JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1205–1212

Received December 22, 2004
Accepted May 3, 2005
DOI 10.1211/jpp.57.9.0016
ISSN 0022-3573

Long-term treatment with Hachimi-jio-gan attenuates kidney damage in spontaneously diabetic WBN/Kob rats

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Abstract

Diabetes mellitus is now the most common cause of end-stage renal failure. In this study, the effects of Hachimi-jio-gan on diabetic kidney damage in spontaneously diabetic WBN/Kob rats were examined. Oral administration of Hachimi-jio-gan to WBN/Kob rats for 25 weeks significantly suppressed urinary protein excretion. It did not affect body weight loss or blood glucose levels, whereas it reversed the increase in kidney weight of WBN/Kob rats. Hachimi-jio-gan also reduced fibronectin and transforming growth factor β_1 (TGF-β_1) protein expression in the renal cortex. Furthermore, renal lipid peroxidation levels of WBN/Kob rats given Hachimi-jio-gan were significantly lower than those of untreated controls. Renal superoxide dismutase activity was elevated by Hachimi-jio-gan treatment in a dose-dependent manner. These results suggested that Hachimi-jio-gan could prevent diabetic kidney damage by reducing renal oxidative injury and expression of fibronectin and TGF-β_1 proteins, which are all involved in the pathophysiology of diabetic nephropathy.

Introduction

Diabetic nephropathy has been postulated to be one of the major problems in diabetic patients. The number of patients started on dialysis therapy for diabetic nephropathy continues to increase every year. Current clinical practice to prevent and treat diabetic nephropathy involves the use of agents such as angiotensin-converting enzyme inhibitors, angiotensin-II receptor blockers and antihypertensive drugs (Parving et al 1983; Preston 1999; Brenner et al 2001), but despite these remedies, large numbers of patients are still suffering from diabetic nephropathy. Therefore, searching for therapeutic interventions that will improve the specific alterations associated with diabetic nephropathy represents one of the most promising areas of research today.

For thousands of years, people have benefited a great deal from herbal medicines, which have been used traditionally to alleviate subjective symptoms of various diseases. They are still valuable for human health and, due to their varied biological activity and low toxicity, have received much attention as potential sources of new therapeutic agents. Hikiami et al (2000) investigated the effects of traditional herbal medicines on the development and progression of diabetic complications, including neuropathy, retinopathy and nephropathy, in 141 patients with type 2 diabetes. They also analysed which kinds of traditional herbal medicines were prescribed, in terms of the phases of diabetic nephropathy and the duration of morbidity associated with diabetes mellitus, and found that Hachimi-jio-gan was prescribed frequently. In animal experiments, we demonstrated the effects of traditional herbal medicines, including Hachimi-jio-gan, Keishi-bukuryo-gan, Ompi-to and Sairei-to, in rats subjected to subtotal nephrectomy followed by streptozotocin (STZ) injection, by measuring the biochemical parameters affected by persistent hyperglycaemia (Nakagawa et al 2001, 2003; Yokozawa et al 2004). In the light of the above findings, Hachimi-jio-gan appeared to be a potential agent to prevent and treat diabetic nephropathy.

We have carried out a long-term study in spontaneously diabetic WBN/Kob rats to determine whether Hachimi-jio-gan could prevent renal pathophysiological changes under diabetic conditions.

