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Review

A novel therapeutic approach of Hachimi-jio-gan to diabetes and its complications

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SUMMARY

Great efforts have been made to improve both the quality of life and life expectancy of diabetes by treating problems associated with chronic complications such as neuropathy, retinopathy and nephropathy. In particular, diabetes is an increased risk of developing several types of kidney disease, and the predominant cause of end-stage renal disease in patients with this disorder is diabetic nephropathy. Therefore, prevention of the occurrence and progression of diabetes and its complications has become a very important issue. The scientific observations of an animal model of streptozotocin-induced diabetes, spontaneously occurring diabetes and diabetic nephropathy in this study suggest that one of the Kampo prescriptions, Hachimi-jio-gan comprising eight constituents, is a novel therapeutic agent.

Key words: Hachimi-jio-gan; Diabetes; Diabetic nephropathy; Rat

Diabetes is a general term referring to disorders characterized by excessive urine excretion and a metabolic disorder induced by high blood glucose levels. Diabetes is primarily characterized by hyperglycemia that is mainly attributed to diabetic oxidative stress caused by several factors. Hyperglycemia leads to the overproduction of free radicals by the nonenzymatic glycation of proteins through Maillard's reaction, and these free radicals exert deleterious effects on the function of β-cells which make them vulnerable to oxidative stress (Brownlee *et al.*, 1984; Njoroge and Monnier, 1989). In addition, hyperglycemia can degrade antioxidant enzyme defenses, thereby allowing reactive oxygen species to cause cellular and tissue damage. In recent years, several workers have suggested that oxygen free radicals are generated as a result of hyperglycemia and cause various complications of diabetes, such as nephropathy, retinopathy and neuropathy (Baynes, 1991; Halliwell *et al.*, 1992; Giugliano *et al.*, 1996; Baynes and Thorpe, 1999). In particular, hyperglycemia increases the activity of protein kinase C in vascular smooth muscle and endothelial cells, which may also contribute to diabetic nephropathy (Larkins and Dunlop, 1992). Therefore, attenuation of oxidative stress by modulating hyperglycemic conditions and decreasing reactive oxygen free radical production may prevent or reverse abnormalities associated with

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diabetes mellitus and its complications.

Hachimi-jio-gan, a traditional Kampo prescription composed of eight constituents, has long been used in Japan and China for the alleviation of subjective symptoms of diabetes and its complications. In particular, Hachimi-jio-gan ameliorates hyperglycemia, so it is used clinically to improve several disorders associated with diabetes (Goto *et al.*, 1989; Furuya *et al.*, 1999). In addition, it has been widely used to treat renal dysfunction in human subjects (Yamada, 1992) and several chronic diseases, including chronic nephritis, sterility and vegetative ataxia (Huang, 1997). Furthermore, in a previous study, we measured the effects of the administration of 4 Kampo prescriptions, Ompi-to, Keishi-bukuryo-gan, Sairei-to and Hachimi-jio-gan, for 5 weeks in an animal model of diabetic nephropathy by evaluating biochemical processes induced by persistent hyperglycemia (Nakagawa *et al.*, 2001). On the basis of the above reports, Hachimi-jio-gan is expected to be a potential novel therapeutic agent for diabetes and its complications. Therefore, we carried out experiments in an animal model of streptozotocin (STZ)-induced diabetes, spontaneously occurring diabetes and diabetic nephropathy to determine whether Hachimi-jio-gan prevents renal pathophysiological changes under diabetic condition.

STZ-induced diabetes

Diabetes mellitus is the most common endocrine disorder characterized by hyperglycemia and long-term complications affecting the eyes, kidneys, nerves and blood vessels. The underlying mechanism responsible for its complications, as well as for diabetes itself, remains unclear, though possible events such as the activation of protein kinase C, the polyol pathway, non-enzymatic glycation and oxidative stress have been suggested (Giugliano *et al.*, 1996; King, 1996; Cooper *et al.*, 1997; Williams *et al.*, 1997; Lu *et al.*, 1998; Sharpe *et al.*, 1998; Ceriello, 2000). Recently, much attention has been focused on the role of oxidative stress and it has been suggested that oxidative stress may constitute the key and common events in the pathogenesis of different diabetic complications (Ceriello, 2000). Therefore, amelioration of diabetic oxidative stress may prevent or reverse abnormalities associated with diabetes and its complications. In the present study we used the STZ-induced diabetic rat model to investigate the effects of Hachimi-jio-gan on diabetic oxidative stress.

The abnormalities of energy utilization and metabolism responsible for the destruction of β-cells and the insulin secretion disorder in the diabetic state could cause abnormal changes in body weight gain and tissue weight. The rats with diabetes induced by STZ showed reduced body weight gain and increased liver and kidney weights. However, the administration of Hachimi-jio-gan attenuated the physiological changes associated with diabetes, implying that Hachimi-jio-gan normalized the energy utilization and metabolism.

