Effect of goreisan on urinary concentrating ability and expression of aquaporin-2 in 5/6 nephrectomized rats

Michiko Jo, a Takako Fujimoto, b Maria Kaneko, a Hiroshi Oka, c and Naotoshi Shibahara a

aDepartment of Kampo Diagnostics, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.,
bEnvironment and Humanity, Faculty of Human development, University of Toyama, 3190 Gofaku, Toyama, 930-8555, Japan.,
cDepartment of Japanese Oriental (Kampo) Medicine, Graduate school of Medicine and Pharmaceutical Science, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. (Received July 5, 2013. Accepted August 26, 2013.)

Abstract

The purpose of this study was to investigate the effect of goreisan, a Kampo (traditional Chinese herbal medicine) formula, on the urinary concentrating ability and the expression of aquaporin 1-3 (AQP1-3) in 5/6 nephrectomized (5/6Nx) rats. Two weeks after 5/6 nephrectomy, the rats were divided into three groups: 0.5% goreisan-treated, 1% goreisan-treated and controls. Additionally, each 5/6Nx group and sham-operated group (sham) was divided into 4- and 8 week-observed groups. The serum potassium concentration in the 1% goreisan-treated group was significantly increased compared with that in the 5/6Nx groups. The remnant kidney weight and urinary protein excretion of 1% goreisan-treatment for 8 weeks were significantly decreased compared with those of controls. The urine osmolality of 1% goreisan treatment for 8 weeks was significantly higher than that of controls. AQP2 expression in the inner medulla, outer medulla and cortex were not decreased by 5/6Nx, but expression in all areas was decreased by goreisan. Furthermore, the expression of AQP3 in the goreisan-treated group in the cortex was significantly increased compared with that in the 5/6Nx groups. The changes in urinary osmolality and the decrease in AQP2 and increase in AQP3 expression by goreisan-treatment in 5/6Nx rats suggest that AQP2 expression is associated with the effects of goreisan on modulation of water balance.

Key words Aquaporin 2, goreisan, 5/6 nephrectomized rat, urine osmolality.

Introduction

Water is the most abundant molecule in the whole body. In individual cells, the plasma membrane separates the interior of the cell from its extracellular environment, but specialized membrane channels facilitate water transport across these biomembranes. These water channels are called aquaporins (AQPs), and are freely permeated by water but not by ions or charged solutes. 1,2) Thirteen mammalian AQPs have been molecularly identified and localized to various epithelial, endothelial and other tissues. 3) Aquaporin-2 and -3 (AQP2, AQP3), the predominant water channel, is abundant in the apical plasma membrane and apical vesicles in the collecting duct principal cells, and is the target for the regulation of collecting duct water reabsorption by the antidiuretic hormone vasopressin. 4-7) Aquaporin-1 (AQP1) is expressed in the proximal tubules and descending thin limb in kidney. 2,3)

The complex interaction between AQP1-3 and impairment of water excretion, and the role of AQP1-3 have been investigated with experimental animals. Many studies have revealed that there is upregulation of AQP2 expression in the kidneys in several diseases associated with urinary concentrating defects such as
acquired nephrogenic diabetes insipidus,3) syndrome of inappropriate secretion of antidiuretic hormone,9) chronic heart failure,10,11) and aged rats with glucocorticoid deficiency,12) as well as chronic renal failure in dehydrated conditions.13,14) On the other hand, AQP2 expression is downregulated in the kidneys in cirrhosis secondary to common bile duct ligation15,16) and from aging.17) Thus, these findings suggest that AQP2 expression is not uniform among pathological conditions, such as different experimental models or different stages of the same disease.9,14,15)

AQP5 plays a central role in urine concentration, and changes in AQP5 have a profound influence on water balance in the whole body, such as dehydration or overhydration to maintain serum osmolality. Water balance abnormalities are considered as “Suitai” (= stasis of body fluids) in Kampo medicine, and various Kampo formulas have been used for the treatment of this pathological concept. In particular, goresian, a well-known Kampo formula originally recorded in an ancient Chinese medicinal book named “Shanghan Lun”, has been used to promote water metabolism to relieve symptoms such as thirst, oliguria and sweating. Goreisan has been widely used to treat edema, such as renal edema, lymphedema, pregnancy edema, cardiac edema and scrotum edema, urine retention, and difficult urination.18-20) With regard to the pharmacological effects of goresian, it prevents renal calcium oxalate crystal deposition in ethylene glycol-fed rats, suppresses the development of nephrocalcinosis in rats fed a high phosphorus diet, suppresses diabetic nephropathy in streptozotocin-induced diabetic rats, and ameliorates podocyte injury in adriamycin-treated rats.21-24) However, the influence of goresian on AQP5 expression in the kidney is unknown.