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Materials and Methods

Preparation of Hachimi-jio-gan extract

The Hachimi-jio-gan extract used was produced by Tsumura Juntendo Inc., Tokyo, Japan. The composition of Hachimi-jio-gan was as follows: Rehmanniae Radix (*Rehmannia glutinosa* Libosch. var. *purpurea* Makino), 6 g; Corni Fructus (*Cornus officinalis* Sieb. et Zucc.), 3 g; Dioscoreae Rhizoma (*Dioscorea japonica* Thunb.), 3 g; Alismatis Rhizoma (*Alisma orientale* Juzep.), 3 g; Hoelen (*Poria cocos* Wolf), 3 g; Moutan Cortex (*Paeonia suffruticosa* Andrews), 2.5 g; Cinnamomi Cortex (*Cinnamomum cassia* Blume), 1 g; and Aconiti Tuber (*Aconitum carmichaeli* Debx.), 0.5 g. These crude drugs were boiled gently in 10-times their volume of water for 60 min and then filtered. The filtrate was spray-dried to obtain the extract at a yield of approximately 10%, by weight, of the original preparation. For the analysis of the components of Hachimi-jio-gan, the aqueous extract (0.5 g) was extracted with 20 mL methanol under ultrasonication for 30 min. The solution was filtered through a membrane filter (0.45 μm) and then subjected to high-performance liquid chromatography (HPLC) analysis using a TSK-GEL ODS-80TS column (φ4.6 $\times$ 250 mm, Tosoh, Japan) with an LC $10AD_{vp}$ pump and a SPD-$M10A_{vp}$ absorbance detector. The elution solvents were (A) 0.05 M AcOH-$AcONH_4$ and (B) CH_3CN, and the column was eluted with a linear gradient of, by volume, 90% A and 10% B changing over 60 min to 100% B. The flow rate was 1.0 mL min^{-1} and the effluent from the column was monitored and processed into three-dimensional data by an SPD-M10A array detector. All assigned peaks were identified by comparing their UV spectral data with those of co-injected authentic samples using the Class LC-10 Version 1.62 software package (Shimadzu, Japan). The three-dimensional HPLC profile of Hachimi-jio-gan extract is shown in Figure 1. The major components of Hachimi-jio-gan detected were morroniside, loganin and paeoniflorin. Penta-*O*-galloylglucose, benzoylmesaconine, benzoylpaeoniflorin, 16-ketoalisol A, paeonol, cinnamic acid and cinnamaldehyde were also observed.

Animals and treatments

The Guidelines for Animal Experimentation approved by Toyama Medical and Pharmaceutical University were followed in these experiments. Male Wistar strain WBN/Kob rats were purchased from Japan SLC Inc. (Hamamatsu, Japan) and kept in an automatically controlled room (temperature approximately 23°C and humidity approximately 60%) with a conventional dark/light cycle. They were fed a diet (LABO MR-DBT) obtained from Nosan Corporation (Yokohama, Japan). The blood glucose levels of the male WBN/Kob rats were monitored continuously and nearly 100% of these rats were diagnosed with diabetes (blood glucose >200 mg dL^{-1}). When they were 50-weeks-old, the diabetic rats were divided into three groups (one control and two treatment groups) of twelve rats, avoiding intergroup differences in blood glucose levels. Hachimi-jio-gan was added to the diet at a concentration of 0.3% or 1%, by weight, and administered for 25 weeks. During this experimental period, all rats were given the diet in a pair-feeding manner and the Hachimi-jio-gan intakes of the 0.3% and 1% Hachimi-jio-gan-treated groups were estimated to be approximately 0.09 and 0.31 g/rat, respectively. The body weights and blood glucose levels were monitored every five weeks over the 25-week experimental period and 24-h urine samples were collected in metabolic cages at 10 and 20 weeks. At the end of the experimental period, the rats were killed, blood samples were obtained from each rat and centrifuged immediately. Subsequently, the required tissues were removed, quickly frozen and kept at -80°C until analysis. Age-matched male Wistar rats ($n = 5$) were used as a normal group.

Determination of blood and urine component levels

Blood glucose, urea nitrogen and creatinine (Cr) levels were determined using commercial reagents (Glucose CII-Test Wako obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan; BUN Kainos and CRE-EN Kainos obtained from Kainos Laboratories Inc., Tokyo, Japan). Urinary protein excretion was determined by the sulfosalicylic acid method (Sakagishi 1968).

Determination of thiobarbituric acid (TBA)-reactive substance levels

Serum TBA-reactive substance levels were measured using the method of Naito & Yamanaka (1978) and renal TBA-reactive substance levels were assayed according to the method of Mihara & Uchiyama (1978).