To investigate the effect of Hachimi-gio-gan on abnormal glucose metabolism, we determined the levels of glucose and glycosylated protein in serum. Hyperglycemia, the primary clinical manifestation of diabetes, is associated with the development of certain diabetic complications. In addition, hyperglycemia and the glycation of proteins are associated with the development of diabetic complications, resulting in the generation of oxygen free radicals (Brownlee and Cerami, 1981). Indeed, excessive glycation of many proteins is detected in humans with diabetes. 5-Hydroxymethylfurfural (5-HMF) is involved in the nonenzymatic browning process and nonenzymatically bound glucose in serum is released as 5-HMF (Bunn *et al.*, 1978; McFarland *et al.*, 1979). Therefore, we evaluated 5-HMF levels to determine the extent of glycosylation of serum protein. As shown in Table 1, the effects on glucose and glycosylated protein levels indicated that the administration of Hachimi-jio-gan might prevent the pathogenesis of

Table 1. Effect of the Hachimi-jio-gan on serum glucose and glycosylated protein of rats with streptozotocin-induced diabetes at 10 days

Group	Dose (mg/kg B.W./day)	Glucose (mg/dl)	Glycosylated protein (nmol/mg protein)
Normal rats	-	166.7 ± 2.2	6.71 ± 0.09
Diabetic rats			
Control	-	560.0 ± 28.4[a]	22.02 ± 1.57[a]
Hachimi-jio-gan	50	521.0 ± 17.5[a,b]	21.57 ± 0.91[a]
Hachimi-jio-gan	100	497.4 ± 14.9[a,c]	20.37 ± 1.10[a]
Hachimi-jio-gan	200	464.5 ± 13.3[a,c]	18.17 ± 0.90[a,c]

[a]$P < 0.001$ vs normal rats; [b]$P < 0.05$, [c]$P < 0.001$ vs diabetic control rats.

diabetic complications caused by impaired glucose metabolism and the glycosylation of serum proteins, eventually resulting in the improvement of the diabetic pathological condition.

Under diabetic conditions, free radicals such as superoxide (O_2^-) and nitric oxide (NO) are produced as a result of the induction of the glycation reaction in β-cells that have been affected by diabetic oxidative stress. It has been well established that the animal model of diabetes induced by STZ injection results in the destruction of β-cells by reactive radicals (Asplund *et al.*, 1984; Oberley, 1988; Kubisch *et al.*, 1997; West, 2000). Our results showed that rats with STZ-induced diabetes had high serum levels of O_2^- and NO (Table 3), indicating that STZ leads to oxidative stress, which

Table 2. Effect of the Hachimi-jio-gan on urine volume and urinary protein of rats with streptozotocin-induced diabetes at 10 days

Group	Dose (mg/kg B.W./day)	Urine volume (ml/day)	Urinary protein (mg/day)
Normal rats	-	12.3 ± 2.6	1.02 ± 0.27
Diabetic rats			
Control	-	97.3 ± 9.4[a]	9.25 ± 0.93[a]
Hachimi-jio-gan	50	96.4 ± 6.3[a]	7.09 ± 0.97[a,b]
Hachimi-jio-gan	100	91.2 ± 12.3[a]	6.88 ± 0.49[a,c]
Hachimi-jio-gan	200	90.8 ± 6.7[a]	6.17 ± 1.08[a,c]

[a]$P < 0.001$ vs normal rats; [b]$P < 0.01$, [c]$P < 0.001$ vs diabetic control rats.

Table 3. Effect of the Hachimi-jio-gan on serum superoxide and nitrite/nitrate levels of rats with streptozotocin-induced diabetes at 10 days

Group	Dose (mg/kg B.W./day)	O_2^- (absorbance)	Nitrite/nitrate (μM)
Normal rats	-	0.312 ± 0.026	2.07 ± 0.07
Diabetic rats			
Control	-	0.383 ± 0.034[b]	2.32 ± 0.27
Hachimi-jio-gan	50	0.381 ± 0.009[b]	2.13 ± 0.14
Hachimi-jio-gan	100	0.340 ± 0.015[c]	1.71 ± 0.15[a,e]
Hachimi-jio-gan	200	0.332 ± 0.009[d]	1.79 ± 0.17[a,e]

[a]$P < 0.05$, [b]$P < 0.001$ vs normal rats; [c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ vs diabetic control rats.