One of the characteristic features of chronic renal failure is associated with polyuria and a urinary concentrating ability develops gradually as the renal failure may be caused by impairment vasopressin-stimulated water reabsorption in the collecting duct.13) 5/6 nephrectomized rats demonstrated low urinary osmolality at basal condition, when free access to normal food and water was provided.14) However, Suzuki et al.13) demonstrated that water restriction in 5/6 nephrectomized rats significantly increased urine osmolality compared with 5/6 nephrectomized rats at basal condition. The present study was aimed to clarify the mechanisms of the effects of goresian, expression of AQP1-3 in the kidney and renal function were investigated in 5/6 nephrectomized rats.

Materials and Methods

Test drug: Spray-dried extract powder of goresian used in this study was kindly provided by Tsumura & Co. (Tokyo, Japan). Goreisan is composed of five crude drugs: Alismatis Rhizoma, the rhizome of Alisma orientale Juzepczuk (Alismataceae); Polyergus, Polyergus umbellatus Fries (Polyporaceae); Poria, Poria cocos Wolf (Polyporaceae); Cinnamomi Cortex, the bark of Cinnamomum cassia Blume (Lauraceae); Atractylodis Lanceae Rhizoma, rhizome of Atractylodes lancea DC (Compositae).

Three-dimensional HPLC analysis: Granules of goresian (1.0 g) were extracted with methanol (20 ml) under ultrasonication for 30 min, and were then centrifuged at 1500 g for 5 min. The supernatant was filtrated with a membrane filter (0.45 μm) and then submitted for HPLC analysis (30 μl). The HPLC apparatus consisted of a Shimadzu LC 10A (analysis system software; CLASS-M10A ver. 1.64, Tokyo, Japan) equipped with a multiple wavelength detector (UV 200-400 nm) (Shimadzu SPD-M10AVP, diode array detector) and an auto injector (Shimadzu CTO-10AC). HPLC conditions were as follows: an ODS column (TSK-GEI 80TS, 250 x 4.6 mm i.d., Tosoh, Tokyo, Japan); eluant: (A) 0.05M AcONH4 (pH 3.6) and (B) 100% CH3CN. A linear gradient of 90% A and 10% B was changed over at 60 min to 0% A and 100% B (100% B was continued for 20 min); temperature, 40°C; flow rate, 1.0 ml/min. Figure 1 shows the chemical profiles of goresian.

Animals and drug treatments: Six-week-old male Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan), and were kept in an automatically controlled room (temperature was approximately 23°C and humidity was 60%) with a conventional light/dark cycle. At the age of 7 weeks, 5/6 nephrectomy was performed under anesthesia with sodium pentobarbital (50 mg/kg body weight, i.p.) by ablation of approximately
2/3 of the left kidney, and then the right kidney was removed by ligation of the renal artery, vein and ureter 1 week later. After acclimatization (after 2 weeks), the 5/6 nephrectomized (5/6Nx) rats were divided by the follow-up period and doses of goreisan into six groups to avoid any intergroup differences in body weight and blood urea nitrogen levels.: untreated for 4 weeks control (n=8), untreated for 8 weeks control (n=8), 0.5% goreisan-treated for 4 weeks (n=7), 1% goreisan-treated for 4 weeks (n=7), 0.5% goreisan-treated for 8 weeks (n=7), and 1% goreisan-treated for 8 weeks (n=7). A sham-operated group matching the 5/6Nx group was also included in the study. Rats underwent a sham operation consisting of laparotomy and manipulation of the renal pedicles, and were divided into two groups: one was followed for 4 weeks and the other was followed for 8 weeks (n=3 and n=5). Sham and 5/6Nx groups were fed a standard chow (Labo MR stock, Nossan Corporation, Yokohama Japan), and 0.5% goreisan-treatment or 1% goreisan-treatment groups were fed a standard chow containing goreisan at a dose of 0.5% (w/w) or 1% (w/w), respectively. Sham-operated group was fed a standard chow containing goreisan at a dose of 0.5% (w/w) or 1% (w/w) for 4 weeks. They had free access to both food and water ad libitum. In this study, rats took the goreisan doses of 0.5% and 1% goreisan-containing pellets at about 30 g rat⁻¹ day⁻¹, a dose was a approximately 5- and 10-fold the clinical dose, respectively. The body weight was measured every 7 days. After 4 or 8 weeks of treatment, the rats were sacrificed and blood and Single Voided Urine samples were obtained from each rat (Fig. 2). The remnant kidneys were rapidly excised and the weight of each kidney was measured. Tissues were quickly frozen and kept at -80 °C until analysis.