Superoxide dismutase (SOD) activity assay

Kidney tissue was homogenized in 9 vols ice-cold physiological saline. The SOD activity of each homogenate was measured using the nitrous acid method described by Elstner & Heupel (1976) and Oyanagui (1984), which is based on inhibition of nitrite formation from hydroxylamine in the presence of superoxide (O_2^-) generators. Protein levels were determined by the method of Itzhaki & Gill (1964) with bovine serum albumin as the standard.

Western blot analyses

Proteins were extracted from the renal cortex with a lysis buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin). The concentration of protein in the extract was determined using a Bio-Rad protein assay kit (Bio-Rad). Samples of the protein extract (10 μg for fibronectin analysis and 40 μg for transforming growth factor β_1 (TGF-β_1) analysis) were

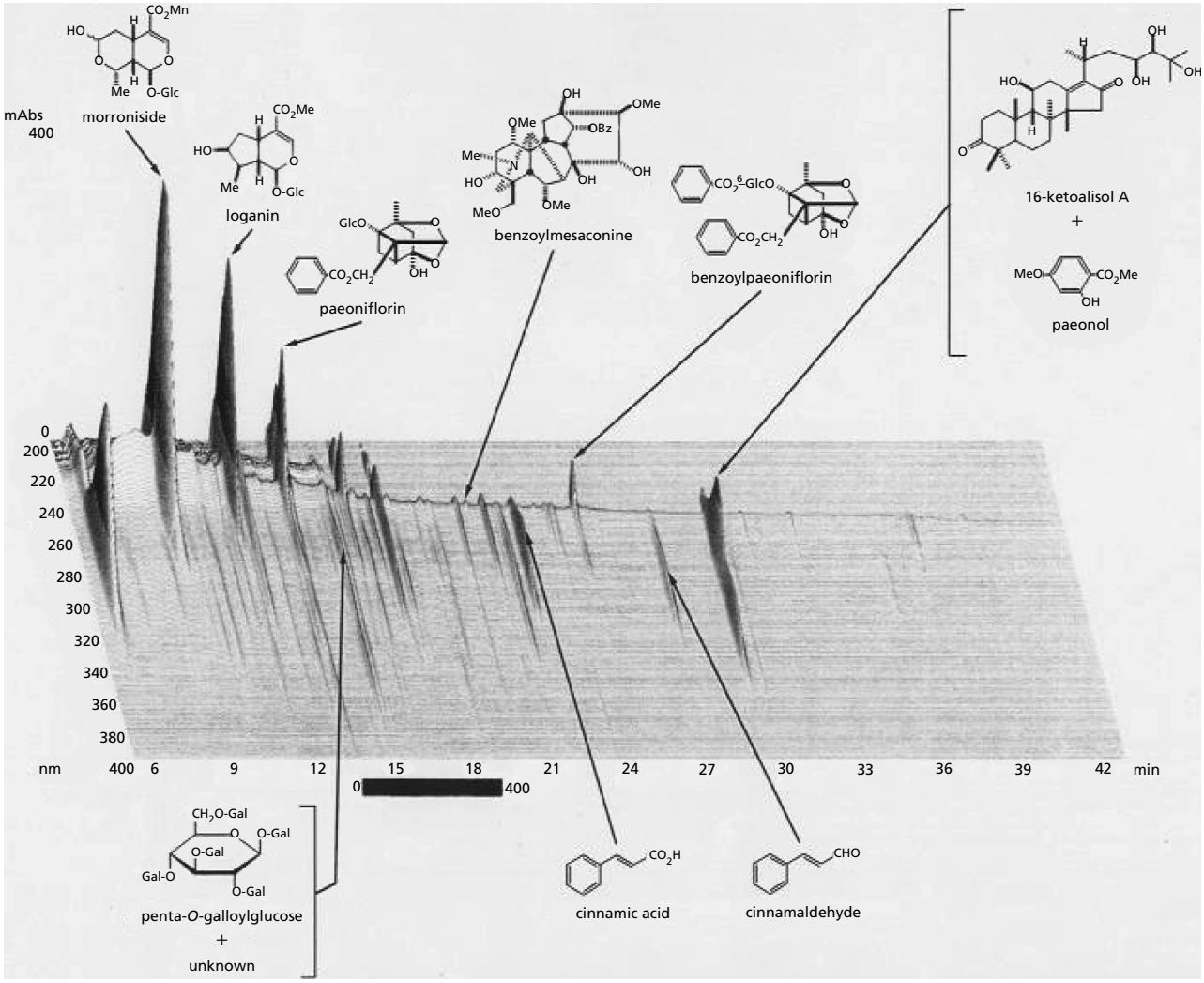

Figure 1 Three-dimensional HPLC profile of Hachimi-jio-gan extract.

separated by electrophoresis using a 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. After blocking with 5% skim milk solution for 1 h, the membrane was reacted with anti-human fibronectin antibody (Dako Co), anti-human TGF-β_1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-mouse β-actin antibody (Sigma, St Louis, MO, USA) overnight at 4°C. Subsequently, it was incubated with horseradish peroxidase-conjugated IgG (Amersham Bioscience, Piscataway, NJ, USA) for 90 min at room temperature and then treated with chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ, USA). Chemiluminescent signals were detected using X-ray film and analysed using the NIH Image program. The data for individual rats were corrected for β-actin. The mean values for fibronectin and TGF-β_1 protein levels of the age-matched Wistar rats were represented as 1 and the corresponding values for the diabetic rats were expressed as ratios of these values.

