (HW) intake in preventing unilateral ureteral obstruction (UUO)-induced renal injury in rats.
METHODS: Male Sprague–Dawley UUO or control rats were administered with distilled water (DW) or HW for 2 weeks post-surgery. Histopathological and immunohistochemical analyses of kidney samples were performed.
RESULTS: Histological changes were not apparent in the sham-operated kidneys. However, UUO kidneys were found to have widened interstitial spaces and tubular dilatation. Compared with the UUO + DW group, HW administration attenuated tubulointerstitial injury and reduced interstitial fibrotic area, causing a substantial decline in the frequency of α-SMA-, ED-1-, and TGF-β1-positive cells in the UUO + HW group. The decrease in the *klotho* mRNA expression in the UUO + HW group was less pronounced than that in the UUO + DW group.
CONCLUSION: Oral HW intake reduced oxidative stress and prevented interstitial fibrosis in UUO kidneys, potentially involving klotho in the underlying mechanism.

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IMPACT:

- Oral intake of hydrogen-rich water (HW) can reduce oxidative stress and suppress interstitial fibrosis in unilateral ureteral obstruction-induced renal injury in rats.
- This mechanism possibly involves klotho, which is known for its antiaging roles.
- The association between molecular hydrogen and klotho in renal fibrosis is well known; this is the first report on the association in a unilateral ureteral obstruction model.
- Drinking HW is a safe and convenient treatment for oxidative stress-induced pathologies, without side effects.
- As a prospect for future research, oral HW intake to treat oxidative stress may improve renal fibrosis in congenital obstructive nephropathy.

INTRODUCTION

Chronic kidney disease (CKD) commonly occurs in children and adolescents with congenital obstructive nephropathy, with almost two-thirds of the patients developing to end-stage renal disease (ESRD). Chronic tissue damage causes progressive extracellular matrix accumulation, renal tubular atrophy, and peritubular capillary loss, resulting in renal fibrosis. Clarification of the underlying molecular mechanisms is important to prevent the progression of CKD to ESRD.

Unilateral ureteral obstruction (UUO) is an accepted model of progressive renal fibrosis due to oxidative stress, inflammation, and apoptosis.[1] UUO increases proinflammatory and fibrogenic cytokine secretion from the obstructed kidneys, leading to macrophage infiltration and interstitial fibrosis. Macrophages and activated α-smooth muscle actin (α-SMA)-positive fibroblasts observed in the tubular and interstitial spaces of UUO kidneys promote renal inflammation and fibrosis.[2]

Several studies have reported that macrophage infiltration, increased reactive oxygen species (ROS) activity, and renin–angiotensin system activation are essential in the pathogenesis of renal fibrosis.[3,4] Oxidative stress is a well-known mechanism leading to the occurrence of chronic fibrosis in UUO kidneys, with damage resulting from ROS accumulation in cells, tissues, and organs.[5] Recently, molecular hydrogen (H_2) was demonstrated to lower oxidative stress, thereby effectively protecting against tissue injury. H_2 selectively lowers the

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production of hydroxyl radical and peroxynitrite, two of the most cytotoxic ROS, and protects against oxidative stress.[6] Another study found that H_2-rich water (HW) and H_2-rich saline significantly attenuated renal ischemia/reperfusion injury and reduced serum levels of 8-oxo-2′-deoxyguanosine, a biomarker of oxidative DNA damage, in rats.[7,8] In addition, several studies on kidney diseases have demonstrated the efficacy of H_2. Specifically, Nakashima et al.[9] reported that H_2 reduced apoptosis and nephrotoxicity in rat models of cisplatin nephrotoxicity. In another study, HW inhibited chronic allograft nephropathy in a renal transplantation model by reducing oxidative stress and suppressing the activation of inflammatory signaling pathways and cytokine production, thereby improving the functioning of the renal allograft and overall survival.[10] In addition, drinking HW rather than inhaling H_2 gas can prevent oxidative damage. Regarding oral HW intake, reducing oxidative stress prevents cognitive impairment and may inhibit the development of cystic kidney disease,[11,12] suggesting that HW reduces oxidative stress and inhibits interstitial fibrosis in the kidneys.

However, whether the reported beneficial effects of HW, especially orally administered HW, on oxidative stress and inflammation can prevent fibrosis after UUO remains unclear. Therefore, we evaluated the efficacy of oral HW intake in preventing UUO-induced renal fibrosis.

METHODS

Animals

Studies were performed in male Sprague–Dawley rats weighing between 200 and 250 g. The rats were kept in temperature- and light-controlled cages, with free access to food and water at Juntendo University Animal Care Facility. The Juntendo University Animal Care Committee approved the study protocol (the approval number: 280262).