All experimental procedures were performed in accordance with the standards established by the ‘Guide for the Care and Use of Laboratory Animals at the University of Toyama and were also approved by the Committee on Animal Experimentation, University of Toyama.

**Protein preparation and western blot analysis:** The cortex, outer medulla, and inner medulla were dissected from the frozen kidney, and were homogenized in buffer solution (20 mM Tris-HCl buffer (pH 7.5), 137
mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin). Large tissue debris and nuclear fragments were removed by centrifuge (1,000 x g for 15 min at 4 °C). The pellet was resuspended in 300 μl of buffer solution and recentrifuged under the same conditions. The pellets were discarded, and the supernatants were pooled and centrifuged at 17,000 x g for 30 min at 4°C. The supernatant was collected and centrifuged at 200,000 x g for 1 hour at 4°C. The pellet was resuspended in 200 μl of buffer solution and retained as the plasma-membrane-enriched fraction. The protein content of each sample was determined by a Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts of protein (20 μg) were separated by electrophoresis using a 4-12% SDS-PAGE gel. The gel was transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and blocked in PBS-T containing 5% nonfat dried milk at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat AQP1, AQP2 and AQP3 antibody 1:1,000 (CHEMICON, Temecula, CA, USA) for 1 hour at room temperature, washed, and incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1,000) in PBS-T for 1 hour. Membranes were subsequently washed three times for 15 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti-β-actin antibodies as an internal control. The reactive bands corresponding to AQP1, AQP2, AQP3 and β-actin were visualized with a chemiluminescence system (ECL, GE Healthcare UK Ltd.). Chemiluminescent signals were detected using X-ray film and analyzed using an NIH image program. The band densities of AQP1, AQP2 and AQP3 were normalized by the corresponding band densities of β-actin and were expressed as percentage of the mean values in shams.

**Analysis of blood and urine samples:** Serum and urine biochemical parameters, urea nitrogen and creatinine, were determined using commercial kits (BUN kainos and CRE-EN kainos; Kainos Laboratories Inc., Tokyo, Japan). Urinary protein excretion was determined by a commercial kit (TP-Test Wako; Wako Pure Chemical Industries Ltd., Osaka, Japan). Serum and urine osmolality, serum sodium, serum potassium, serum chloride and serum arginine vasopressin were measured by Bio Medical Laboratories (Tokyo, Japan).

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**Figure 2** Experimental protocol. 5/6 nephrectomy (5/6Nx) was performed in Wistar rats by right 2/3 nephrectomy followed by contralateral nephrectomy. Sham-operated rats were matched to Nx rats. Goreisan-treated Nx rats were fed a standard chow containing goreisan at a dose of 0.5% (w/w) or 1% (w/w) for 4 weeks or 8 weeks after 5/6Nx. Sham and untreated control rats were fed a standard chow. All groups had free access to both food and water ad libitum.
Statistical analysis: Values are presented as mean ± S.E. Statistical differences were determined by one-way analysis of variance with Dunnett’s test for multiple comparisons and those at $P < 0.05$ were accepted as significant.

Results

Body and remnant kidney weights: Changes in body and remnant kidney weights are summarized in Table 1. The body weight was significantly decreased in the 5/6Nx groups compared with the sham groups with the 4 weeks treatment duration. There were no significant differences in weights among the three 5/6Nx groups for both the 4-week and 8-week treatment duration. The remnant kidney weight did not change among the three 5/6Nx groups with the 4-week treatment duration. In contrast, the remnant kidney weight of the 1% gorenisan-treated group for 8 weeks was significantly lower ($P < 0.05$) compared with that of the control group for 8 weeks.

Blood and urine biochemical parameters: Table 2 shows the effects of gorenisan on blood and urine biochemical parameters. The serum levels of urea nitrogen and creatinine in the 5/6Nx groups were significantly higher ($P < 0.05$) than those in the sham groups, but there was no significant difference among the 5/6Nx groups with the 4-week and 8-week treatment duration. The urinary excretion of protein in all 5/6Nx groups was increased ($P < 0.05$) compared with that in the sham groups. The urinary excretion of protein in the 1% gorenisan-treated group for 4 weeks was increased, and that of the 1% gorenisan-treated for 8 weeks group was significantly decreased ($P < 0.05$) compared with that of