Statistics

Values were presented as means ± s.e. The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were analysed statistically by Dunnett's test and those at $P < 0.05$ were accepted as significant.

Results

Body and kidney weights, urine volume and blood glucose levels

The changes in the body and kidney weights and urine volumes of each group are summarized in Table 1. The initial and final body weights and body weight gains of the rats with spontaneous diabetes were significantly lower than those of the normal rats. Oral administration of Hachimi-jio-gan for 25 weeks did not change the body weight parameters. However, the kidney weight of the

Table 1 Body and kidney weights, and urine volume for the different treatment groups

Group	Body weight			Kidney weight (g/100 g)	Urine volume (mL/day)
	Initial (g)	Final (g)	Gain (g)		
Wistar rats	541.6 ± 14.4	639.6 ± 27.1	98.0 ± 22.0	0.527 ± 0.021	14.6 ± 1.8
WBN/Kob rats					
Control	370.0 ± 13.2*	349.6 ± 8.8*	−20.2 ± 6.3*	1.015 ± 0.033*	210.8 ± 15.5*
0.3% Hachimi-jio-gan	379.3 ± 9.3*	346.2 ± 8.1*	−33.2 ± 11.7*	0.943 ± 0.048*	198.8 ± 27.8*
1.0% Hachimi-jio-gan	376.5 ± 11.9*	356.0 ± 8.9*	−20.5 ± 12.3*	0.931 ± 0.051*#	177.1 ± 27.0*

Values are mean ± s.e. *$P < 0.001$ vs Wistar rats; #$P < 0.05$ vs WBN/Kob control rats.

control diabetic WBN/Kob rats increased significantly compared with that of the normal rats, whereas Hachimi-jio-gan treatment reduced the kidney weight significantly. The urine volume of the control diabetic WBN/Kob rats also increased markedly. Hachimi-jio-gan treatment tended to reduce it, although this effect failed to reach significance. As shown in Figure 2, the blood glucose levels of control diabetic WBN/Kob rats were significantly higher than those of normal Wistar rats during the whole experimental period. Administration of 1.0% Hachimi-jio-gan from 5 to 15 weeks reduced these levels significantly.