Experimental design

UUO was established according to a previously described method.[13] After administering light anesthesia with isoflurane, the left renal and urogenital system was exposed through a midline incision in the abdominal cavity. The left ureter was ligated with 5-0 silk sutures at two points next to the ureteropelvic junction. Similar surgical procedures were performed on the sham-operated rats; however, the left ureter was not ligated. The rats were randomized into four groups, with five rats in each group, as follows: sham operation + distilled water (DW), sham operation + HW, UUO + DW, and UUO + HW. The rats in the UUO group underwent unilateral ureteral ligation.

Postoperatively, the sham + DW and UUO + DW groups were provided oral DW for 14 days, whereas the sham + HW and UUO + HW groups were provided oral HW for 14 days. The Aquela kit (Ecomo International, Fukuoka, Japan) was used to prepare HW. In accordance with the methodology previously described, H_2 gas was produced in an acrylic resin tube using H_2-producing material placed in a polyethylene terephthalate bottle.[14] Molecular H_2 was dissolved in water to a supersaturated level under the high pressure of the generated H_2 gas in a bottle with no air gap, allowing the highest H_2 concentration to be present. HW was delivered in aluminum bottles that were replaced twice a day and adjusted to contain >2.0 ppm H_2. The concentration of dissolved H_2 was measured using the concentration detector M687DH-X10E (Shiro Industry, Osaka, Japan) before and after replacement.

The rats were sacrificed on postoperative day 14, and the left kidneys were excised for biochemical and histological analyses. In addition, whole-blood samples were collected from the abdominal aorta of the rats.

Histopathological and immunohistochemical analyses

The kidneys were fixed with 10% formalin in phosphate-buffered saline and embedded in paraffin before sectioning. Hematoxylin and eosin or Sirius Red were used for the staining the prepared sections in order to ascertain the presence of interstitial collagen deposition and the level of tubulointerstitial damage. The tubulointerstitial injury score was assessed using previous methods,[15] based on the extent tubular dilatation, distortion of tubular basement membranes, and atrophy, with the score ranging from 0 to 5 (grade 0, no morphological deformities; grade 1, <10% deformity; grade 2, 10–25% deformity; grade 3, 25–50% deformity; grade 4, 50–75% deformity; and grade 5, ≥75% deformity). Ten nonoverlapping fields at ×200 magnification were photographed from each renal cortical section. Interstitial fibrosis volume was assessed using Sirius Red staining. The interstitial area superimposed on the photograph was measured using KS400 image analysis system (Carl Zeiss, Oberkochen, Germany), and the percentage of interstitium per unit area was estimated.

Immunohistochemical studies were performed using 4-μm-thick paraffin-embedded sections as described previously.[13,15] The samples were stained with ED-1, α-SMA, and transforming growth factor beta 1 (TGF-β1) using the UltraView DAB detection kit and a Ventana BenchMark XT processor (Ventana Medical Systems, Tucson, AZ). ED-1 is the monoclonal antibody clone raised against rat CD68 protein. A mouse monoclonal anti-rat ED-1 antibody (final dilution, 1:100; MCA341GA, Bio-Rad Laboratories, Hercules, CA) was used to stain the sections to further evaluate interstitial monocytes and infiltrating macrophages, a monoclonal mouse anti-human α-SMA antibody (final dilution, 1:200; M0851, Dako, Carpinteria, CA) to assess myofibroblast differentiation, or a rabbit polyclonal antibody to TGF-β1 (1:200; ab25121, Abcam, Cambridge) was used to determine TGF-β1 expression. Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and biotinylated goat anti-rabbit IgG (Vector Laboratories) were used as secondary antibodies. Finally, the 3,3′-DAB reaction was performed using a commercially available kit (Dako), and the sections were counterstained with hematoxylin. The KS400 image analysis system was used to evaluate all stained sections. The frequency of ED-1-positive cells was calculated under a microscope at ×200 magnification. The areas stained for α-SMA and TGF-β1 were expressed as the percentage of total area observed under ×400 magnification. A total of 10 renal cortical fields per section were evaluated. All histopathological and immunohistochemical assessments were performed by two independent pathologists who were blinded to the study.