<table>
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<tr>
<th>Table 1</th>
<th>Effects of gorenisan on body and tissue weights.</th>
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<td>Body Weight (g)</td>
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<td>4 weeks treatment</td>
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<tr>
<td>Sham</td>
<td>347 ± 21</td>
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<tr>
<td>5/6Nx</td>
<td>301 ± 16*</td>
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<tr>
<td>0.5% Gorenisan</td>
<td>316 ± 34*</td>
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<tr>
<td>1% Gorenisan</td>
<td>313 ± 15*</td>
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<tr>
<td>8 weeks treatment</td>
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<tr>
<td>Sham</td>
<td>381 ± 25</td>
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<tr>
<td>5/6Nx</td>
<td>367 ± 20</td>
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<tr>
<td>0.5% Gorenisan</td>
<td>362 ± 38</td>
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<tr>
<td>1% Gorenisan</td>
<td>380 ± 60</td>
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Data represent mean ± S.E. (n=3-8). Sham-operated rats (Sham, n=3), untreated rats 4 weeks after 5/6 nephrectomy (5/6Nx, n=6), 0.5% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (0.5% gorenisan, n=6), and 1.0% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (1% gorenisan, n=6). Sham-operated rats (Sham, n=4), untreated rats 8 weeks after 5/6 nephrectomy (5/6Nx, n=6), 0.5% gorenisan-treated rats 8 weeks after 5/6 nephrectomy (0.5% gorenisan, n=6), and 1.0% gorenisan-treated rats 8 weeks after 5/6 nephrectomy (1% gorenisan, n=6). *P<0.05 compared with sham; ‡P<0.05 compared with 5/6Nx group.

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<th>Table 2</th>
<th>Effects of gorenisan on biochemical parameters.</th>
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<td>Serum urea nitrogen (mg/dl)</td>
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<td>4 weeks treatment</td>
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<tr>
<td>Sham</td>
<td>20.6 ± 0.15</td>
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<tr>
<td>5/6Nx</td>
<td>32.8 ± 2.41*</td>
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<tr>
<td>0.5% Gorenisan</td>
<td>36.0 ± 1.45*</td>
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<tr>
<td>1% Gorenisan</td>
<td>36.6 ± 2.82*</td>
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</table>

8 weeks treatment

|          | Serum urea nitrogen (mg/dl) | Serum creatinine (mg/dl) | Urinary protein (mg/mg Cr) | Serum sodium (mEq/l) | Serum chloride (mEq/l) | Serum potassium (mEq/l) | Serum osmolality (mosm/kg H2O) | Urinary osmolality (mosm/kg H2O) | Serum arginine vasopressin (pg/ml) |
| Sham | 21.4 ± 1.03 | 0.376 ± 0.035 | 2.53 ± 0.69 | 137.6 ± 1.5 | 101.2 ± 0.8 | 7.6 ± 0.5 | 289.8 ± 1.8 | 1159.3 ± 252.7 | 73.7 ± 9.3 |
| 5/6Nx | 29.1 ± 1.15* | 0.595 ± 0.030* | 4.16 ± 1.69* | 138.8 ± 1.3 | 100.5 ± 1.2 | 7.3 ± 0.5 | 292.3 ± 3.8 | 737.3 ± 187.3* | 78.4 ± 6.9 |
| 0.5% Gorenisan | 28.0 ± 1.28* | 0.569 ± 0.011* | 2.67 ± 0.17 | 140.6 ± 1.0 | 102.4 ± 1.3 | 7.0 ± 0.4 | 292.4 ± 3.4 | 1013.8 ± 109.6 | 52.4 ± 11.2 |
| 1% Gorenisan | 26.5 ± 1.46* | 0.608 ± 0.012* | 2.73 ± 0.80* | 140.3 ± 4.4 | 100.3 ± 3.4 | 5.9 ± 0.2* | 289.9 ± 6.7 | 1035.0 ± 284.0* | 48.6 ± 7.1 |

Data represent mean ± S.E. Sham-operated rats (Sham, n=3), untreated rats 4 weeks after 5/6 nephrectomy (5/6Nx, n=6), 0.5% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (0.5% gorenisan, n=6), and 1.0% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (1% gorenisan, n=6). Sham-operated rats (Sham, n=4), untreated rats 8 weeks after 5/6 nephrectomy (5/6Nx, n=6), 0.5% gorenisan-treated rats 8 weeks after 5/6 nephrectomy (0.5% gorenisan, n=6), and 1.0% gorenisan-treated rats 8 weeks after 5/6 nephrectomy (1% gorenisan, n=6). *P<0.05 compared with sham; ‡P<0.05 compared with 5/6Nx.
the 5/6Nx groups. With regard to serum electrolytes, serum sodium and chloride concentrations were similar among the groups, whereas the serum potassium concentration in the 1% goreisan-treated group was significantly increased compared with that in the 5/6Nx groups. There was no significant difference in serum osmolality among all the groups. The arginine vasopressin concentration in the 0.5% and 1% gorenisan-treated group was significantly increased ($P < 0.05$) compared with that of sham-operated group, although there was no significant difference among the 5/6Nx groups.