Serum urea nitrogen and creatinine levels, and urinary protein excretion

Table 2 shows the effects of Hachimi-jio-gan on serum levels of urea nitrogen and Cr. After oral administration of Hachimi-jio-gan at the 0.3% and 1.0% doses for 25 weeks, urea nitrogen levels were significantly reduced from 16.3 mg dL^{-1} to 14.8 and 14.9 mg dL^{-1}, respectively. Serum Cr levels were not changed by Hachimi-jio-gan administration. As shown in Figure 3, at 10 weeks, the urinary protein excretion rates of normal Wistar rats and control diabetic WBN/Kob rats were 10.0 and 18.3 mg/day, respectively, but there were no significant differences between the untreated and Hachimi-jio-gan-treated groups of WBN/Kob rats. After 20 weeks, the urinary protein content of the control diabetic rats was approximately 2.1-times higher than that after 10 weeks, reflecting the progression of diabetic kidney damage. However, 1.0% Hachimi-jio-gan treatment inhibited this increase significantly.

TBA-reactive substance levels in the serum and kidney, and renal SOD activity

Table 2 shows the effects of Hachimi-jio-gan on the lipid peroxidation levels. These levels were significantly elevated in diabetic WBN/Kob rats. Hachimi-jio-gan administration for 25 weeks tended to reduce the serum lipid peroxidation levels, but the reductions were not significant, whereas the renal lipid peroxidation level declined significantly from 1.99 to 1.74 nmol (mg protein)$^{-1}$ (13%

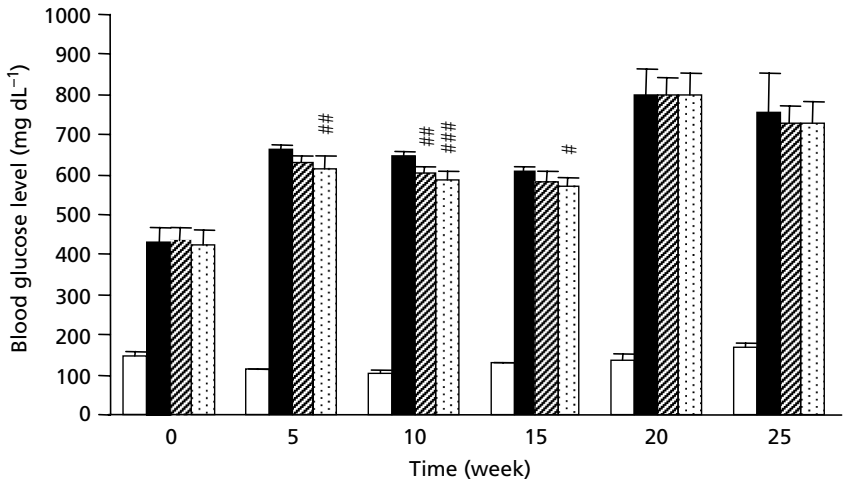

Figure 2 Blood glucose levels in Wistar rats (□) and in diabetic WBN/Kob rats (control, ■; 0.3% Hachimi-jio-gan, ▨; 1.0% Hachimi-jio-gan, ⬚). #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs WBN/Kob control group.

Table 2 Biochemical parameters in serum and kidney at 25 weeks for the different treatment groups

Group	Serum urea nitrogen ($mg\,dL^{-1}$)	Serum creatinine ($mg\,dL^{-1}$)	Serum TBA-reactive substance ($nmol\,mL^{-1}$)	Renal TBA-reactive substance (nmol (mg protein)$^{-1}$)	Renal SOD activity (U (mg protein)$^{-1}$)
Wistar rats	11.2 ± 0.5	0.553 ± 0.033	2.76 ± 0.18	1.47 ± 0.03	31.3 ± 2.9
WBN/Kob rats					
Control	16.3 ± 1.0***	0.419 ± 0.026***	4.08 ± 0.40***	1.99 ± 0.17***	20.2 ± 2.4***
0.3% Hachimi-jio-gan	14.8 ± 0.3***$^{\#}$	0.388 ± 0.027***	3.90 ± 0.36***	1.74 ± 0.12*$^{\#}$	22.6 ± 3.1**
1.0% Hachimi-jio-gan	14.9 ± 0.3***$^{\#}$	0.385 ± 0.017***	3.60 ± 0.32*	$1.54 \pm 0.07^{\#\#}$	25.4 ± 3.4*$^{\#}$

Values are mean $\pm$ s.e. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs Wistar rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs WBN/Kob control rats.