Real-time polymerase chain reaction

In the renal cortex, the expression levels of *α-Sma*, *Tgf-β1*, tumor necrosis factor-α (*Tnf-α*), and *klotho* were assessed by real-time polymerase chain (RT-PCR) reaction using the TaqMan system according to the manufacturer's instruction. Briefly, cDNA was synthesized from kidney RNA samples using the High-Capacity cDNA reverse transcription kit and analyzed using the default protocols of the 7500 Fast Real-Time PCR system (Life Technologies, Carlsbad, CA). The expression of each gene was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase gene using the standard curve method. Primers and probes for *α-Sma* (Rn01759928_g1), *Tgf-β1* (Rn00572010_m1), *Tnf-α* (Rn99999017_m1), and *klotho* (Rn00580123_m1) were prepared using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The cycling conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s. The polymerase chain reaction primer sequences were as follows: *α-Sma*, 5′-CACCATGAAGATCAAGATCATTGCC-3′ (forward) and 5′-GGTAGACAGC-GAAGCCAGGA-3′ (reverse); *Tgf-β1*, 5′-CTTCAGCTCCACAGAGAACTGC-3′ (forward) and 5′-CACGATCATGTTGGACAACTGCTCC-3′ (reverse); and *Tnf-α*, 5′-CAGCCTCTTCTCATTCCTGC-3′ (forward) and 5′-GGTCTGGGCCATAGAACT GA-3′ (reverse).

Statistical analyses

F tests were used to determine whether the data were parametric, which confirmed the absence of an equality of variance. Therefore, data were reported using medians and interquartile ranges (IQRs). In addition, the nonparametric Kruskal–Wallis test was applied to compare between two groups, and the Steel–Dwass–Critchlow–Fligner procedure was used to compute multiple comparisons. Statistical analysis was carried out using R version 3.4.3 (R Foundation for Statistical Computing, http://www.R-project.org/). In all the analyses, $P < 0.05$ was considered statistically significant.

RESULTS

HW attenuates postoperative increases in kidney weight and diameter in UUO kidneys

The preoperative median (IQRs) body weights were 242.9 (236.9–246.8), 239.8 (232.8–244.2), 242.2 (237.0–248.9), and 240.4 (230.5–253.5) g in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively (Fig. 1a). The median (IQRs) body weights postoperatively were 318.7 (312.2–331.8), 323.8 (316.2–335.6), 290.0 (267.9–306.6), and 300.4 (269.6–312.1) g in

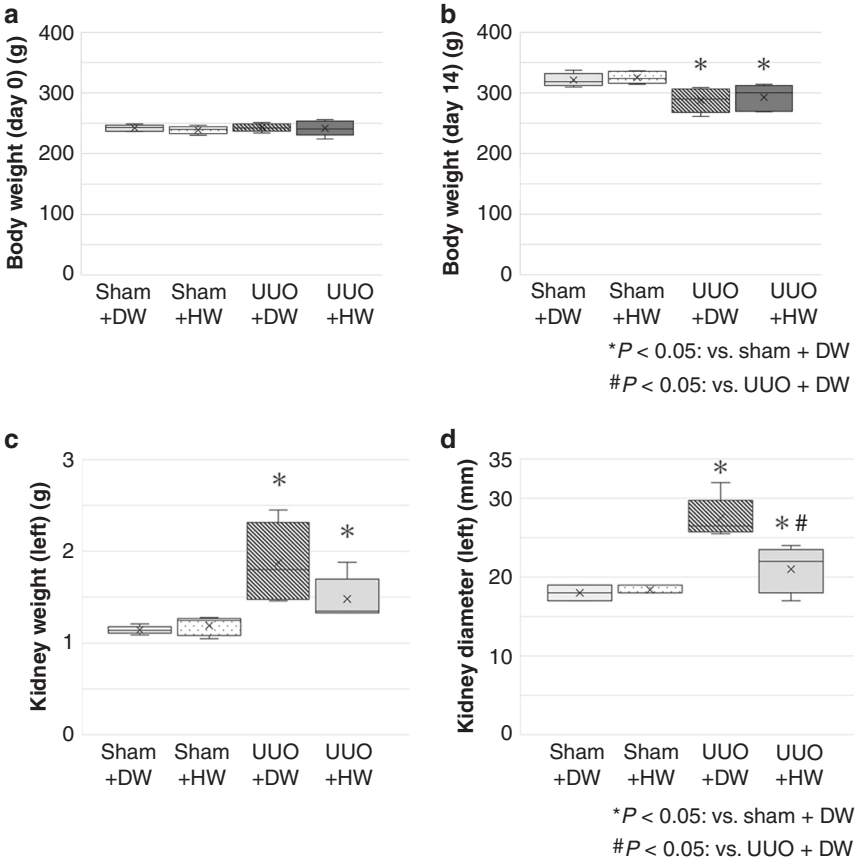

Fig. 1 HW administration attenuates postoperative gain in kidney weight and diameter. Changes in kidney weight and diameter were determined to assess the degree of hydronephrosis. Statistical analyses of body weight before (**a**) and postoperatively (**b**), and changes in left kidney weight postoperatively (**c**) and in left kidney diameter postoperatively (**d**). *$P < 0.05$ vs. sham + DW group, #$P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as medians and interquartile ranges (IQRs). S sham, DW distilled water, HW H_2-rich water, UUO unilateral ureteral obstruction.

the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively (Fig. 1b). No significant differences were noted in body weight preoperatively between the sham and UUO groups (Fig. 1a). However, the UUO groups (UUO + DW and UUO + HW) had lower body weight than the sham groups (sham + DW and sham + HW) on postoperative day 14 ($P < 0.05$; Fig. 1b).