However, urine osmolality in the 0.5% and 1% gorenisan-treated groups tended to be lower than that of the control group with 4 weeks treatment duration. Moreover, with 8 weeks treatment duration, the urine osmolality in the 5/6Nx group was significantly decreased ($P < 0.05$) compared with that in the sham group, and that in the 1% gorenisan-treated group was significantly increased ($P < 0.05$) compared with that in the 5/6Nx group.

**Aquaporin 1 expression:** The anti-AQP1 antibody recognize 29 and 35 to 50 kD bands, corresponding to non-glycosylated and glycosylated AQP1, respectively.25 Figure 3, A-C showed an expression of AQP1, did not

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**Figure 3** Effect of gorenisan on AQP1 expression in the kidney.

A: AQP1 expression in the renal inner medulla of sham-operated rats (sham; open bar, n=3), untreated rats 4 weeks after 5/6 nephrectomy (5/6Nx; closed bar, n=6), 0.5% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (0.5%; gray bar, n=6), and 1.0% gorenisan-treated rats 4 weeks after 5/6 nephrectomy (1%; hatched bar, n=6). B: AQP1 expression in the renal outer medulla in 4 weeks. C: AQP1 expression in the renal cortex in 4 weeks. D: AQP1 expression in renal inner medulla in 8 weeks. E: AQP1 expression in the renal outer medulla in 8 weeks. F: AQP1 expression in the renal cortex in 8 weeks. Data are mean ± S.E. *$P<0.05$ compared with sham; **$P<0.05$ compared with 5/6Nx at 4 weeks and 8 weeks, respectively.
significantly differ between sham group and 5/6Nx group in 4 weeks. There was statistically decreased expression of AQP1 in the inner medulla and cortex in 1% goreisan in 4 weeks compared with 5/6Nx group. On the other hands, there was major decreased in AQP1 expression in the outer medulla in 5/6Nx group, when compared with sham group, whereas there was no significant differences in the inner medulla and the cortex expression of AQP1 between sham group and 5/6Nx group with 8 weeks treatment duration (Fig. 3, D-F).

Aquaporin 2 expression: Two immunoreactive bands of 29 and 35 to 50 kD, corresponding to non-glycosylated and glycosylated forms of AQP2, respectively, were recognized as AQP2.

AQP2 expression in the renal inner medulla in the 1% goreisan-treated group was significantly decreased compared with that in the 5/6Nx group (Fig. 4A). In the renal outer medulla, AQP2 expression in the 5/6Nx group was lower than that in the sham group and that in the 0.5% goreisan-treated group was significantly lower than that in the 5/6Nx group (Fig. 4B). AQP2 expression in the renal cortex in the control group was higher than that in the sham group. AQP2 expression in the

Figure 4 Effect of goreisan on AQP2 expression in the kidney.
A: AQP2 expression in the renal inner medulla of sham-operated rats (sham; open bar, n=3), untreated rats 4 weeks after 5/6 nephrectomy (5/6Nx; closed bar, n=6), 0.5% goreisan-treated rats 4 weeks after 5/6 nephrectomy (0.5%; gray bar, n=6), and 1.0% goreisan-treated rats 4 weeks after 5/6 nephrectomy (1%; hatched bar, n=6). B: AQP2 expression in the renal outer medulla in 4 weeks. C: AQP2 expression in the renal cortex in 4 weeks. D: AQP2 expression in renal inner medulla in 8 weeks. E: AQP2 expression in the renal outer medulla in 8 weeks. F: AQP2 expression in the renal cortex in 8 weeks. Data are mean ± S.E. *P<0.05 compared with sham; **P<0.05 compared with 5/6Nx at 4 weeks and 8 weeks, respectively.
renal cortex in the 1% goreisan-treated group was significantly lower than that in the 5/6Nx group (Fig. 4C).

Figure 4, D-F shows AQP2 expression in the kidney with 8 weeks treatment duration. AQP2 expression in the 5/6Nx group was higher than that in the sham group in the renal inner medulla and outer medulla, although this change was not significant. AQP2 expression in the 5/6Nx group in the cortex was significantly higher ($P < 0.05$) than that in the sham group. AQP2 expression in the 0.5% and 1% goreisan-treated groups in renal inner and outer medulla, and that in the 0.5% goreisan-treated group in the cortex was significantly decreased ($P < 0.05$) compared with that in the 5/6Nx group.