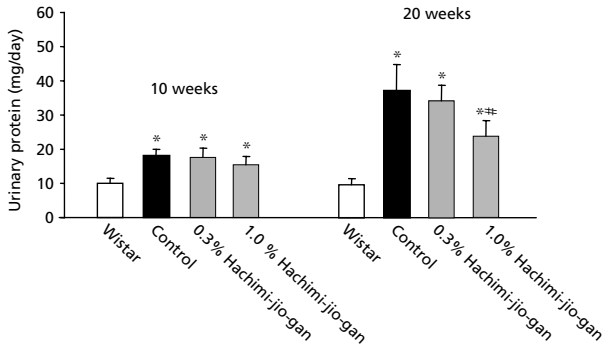

Figure 3 Urinary protein excretion. *$P < 0.001$ vs Wistar rats; $^{\#}P < 0.05$ vs WBN/Kob control rats.

decrease, $P < 0.05$) and 1.54 nmol (mg protein)$^{-1}$ (23% decrease, $P < 0.001$) after administration of the 0.3% and 1.0% doses, respectively. As shown in Table 2, the renal SOD activity of the untreated diabetic WBN/Kob rats was significantly lower compared with normal Wistar rats. The renal SOD activity was increased significantly from 20.2 to 25.4 U (mg protein)$^{-1}$ by 1.0% Hachimi-jio-gan treatment.

Fibronectin and TGF-β_1 protein expression

The expression of fibronectin and TGF-β_1 proteins, at 25 weeks of the administration period, increased significantly in the diabetic renal cortex (Figure 4). Administration of 0.3% Hachimi-jio-gan did not change the fibronectin protein levels, whereas a significant reduction was observed after treatment with 1.0% Hachimi-jio-gan (Figure 4A). Similarly, the increase in TGF-β_1 protein expression was reduced by 1.0% Hachimi-jio-gan administration (Figure 4B).

Discussion

The WBN/Kob rat is an animal model of spontaneously developing diabetes mellitus and, without insulin therapy, it lives for a long time with hyperglycaemia. As a result, several complications have been observed in these rats (Ishizaki et al 1987; Mori et al 1992). In this study, we have investigated the effects of Hachimi-jio-gan on the development of diabetic kidney damage in WBN/Kob rats.

Our WBN/Kob rats had typical characteristics of diabetes mellitus, such as hyperglycaemia, polyuria and growth retardation. Oral administration of Hachimi-jio-gan was not observed to have an obvious effect on blood glucose levels. Body weight gain during the 25-week treatment period was not affected by Hachimi-jio-gan, whereas polyuria showed a tendency to be suppressed, although not significantly. Proteinuria is a powerful predictor of nephropathy in diabetic patients. The appearance of increased urinary protein excretion results from a lesion in the glomerular basement membrane and is thus a sign of renal disorders. To examine the effect of Hachimi-jio-gan, urinary protein excretion was determined after 10 and 20 weeks of treatment. In comparison with age-matched Wistar rats, the urinary protein content of WBN/Kob rats increased as the experimental period progressed, indicating that renal function had deteriorated during the long-term morbidity period of diabetes mellitus. After 10 weeks of treatment, there were no significant differences among the urinary protein levels of the three WBN/Kob groups. However, after oral administration for 20 weeks, Hachimi-jio-gan suppressed the increase in proteinuria significantly. As the additional parameters of renal function, we examined serum urea nitrogen, a waste product of the kidney, and serum Cr levels, representing the glomerular filtration rate. Hachimi-jio-gan significantly improved urea nitrogen levels, whereas Cr levels did not change significantly.