will eventually affect the function of β-cells. O_2^- is an attractive candidate for a mediator of endothelial dysfunction in diabetes (Tesfamariam, 1994). Under conditions of diabetes, the increased production of O_2^- occurs via hyperglycemia, auto-oxidation of glucose and/or nonenzymatic protein glycation. The present study showed that Hachimi-jio-gan led to a decrease in the generation of O_2^- induced by diabetes, as shown in Table 3. However, we also have to consider that the O_2^- level may reflect the O_2^- generation activity under the condition of 20% oxygen in the air and not the exact *in vivo* O_2^- generation, which is difficult to measure because of its short half life. NO is also responsible for deleterious effects on β-cell function and it interacts with O_2^- to form the highly reactive hydroxyl radical that leads to reactive oxidative damage under conditions of diabetes (Oberley, 1988; Beckman *et al.*, 1990; Mandrup-Poulsen *et al.*, 1990). In addition, NO targets intracellular antioxidative enzymes, resulting in the loss of their function (Corbett and McDaniel, 1992). Hachimi-jio-gan scavenged O_2^- and NO resulting from diabetic oxidative stress (Table 3). The free radical scavenging property of Hachimi-jio-gan suggests that Hachimi-jio-gan might protect against diabetic oxidative stress.

Oxidative stress is associated with the peroxidation of lipids, which is determined by measuring thiobarbituric acid (TBA)-reactive substance levels. The concentration of lipid peroxidation products may also reflect the oxidative stress associated with the diabetic condition. Baynes (1991) and Kakkar *et al.* (1995) reported that tissue and blood malondialdehyde levels of rats with STZ-induced diabetes increased due to lipid peroxidation. Therefore, the measurement of TBA-reactive substance is frequently used to determine oxidative stress in diabetes. In the present study, the TBA-reactive substance concentrations of hepatic and renal mitochondria as well as serum significantly increased after the induction of diabetes in rats (Table 4). Consistent with our results, several other studies in which TBA-reactive substance levels were assayed in human and animal models have also reported increased lipid peroxidation in the plasma, liver and kidneys of diabetic subjects (Sato *et al.*, 1979; Karpen *et al.*, 1982; Nourooz-Zadeh *et al.*, 1997; Stanely Mainzen Prince and Menon, 2001). The increase of lipid peroxidation in tissues such as the liver and kidneys implies a susceptibility to diabetic oxidative stress, leading to diabetic complications. From this view point, the prevention of lipid peroxidation resulting from oxidative stress is considered to play a crucial role in protecting against the disorders involved in diabetes. The administration of Hachimi-jio-gan resulted in the efficient inhibition of lipid peroxidation in the liver and kidneys as well as in the serum (Table 4), suggesting the alleviation of oxidative stress in the diabetic pathological condition through the

Table 4. Effect of the Hachimi-jio-gan on TBA-reactive substance levels of rats with streptozotocin-induced diabetes at 10 days

Group	Dose (mg/kg B.W./day)	Serum (nmol/mg protein)	Hepatic mitochondria (nmol/mg protein)	Renal mitochondria (nmol/mg protein)
Normal rats	-	0.055 ± 0.006	0.421 ± 0.014	1.743 ± 0.083
Diabetic rats				
Control	-	0.166 ± 0.034[a]	0.596 ± 0.052[a]	1.860 ± 0.072[a]
Hachimi-jio-gan	50	0.083 ± 0.005[a,b]	0.512 ± 0.026[a,b]	1.653 ± 0.123[a,c]
Hachimi-jio-gan	100	0.081 ± 0.005[a,c]	0.412 ± 0.034[a,c]	1.527 ± 0.062[a,c]
Hachimi-jio-gan	200	0.073 ± 0.008[a,c]	0.344 ± 0.017[a,c]	1.414 ± 0.074[a,c]

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs normal rats; [d]$P < 0.01$, [e]$P < 0.001$ vs diabetic control rats.

inhibition of lipid peroxidation.

Over the experimental period, urine volume and urinary protein excretion were markedly elevated in the rat model used, reflecting the physiological abnormalities associated with diabetes. However, the administration of Hachimi-jio-gan for 10 days reduced urinary protein significantly. The urine volume of rats with STZ-induced diabetes given Hachimi-jio-gan orally showed a tendency to decline without significance (Table 2). On the basis of these results, we would expect Hachimi-jio-gan to normalize the physiological changes associated with diabetes and prevent the development of diabetes and its complications.

The results of this study imply that Hachimi-jio-gan plays a role in ameliorating glucose metabolism and attenuating oxidative stress in diabetes through scavenging free radicals and inhibiting lipid peroxidation. Hachimi-jio-gan may be a beneficial therapy for the pathological conditions associated with diabetic oxidative stress. On the basis of the present study, the protective potential of individual ingredients and major compounds in Hachimi-jio-gan against oxidative stress related to diabetes has to be investigated with the mutual and synergistic protective mechanism among them.