Aquaporin 3 expression: The anti-AQP3 antibodies recognize 27 and 33 to 40 kD bands, corresponding to non-glycosylated and glycosylated AQP3, respectively. As shown in Figure 5, A-C, 5/6Nx group demonstrated a significant increase in AQP3 in the outer medulla, when compared with sham group with 4 weeks treatment duration, but the reduction was not significant in the 0.5% and 1% goreisan-treated groups. In contrast, expression of AQP3, at the 4 weeks of treatment period, significantly increased in cortex compared with 5/6Nx group, but markedly restored by administration of 0.5%- and 1% goreisan. Figure 5, D-F shows the expression of AQP3, at the 8 weeks of treatment period, in cortex was

![Figure 5](image)

**Figure 5** Effect of goreisan on AQP3 expression in the kidney.
A: AQP3 expression in the renal inner medulla of sham-operated rats (sham; open bar, n=3), untreated rats 4 weeks after 5/6 nephrectomy (5/6Nx; closed bar, n=6), 0.5% goreisan-treated rats 4 weeks after 5/6 nephrectomy (0.5%; gray bar, n=6), and 1.0% goreisan-treated rats 4 weeks after 5/6 nephrectomy (1%; hatched bar, n=6). B: AQP3 expression in the renal outer medulla in 4 weeks. C: AQP3 expression in the renal cortex in 4 weeks. D: AQP3 expression in renal inner medulla in 8 weeks. E: AQP3 expression in the renal outer medulla in 8 weeks. F: AQP3 expression in the renal cortex in 8 weeks. Data are mean ± S.E. *$P<0.05$ compared with sham; **$P<0.05$ compared with 5/6Nx at 4 weeks and 8 weeks, respectively.
significantly decreased compared with sham-operated group. This down-regulation in AQP3 was markedly reverted in the inner medulla by administration of 0.5% - and 1% goreisan, with the exception of the cortex in 0.5% goreisan treatment. On the other hand, the expression level of AQP3 in outer medulla was significantly greater in response to goreisan than that of 5/6Nx.

Aquaporin 1-3 expression in sham-operated groups:
Sham-operated group was fed a standard chow containing goreisan at a dose of 0.5% or 1% for 4 weeks. Table 3 shows AQP1-3 expression in the renal inner medulla, outer medulla and cortex with 4 weeks treatment duration. There was increased (P < 0.05) in AQP1 expression in the outer medulla in the 1% goreisan-treated group, when compared with sham-operated group, whereas there was no significant differences in protein prepared from the inner medulla and the cortex expression of AQP1 between sham-operated group. There were no significant differences in the expression level of AQP2 and AQP3 between sham-operated group, and goreisan-treated group.

Table 3 Effect of goreisan in sham-operated rats for 4 weeks on AQP1-3 expression in the kidney

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<tr>
<td>AQP1</td>
<td></td>
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<tr>
<td>Sham</td>
<td>1.00 ± 1.19</td>
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<td>0.5% Goreisan</td>
<td>2.89 ± 0.56</td>
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<td>1% Goreisan</td>
<td>2.61 ± 0.47</td>
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<td>AQP2</td>
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<td></td>
<td></td>
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<tr>
<td>Sham</td>
<td>1.00 ± 0.22</td>
<td>1.00 ± 0.36</td>
<td>1.00 ± 0.18</td>
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<tr>
<td>0.5% Goreisan</td>
<td>2.41 ± 0.90</td>
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<td>1% Goreisan</td>
<td>3.53 ± 0.89</td>
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<th>Inner medulla</th>
<th>Outer medulla</th>
<th>Cortex</th>
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<td>AQP3</td>
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<tr>
<td>Sham</td>
<td>1.00 ± 0.68</td>
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<td>0.5% Goreisan</td>
<td>1.05 ± 0.11</td>
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<td>1% Goreisan</td>
<td>2.21 ± 1.09</td>
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Data represent mean ± S.E. AQP1-3 expression in renal inner medulla, outer medulla and cortex of sham-operated rats (Sham, n=3), 0.5% goreisan-treated rats 4 weeks after sham operation, (0.5% goreisan, n=5), and 1% goreisan-treated rats 4 weeks after sham operation, (1% goreisan, n=5). *P<0.05 compared with sham.