In the diabetic kidney, excessive deposition of extracellular matrix (ECM) proteins, such as type IV collagen, laminin and fibronectin, and subsequent mesangial expansion were frequently observed as the duration of diabetes increased (Mauer et al 1984; Steffes et al 1989). These structural changes contribute to deterioration of renal function and glomerulosclerosis. TGF-β_1 is a cytokine that regulates the production of ECM proteins. It has been reported that the expression of TGF-β_1 and ECM proteins is increased with the high glucose-stimulated renal cells and diabetic animal models, and TGF-β_1 has been widely accepted as playing a central role in these pathophysiological processes (Ignotz

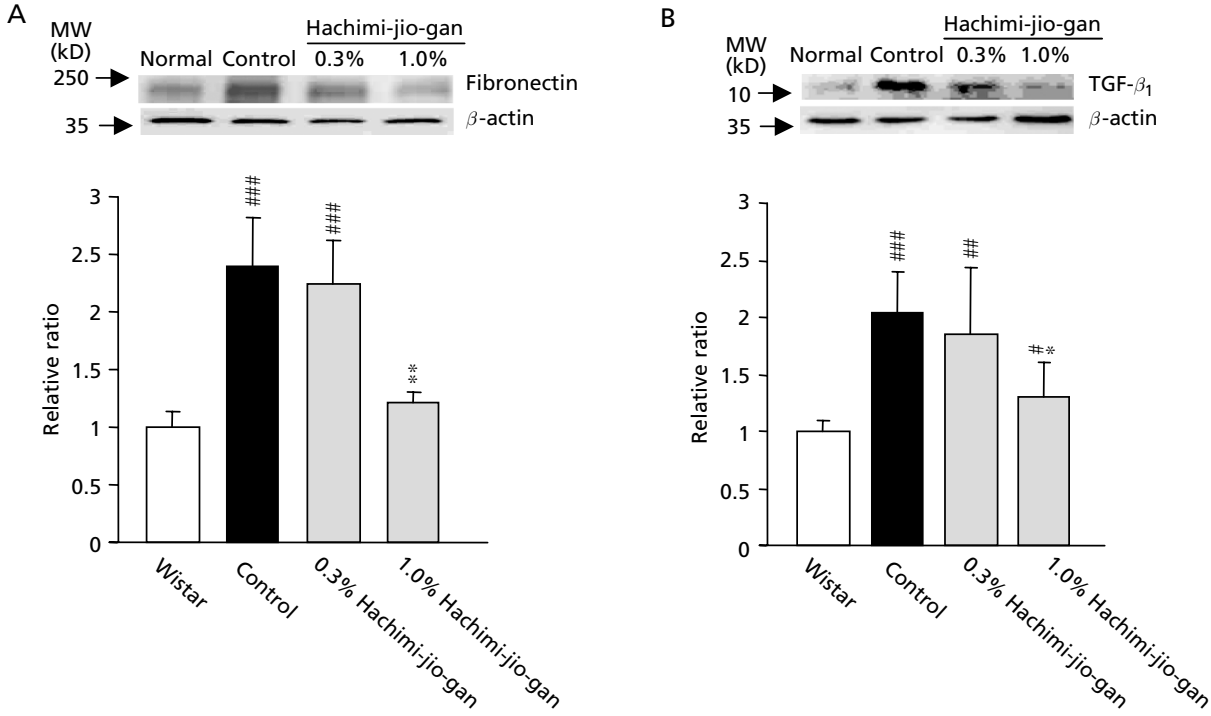

Figure 4 Western blot analysis of fibronectin (A) and TGF-β_1 (B) in renal cortex at 25 weeks. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Wistar rats; $^{*}P < 0.05$, $^{**}P < 0.001$ vs WBN/Kob control rats.

et al 1987; Ziyadeh et al 1994; Sharma & Ziyadeh 1995). In this study, the amounts of fibronectin and TGF-β_1 protein in the renal cortices of WBN/Kob rats also increased as the experiment progressed. Hachimi-jio-gan treatment significantly suppressed fibronectin and TGF-β_1 protein expression. The results confirmed the usefulness of the WBN/Kob rat as a model of diabetic nephropathy and showed that long-term treatment with Hachimi-jio-gan delayed the deterioration of renal function and fibronectin deposition in which TGF-β_1 is closely involved.