The postoperative left kidney weights were 1.14 (1.11–1.18), 1.25 (1.09–1.27), 1.80 (1.48–2.32), and 1.35 (1.33–1.70) g in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively (Fig. 1c). In addition, the left kidney diameters were 18.0 (17.0–19.0), 18.0 (18.0–19.0), 26.5 (25.8–29.8), and 22.0 (18.0–23.5) mm in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively (Fig. 1d). The left kidney weight and diameter were higher in the UUO groups compared to the sham groups. In contrast, after the 2-week treatment, the left kidney diameter in the rats with UUO treated with HW was lower than that in rats with UUO treated with DW ($P < 0.05$; Fig. 1c, d).

HW reduces serum creatinine levels in UUO kidneys

The renal function was assessed using serum creatinine (Cr) levels. Significant increases in serum Cr levels were observed in the rats with UUO than in the sham-operated rats. These increases were significantly reduced by HW administration in the rats with UUO (0.28 [0.27–0.31], 0.28 [0.25–0.31], 0.42 [0.40–0.44], and 0.34 [0.29–0.37] mg/dL in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$). However, difference in bun levels between the rats that were sham-operated and those with UUO did not reach statistical significance (21.1 [20.6–22.9], 20.7 [19.8–22.0], 22.6 [21.2–24.2], and 21.4 [20.2–22.4] mg/dL in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively).

HW attenuates histological changes in UUO kidneys

The sham-operated kidneys did not reveal any histological changes. Conversely, the kidneys of rats with UUO demonstrated widened interstitial spaces, tubular dilatation, tubular atrophy, and interstitial volume, accompanied with a larger number of interstitial cells and infiltrating leukocytes; the tubulointerstitial injury score was significantly higher in the UUO kidneys compared with the sham-operated kidneys. HW administration improved the histological findings; therefore, the tubulointerstitial volume score was lower in the UUO + HW group than in the UUO + DW group (0.30 [0.20–0.35], 0.45 [0.28–0.55], 3.8 [3.60–4.10], and 2.0 [1.65–2.25] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$; Fig. 2a, b).

Extensive interstitial collagen deposition, a typical index of fibrosis, was examined using Sirius Red staining in the UUO kidneys, which revealed that the extent of renal interstitial fibrosis was significantly decreased with HW administration. The interstitial fibrotic area was smaller in the UUO + HW group than in the UUO + DW group (4.9% [3.6–4.9%], 4.2% [3.1–4.7%], 35.5% [31.5–57.7%], and 10.4% [6.9–11.9%] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$; Fig. 2c, d).

HW reduces α-SMA expression in UUO kidneys

α-SMA is produced primarily in vascular smooth muscle cells, and α-SMA-positive myofibroblasts are responsible for collagen synthesis and participation in cell activity in fibrotic disease. The