Discussion

In this study, 5/6 nephrectomized rats were studied to investigate the effects of goreisan on AQP2 expression. The 5/6 nephrectomy model is frequently used as an animal model, which induces chronic renal failure (CRF). The underlying concept of this model is that the overload to the remaining nephrons after reduction of kidney mass results in progressive renal damage leading to terminal renal failure.26 The time course of this progression in the 5/6Nx model is divided into three phases with an early phase, a stable phase, and a late phase. The early phase (1-2 weeks after 5/6Nx) is characterized by a significant and rapid rise of serum creatinine and blood urea nitrogen (BUN) levels. A steady deterioration of renal function is observed in the stable phase (3-10 weeks after 5/6Nx). In the late phase (after the 10th week of 5/6Nx), further impaired renal function is observed.27,28 In humans, water balance disorders showing various symptoms such as edema and polyuria, are observed from the early stage to end-stage of renal disease (ESRD). Moreover, goreisan has been frequently used to treat the early or the progressive stage of renal disease, but not the end-stage of renal disease (except for patients accepting dialysis). Therefore, in this study, we chose a goreisan treatment duration of 4 and 8 weeks, and these times corresponded to the in-between stage and end stage of the stable phase.

The urinary concentrating ability in the kidney is closely related to water balance of the whole body. In the present study, the serum and the urinary osmolalities were measured as markers of the urinary concentrating ability. The serum osmolality was not changed by 5/6Nx, and no significant influence with goreisan-treatment was observed. On the other hand, urinary osmolality in 5/6Nx rats was decreased, and that in 8-week treatment group was significantly decreased. Our results of urine osmolality in 5/6Nx rats are similar to previous reports.13,14,29-32 The influence of goreisan on urinary osmolality varied from 4 weeks to 8 weeks of goreisan-treatment. Urine osmolality that was decreased by 5/6Nx was further decreased by goreisan-treatment for 4 weeks, although there was no significant difference. In contrast, with goreisan-treatment for 8 weeks, urine osmolality was increased, and a significant increase was obtained with 1% goreisan-treatment.
compared with that in the control group. The reason for this finding could be due to the histological differences of remnant kidneys that might have influenced the effect of goreisan. The 5/6Nx rat is a CRF model in which glomerulosclerosis and the tubulointerstitial fibrosis gradually progress after 5/6Nx. Therefore, different conditions of the targets of goreisan might influence the effect of goreisan on urinary osmolality. On the other hand, goreisan is not a simple diuretic drug, but is a water balance modulator ("risuizai" in Kampo medicine) and it has been reported to show different effects with different conditions of water balance such as dehydration and overhydration. Therefore, the different conditions of water balance between 4 weeks and 8 weeks of goreisan-treatment might be associated with the different effects of goreisan. To clarify the mechanisms of the different effects of goreisan on urinary osmolality over time, it is necessary to examine water balance in the whole body at various elapsed times.

Collecting duct water reabsorption has an important part to play in urinary concentrating ability, and it is regulated by vasopressin. AQP2 is the primary target for vasopressin regulation of collecting duct water reabsorption. Previous studies examining AQP2 expression in 5/6Nx rats reported that AQP2 expression was decreased while another study reported that AQP2 expression did not change. In this study, serum vasopressin did not change. In the former reports, AQP2 expression was examined at 2 weeks after 5/6Nx, and in the latter report, AQP2 expression was examined at 7 weeks after 5/6Nx. It is possible that the difference in AQP2 expression after 5/6Nx between the previous studies was caused by the difference in elapsed time after 5/6Nx. In the present study, AQP2 expression in the renal inner medulla, outer medulla and cortex were investigated by western blotting. Although it was not significant, AQP2 expression in the inner medulla and cortex tended to be increased, and that in the outer medulla was decreased 4 weeks after 5/6Nx. On the other hand, AQP2 expression in all areas was increased 8 weeks after 5/6Nx compared with that in the sham group, and there was a significant increase in AQP2 expression in the cortex. Our study was the first to determine the influence of 5/6Nx on AQP2 expression in the inner medulla, outer medulla, and cortex.

We found that AQP2 expression in all areas was decreased both by goreisan-treatment for 4 weeks and by goreisan-treatment for 8 weeks. It is unclear what the mechanism is for the reduction in AQP2 expression by goreisan. AQP2 is regulated mainly by vasopressin through the vasopressin V2 receptor (V2R). It has been reported that AQP2 and V2R mRNA expressions in the normal rat kidney are decreased by the aqueous extract of Polyporus, which is one of the crude drug components of goreisan, while AQP1 and AQP3 mRNA expression are unchanged. In addition, vasopressin-stimulated cAMP production is attenuated in renal tissue in rats treated with saireito, which is composed of shosaikoto and goreisan, and stimulation of V2R might be inhibited by saireito. It is possible that the decreased V2R mRNA expression associated with the decrease in AQP2 expression by goreisan, although V2R mRNA expression was not measured in our study.