The pathogenesis of diabetic nephropathy has been discussed extensively for years. Numerous studies have indicated that large amounts of reactive oxygen species (ROS) are produced in the diabetic body and mediate serious damage to lipids, proteins and DNA, resulting in cellular and tissue injury (Baynes 1991; Ha & Kim 1995; Giugliano et al 1996). Indeed, increased levels of biomarkers of oxidative damage, including lipid peroxidation, advanced glycation end products and 8-hydroxy-2′-deoxyguanosine, were observed in the serum, kidneys and urine of diabetic subjects (Sato et al 1979; Kakimoto et al 2002). Ha & Kim (1995) demonstrated that the concentrations of lipid peroxides in the blood plasma and urine of rats with STZ-induced diabetes were higher than those of non-diabetic rats and, concurrently, proteinuria increased in the former. They noted that a correlation between proteinuria and plasma glucose levels was lacking, but there was a strong correlation between proteinuria and lipid peroxidation levels, suggesting that oxidative stress caused the development of diabetic nephropathy. Additionally, Nath et al (1998) reported that a chronic pro-oxidant state induced renal expression of mRNAs for ECM proteins and TGF-β_1 in-vivo and in-vitro. Vitamin E, an antioxidant, has been shown to reduce urinary albumin excretion and prevent the increase in glomerular TGF-β_1 immunoreactivity in diabetic rats (Craven et al 1997; Koya et al 1997). Moreover, ECM protein synthesis induced by high glucose levels was effectively attenuated by antioxidants (Trachtman et al 1993, 1995; Trachtman 1994). Based on the above reports, oxidative stress induced by diabetes has been considered to be a common pathogenetic factor of diabetic nephropathy, and the attenuation of oxidative stress may improve pathological conditions and prevent further development of diabetic nephropathy. In view of these findings, the use of antioxidants without toxicity, such as traditional herbal medicines and crude drug and food components, for people with diabetes is receiving much attention. Therefore, we examined the antioxidative effects of Hachimi-jio-gan in spontaneously diabetic rats.

In this study, we measured the serum and renal TBA-reactive substance levels, which are well-accepted biomarkers for lipid oxidative damage. Lipid peroxidation levels in the serum and kidney have been shown to be higher in untreated diabetic WBN/Kob rats than age-matched Wistar rats. Following treatment with Hachimi-jio-gan, serum lipid peroxidation levels showed a tendency to decrease, whereas in the kidney they were reduced significantly in a dose-dependent manner. In particular, the level of the 1.0%-treated group declined to near the normal level, indicating that Hachimi-jio-gan exerted antioxidative activity in the kidneys of diabetic rats. Similar

results were observed in our previous study in rats subjected to sub-total nephrectomy followed by STZ injection, which resulted in progressive renal lesions (Yokozawa et al 2004). Additionally, we found that Hachimi-jio-gan treatment resulted in significantly elevated SOD activity compared with the untreated control group. SOD, which is a scavenger of O_2^-, plays a key role in the endogenous defense system against ROS. Several studies have demonstrated that renal SOD activity of rats with STZ-induced diabetes was lower than that of non-diabetic rats, indicating that reduced SOD activity was associated with enhanced oxidative stress in diabetes (Loven et al 1986; Wohaieb & Godin 1987). These results suggested that the increased SOD activity after Hachimi-jio-gan treatment contributed to enhancement of the antioxidative defense system, thereby reducing lipid oxidative damage.

In summary, in spontaneously diabetic rats Hachimi-jio-gan preserved renal function and suppressed expression of fibronectin and TGF-β_1 proteins in the kidney, resulting in retardation of diabetic nephropathy. Our results suggested that antioxidative properties of Hachimi-jio-gan were involved in its renoprotective effects. These findings indicated that this herbal medicine might have potential as a therapeutic agent for diabetic nephropathy, although further studies are needed to fully understand the pharmacological mechanisms responsible for its effects.

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