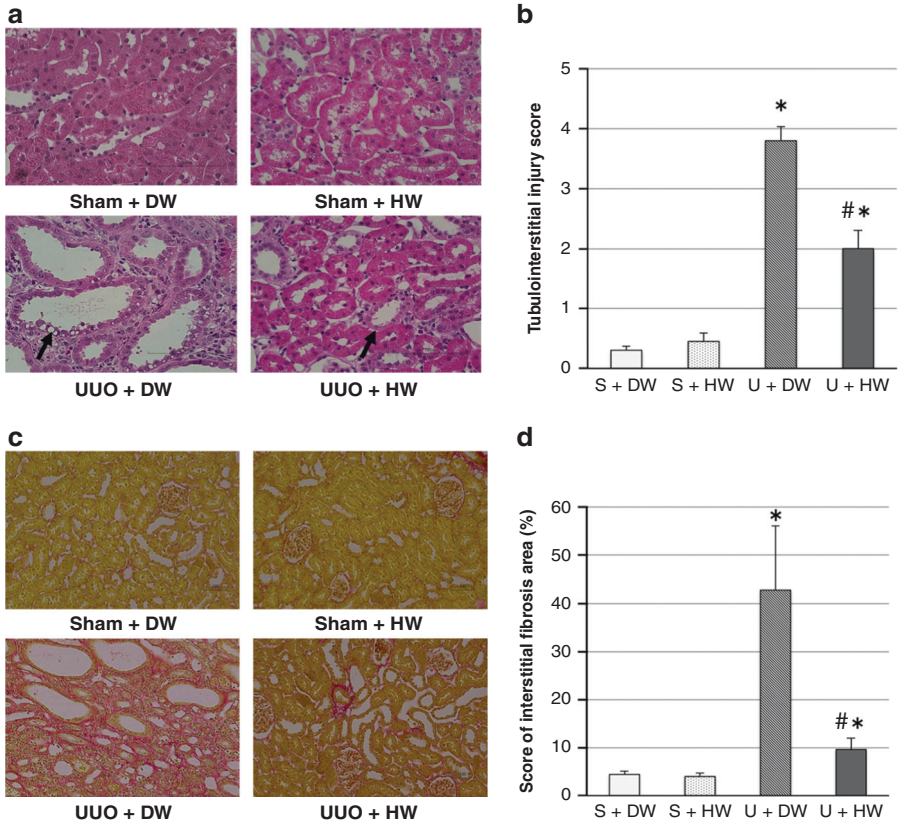

Fig. 2 HW administration attenuates tubulointerstitial injury as well as interstitial collagen deposition and fibrosis in obstructed kidneys. **a** Representative micrographs showing hematoxylin and eosin staining of sections from kidneys collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. Original magnification, ×200. **b** Statistical analysis of the tubulointerstitial injury score postoperatively. *$P < 0.05$ vs. sham + DW group, #$P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are expressed as medians and interquartile ranges (IQRs). **c** Representative micrographs showing Sirius Red staining of sections from kidneys collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. Original magnification, ×200. **d** Statistical analysis of the area of interstitial fibrosis postoperatively. *$P < 0.05$ vs. sham + DW group, #$P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as medians and interquartile ranges (IQRs).

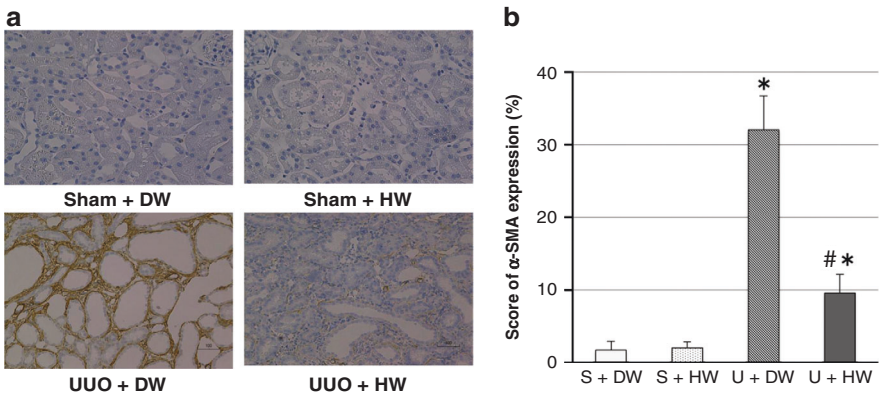

Fig. 3 HW administration reduces α-SMA expression in obstructed kidneys. **a** Representative micrographs showing α-SMA staining sections from kidneys collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. Original magnification, ×200. **b** Statistical analysis of α-SMA expression postoperatively. *$P < 0.05$ vs. sham + DW group. #$P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as medians and interquartile ranges (IQRs).

number of α-SMA-producing cells surrounding the peritubular and periglomerular spaces was significantly higher in the UUO group than in the sham group. However, the rats with UUO that were given HW showed a marked reduction in α-SMA expression level (1.5% [0.7–2.8%], 1.8% [1.4–2.7%], 32.0% [27.7–36.4%], and 8.1% [7.5–12.4%] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$; Fig. 3).

HW attenuates monocyte/macrophage infiltration in UUO kidneys

Macrophage infiltration was assessed by determining the frequency of ED-1-positive cells in the renal cortex. Compared with the presence of rare ED-1-positive cells in the sham-operated kidneys, the frequency of ED-1-positive cells in the interstitium was significantly higher in the UUO kidneys. However, the

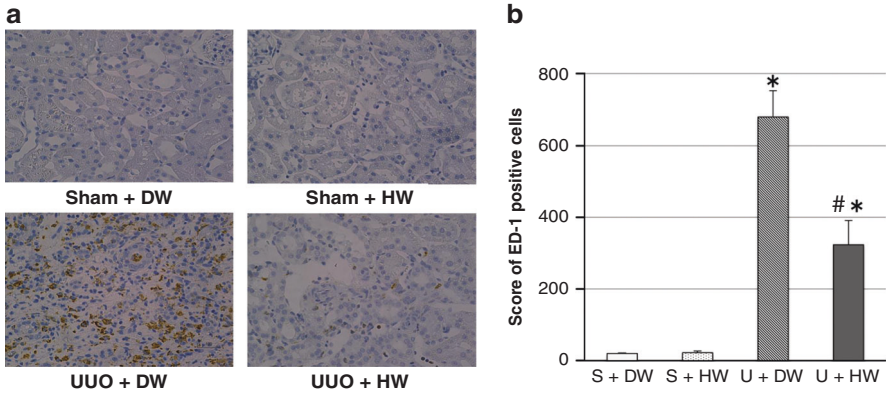

Fig. 4 HW administration reduces ED-1 expression in obstructed kidneys. a Representative micrographs showing ED-1 staining sections from kidneys collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. Original magnification, ×200. **b** Statistical analysis of ED-1 expression postoperatively. $^{*}P < 0.05$ vs. sham + DW group, $^{\#}P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as median and interquartile ranges (IQRs).