With regard to the relationship between the change in urinary osmolality and decreased AQP2 expression by goreisan-treatment, the decrease in AQP2 by goreisan-treatment for 4 weeks could explain the decrease in urine osmolality. However, when goreisan-treatment was given for 8 weeks, we did not observe an increase in AQP2 expression as expected from the results of increased urinary osmolality, but observed a decrease instead. The reason for this discrepancy could be because not only AQP2 but also other aquaporins, Na+-K+ ATPase, and urea transporter have important roles to play in urinary dilution and urinary concentration. Therefore, it is possible that these transporters, except for AQP2, were associated with the increased urinary osmolality by goreisan-treatment for 8 weeks. Furthermore, we demonstrated that AQP1 and AQP3 expressions were determined in the renal inner medulla, outer medulla and cortex by western blotting. There were no significant differences in AQP1 among the three 5/6Nx groups for both the 4-week and 8-week treatment duration. The expression level of AQP1 was lower in the outer medulla in treated with goreisan in 4 weeks than 5/6Nx, did not appear markedly different compared with 5/6Nx group. In contract, expression of AQP3, at the 4 weeks of treatment period, significantly increased in cortex compared with 5/6Nx group treated with 0.5% and 1% goreisan. The results in the present study suggest that goreisan have a major effect to change AQP3 expression to play an important role in cortex.
Nevertheless, further studies are required to clarify the reasons for this discrepancy in our results.

The effects of goreisan-treatment for 4 weeks on AQP2 expression in the inner medulla and the outer medulla in 1% goreisan-treated group was higher than in 0.5% goreisan-treated group, and those for 8 weeks on AQP2 expression in the cortex were also. Goreisan is not used as a simple diuretic but as a water balance modulator. Because it is necessary to increase and decrease urine volume for modulating water balance in the whole body, goreisan has to contain both the components that increase AQP2 expression as well as the components that decrease AQP2 expression. Moreover, each target threshold of goreisan’s components may be different. Therefore, the dose-independent effect of goreisan on AQP2 expression might be caused by many components and different target thresholds.

Furthermore, we demonstrated that AQP1-3 expression in sham-oparated rats by administration of 0.5% and 1% goreisan for 4 weeks. Although, there was increased in AQP1 expression in the outer medulla in the 1% goreisan-treated group, when compared with sham group, the expression level of AQP2 and AQP3 were no significant differences between sham group and goreisan-treated group. The results suggest that AQP expression is not concerned with goreisan treatment in normal conditions.

To examine the influence of goreisan on the renal function of 5/6Nx rats, we measured the remnant kidney weight, BUN, serum creatinine and urinary protein. The remnant kidney weight was significantly decreased by administration of 1% goreisan for 8 weeks. Moreover, there was no significant change in BUN and serum creatinine levels. As mentioned previously, an increased remnant kidney weight after 5/6Nx might be due to compensatory hypertrophy in 2-4 weeks, minimal changes in residual renal tissue in 4-10 weeks, and glomerulosclerosis and interstitial fibrosis beyond 10 weeks after 5/6Nx.26,27 Because the time point after 8 weeks of goreisan-treatment in our study corresponds to just before the late phase, our findings of an unchanged remnant kidney weight and stationary BUN and serum creatinine levels suggested that goreisan had not deteriorated renal function.

Proteinuria is commonly used to evaluate the clinical course and to investigate the pathogenic mechanisms of CRF. In the present study, the urinary excretion of protein was significantly changed by 1% goreisan-treatment. The increase in urinary excretion of protein by 1% goreisan-treatment for 4 weeks suggests that goreisan might deteriorate renal function in 5/6Nx rats. However, a significant decrease in urinary excretion of protein was observed with 1% goreisan-treatment for 8 weeks. We believe that goreisan may ameliorate renal dysfunction, taking into account the results of proteinuria, remnant kidney weight, and BUN and serum creatinine levels.

In conclusion, we determined that goreisan-treatment for 4 and 8 weeks changed urinary osmolality and decreased AQP2 and increased AQP3 expression in cortex in 5/6Nx rats without causing renal dysfunction. The results in the present study suggest that AQP expression is associated with the effects of goreisan on the modulation of water balance.

References

7) Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F. and Sasaki, S.: Cloning and expression of apical