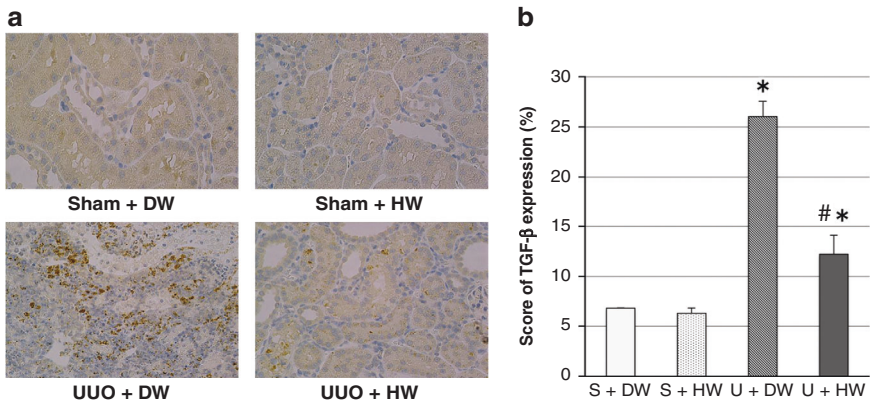

Fig. 5 HW administration reduces TGF-β1 expression in obstructed kidneys. a Representative micrographs showing TGF-β1 staining in sections from kidneys collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. Original magnification, ×200. **b** Statistical analysis of TGF-β1 expression postoperatively. $^{*}P < 0.05$ vs. sham + DW group, $^{\#}P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as medians and interquartile ranges (IQRs).

administration of HW led to a significant reduction in the frequency of infiltrating ED-1-positive cells in the rats with UUO in comparison with that in the rats with UUO that were given DW (21.0 [19.0–21.5], 25.0 [16.0–28.0], 681.0 [600.0–759.0], and 305.0 [270.5–386] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$; Fig. 4).

HW reduces TGF-β1 expression in UUO kidneys

The frequency of TGF-β1-expressing cells significantly increased with UUO compared with the sham-operated rats. However, the number of TGF-β1-expressing cells was markedly reduced with HW administration in rats with UUO than in those with UUO that were administered DW (6.8% [6.0–6.9%], 6.3% [5.7–6.8%], 26.1% [24.7–28.1%], and 12.2 [10.5–14.3%] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; $P < 0.05$; Fig. 5).

Effect of HW on *α-Sma*, *Tgf-β1*, *Tnf-α*, and *klotho* mRNA expression levels in UUO kidneys

The RT-PCR analysis of the kidney tissue revealed significant increases in the *α-Sma*, *Tgf-β1*, and *Tnf-α* expression levels in the UUO kidneys compared with that in the sham-operated kidneys. However, HW administration in the rats with UUO tended to attenuate the observed increase in *α-Sma*, *Tgf-β1*, and *Tnf-α* expression levels (Fig. 6). Moreover, the immunohistochemical findings and gene expression levels did not significantly differ in the sham + HW and UUO + HW groups. Furthermore, the observed decrease in the *klotho* mRNA expression level in the UUO + HW group was less pronounced than those of the UUO + DW group (1.10 [1.04–1.37], 1.17 [1.05–1.45], 0.32 [0.04–0.74], and 0.84 [0.71–1.25] in the sham + DW, sham + HW, UUO + DW, and UUO + HW groups, respectively; Fig. 6).

DISCUSSION

In the present study, oral HW intake significantly improved UUO-induced histopathological and pathogenic changes, demonstrating that oral HW intake reduced oxidative stress and was beneficial in preventing UUO-induced renal fibrosis.

The occurrence of renal fibrosis is the most common pathological pathway through which most renal diseases progress to ESRD regardless of the underlying disease. Furthermore, renal dysfunction correlates more strongly with the degree of tubulointerstitial injury compared to glomerular injury. Tubulointerstitial inflammation is observed in CKD, and persistent inflammatory reactions in the renal system influences the occurrence of tubulointerstitial fibrosis with impaired renal function.[16]

Oxidative stress, which has been extensively reported in diseases that involve ROS generation and oxidative modification of various substrates during inflammatory processes, is a primary factor responsible for the occurrence of inflammatory conditions such as UUO; therefore, attenuation of oxidative stress is important in preventing fibrotic tissue formation.

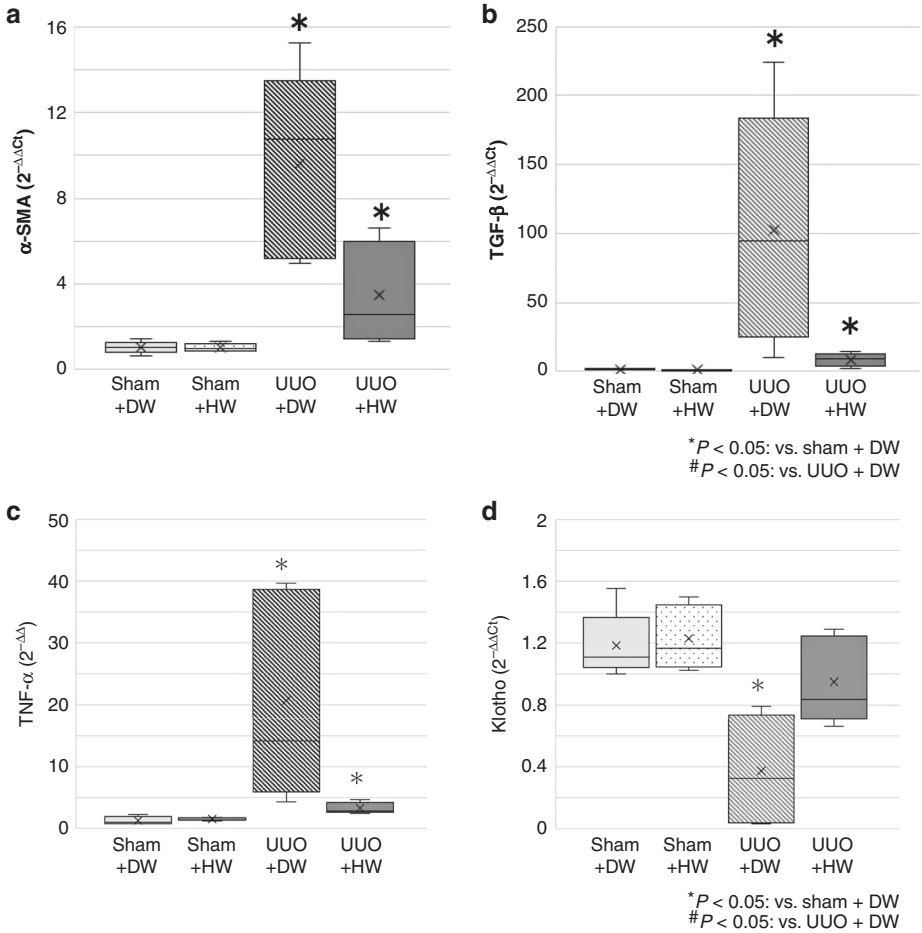

Fig. 6 HW administration tends to reduce the expression levels of *α-Sma*, *Tgf-β1*, and *Tnf-α* in obstructed kidneys and attenuate the reduction in *klotho* gene expression in obstructed kidneys. Expression of *α-Sma* (**a**), *Tgf-β1* (**b**), *Tnf-α* (**c**), and *klotho* (**d**) in kidney sections collected from sham-operated normal rats and UUO rats on postoperative day 14. Rats were administered DW or HW postoperatively. $^{*}P < 0.05$ vs. sham + DW group, $^{\#}P < 0.05$ vs. UUO + DW group; $n = 5$ per group. Data are reported as medians and interquartile ranges (IQRs).

In recent years, many studies have shown the beneficial effects of H_2 in various disease and physiological states. H_2 selectively scavenges harmful ROS, such as hydroxyl radicals and peroxynitrite, thereby reducing nucleic acid oxidation and lipid peroxidation and protecting cells and tissues from oxidant stress and apoptotic injury.[6] Moreover, H_2 increases the effects of antioxidant enzymes, including superoxide dismutase and catalase, and suppresses inflammation by inhibiting interleukin (TNF-α) and interleukin (IL)-6.[17] According to recent studies, in addition to directly neutralizing ROS, H_2 indirectly lowers oxidative stress by inhibiting the expression of related genes. Therefore, H_2 might improve various pathogenic states by regulating gene expression.

Our data demonstrated that oral HW intake suppressed renal gene expression and TGF-β1 and α-SMA immunoreactivity and reduced tubulointerstitial fibrosis, as indicated by Sirius Red staining, in UUO kidneys. TGF-β is a primary factor in the critical regulation of interstitial fibrosis and is essential in numerous aspects of renal fibrosis.[18] TGF-β1 promotes α-SMA expression, thereby leading to the transformation of fibroblasts into myofibroblasts, which leads to massive fibrosis through the production and release of collagen and fibronectin.[19]

UUO-induced renal injury promotes the release of proinflammatory cytokines, such as TNF-α, IL-6, and IL-18, that contributes to neutrophil activation and infiltration as well as organ and tissue injury.[20] Additionally, ROS activates TNF-α expression by upregulating the NF-κB signaling.[21] In this study, the gene expression levels of proinflammatory cytokines such as *Tnf-α* were significantly elevated in the UUO kidneys. However, oral HW intake showed a tendency to reduce the levels of inflammatory mediators while protecting renal tissue from UUO-induced oxidative stress.

In addition, UUO generally induces renal fibrosis through ROS accumulation, macrophage infiltration, and nitric oxide inactivation. In a rat model of CKD, H_2 was shown to protect histological changes due to ischemia-induced cardiorenal injury, partly through the inhibition of nitric oxide inactivation,[22] providing further support that the observed beneficial effects of H_2 might involve the attenuation of oxidative stress.

Klotho is an antiaging gene encoding a type 1 transmembrane protein that forms a complex with multiple fibroblast growth factor receptors. It is expressed mainly in the distal tubules of the kidney and in the brain. *Klotho* gene was identified as a cause of premature aging in mutant mice. Mice deficient in the *klotho* develop growth disorders, atrophy, and fibrosis in various organs, arteriosclerosis, ectopic calcification, and dementia, typically dying at 8–9 weeks of age.[23] Conversely, mice overexpressing *klotho* gene are highly cognitive and resistant to CKD and have an improved life expectancy.[24] According to recent studies, phosphorus metabolism contributes to the mechanism of antiaging effects of klotho.[25] The decrease in klotho expression in early-stage CKD is considered to cause renal fibrosis in CKD.

In the present study, the decrease in *klotho* gene expression, which was observed in the UUO + DW group, was less pronounced in the UUO + HW group. Chen et al. observed that

the administration of H_2-rich saline could inhibit renal fibrosis after ischemia/reperfusion-induced acute kidney injury by increasing klotho expression and stimulating autophagy. Additionally, the authors reported that H_2 might be protective against kidney injury through a reduction in the methylation of *klotho*.[26] Therefore, our findings suggest that klotho might be involved in molecular H_2-mediated suppression of renal fibrosis and that the epigenetic regulation of *klotho* might be related to these effects.

H_2 can be administered via several routes such as H_2 gas inhalation, injection of H_2-enriched saline, H_2 bath, topical application of H_2-enriched in eyes, and oral ingestion of HW.[27] Most hydrophilic antioxidants cannot penetrate biomembranes but remain on the membrane surface, whereas H_2 can be distributed rapidly into lipids and cytosol and has no cytotoxicity, even at high concentrations.[28] A study reported that the inhalation of H_2 gas had no effect on physiological parameters such as pH and blood electrolytes and had no adverse effects.[29] In addition, oral HW intake is a useful route for H_2 administration because it is portable, safe, and does not alter the physico-chemical properties like taste, smell, or pH of foods, drinks, and drugs.

The efficacy of oral HW intake was assessed in in vivo animal models and in clinical settings. For example, consumption of HW was reported to improve lipid and glucose metabolism in patients with type II diabetes.[30] Furthermore, HW administration for 48 weeks was shown to improve the total scores in the unified Parkinson's disease rating scale in individuals with Parkinson's disease.[31] These findings suggest the potential utility of oral HW intake for the management of oxidative stress-induced pathologies.

Our findings demonstrate the protective effect of oral HW intake on UUO-induced renal fibrosis. Previous studies have confirmed the beneficial effects of HW; however, few reports to date have evaluated oral HW intake in a UUO model of renal fibrosis. We advise that oral HW intake might not completely inhibit fibrosis but may delay its progression. Although the limitation of this study may be the lack of statistical power from the small number of animals, our findings show a significant benefit of HW in UUO. However, studies with bigger sample sizes are warranted to confirm these findings. Renal fibrosis is a chronic pathology that progresses to CKD and eventually to ESRD in children and adolescents. Drinking HW is a safe and a convenient route of H_2 administration; it does not affect taste or drug efficacy.[27] As a prospect for future research, HW may improve the quality of life in children with renal fibrosis such as congenital obstructive nephropathy.

In conclusion, oral HW intake can potentially reduce oxidative stress and suppress interstitial fibrosis in UUO kidneys, possibly through a *klotho*-mediated mechanism.

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AUTHOR CONTRIBUTIONS

Study conception or design and data acquisition, analysis, or interpretation; final approval of the manuscript to be published; and agreement to be accountable for all

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COMPETING INTERESTS

The authors declare no competing interests.

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