Spontaneously occurring diabetes

The WBN/Kob rat is an animal model of spontaneously developing diabetes mellitus and, without insulin therapy, it lives for a long time with hyperglycemia. As a result, several complications have been observed in these rats (Ishizaki *et al.*, 1987; Mori *et al.*, 1992). In the present study, we 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 hyperglycemia, polyuria and growth retardation. In this study, 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 (Fig. 1), 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 also serum creatinine (Cr) levels representing the glomerular filtration rate. As shown in Table 5, Hachimi-jio-gan significantly improved urea nitrogen levels, whereas Cr levels were slightly, but not significantly, lower than the

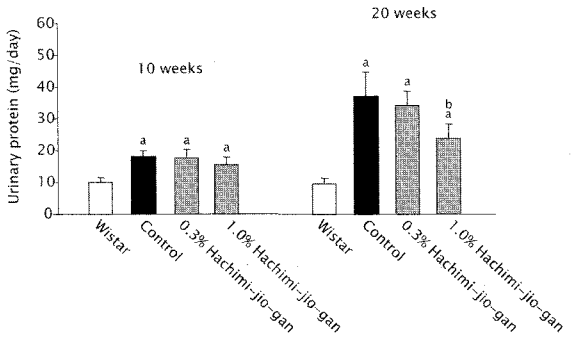

Fig. 1. Urinary protein excretion of spontaneously diabetic WBN/Kob rats. $^{a}P < 0.001$ vs Wistar rats; $^{b}P < 0.05$ vs WBN/Kob control rats.

Table 5. Effect of the Hachimi-jio-gan on serum urea nitrogen and creatinine of spontaneously diabetic WBN/Kob rats at 25 weeks

Group	Urea nitrogen (mg/dl)	Cr (mg/dl)
Wistar rats	11.2 ± 0.5	0.553 ± 0.033
WBN/Kob rats		
Control	16.3 ± 1.0[a]	0.419 ± 0.026[a]
0.3% Hachimi-jio-gan	14.8 ± 0.3[a,b]	0.388 ± 0.027[a]
1.0% Hachimi-jio-gan	14.9 ± 0.3[a,b]	0.385 ± 0.017[a]

[a]$P < 0.001$ vs Wistar rats; [b]$P < 0.05$ vs WBN/Kob control rats.

control value.

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 the deterioration of renal function and glomerulosclerosis. Transforming growth factor-β_1 (TGF-β_1) is a cytokine that regulates the production of ECM proteins. It has been reported that expressions of TGF-β_1 and ECM proteins are 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 *et al.*, 1987; Ziyadeh *et al.*, 1994; Sharma and Ziyadeh, 1995). In the present 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 both fibronectin and TGF-β_1 protein expressions, as shown in Fig. 2. These results confirm that the WBN/Kob rat is a useful model of diabetic nephropathy and show that long-term treatment with Hachimi-jio-gan delays 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

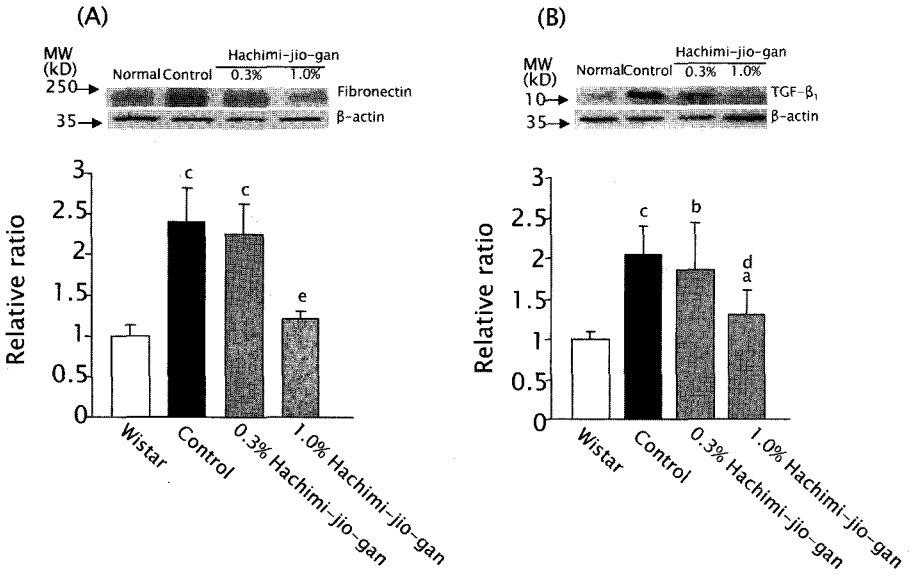

Fig. 2. Western blot analysis of fibronectin (A) and TGF-β_1 (B) in renal cortex of spontaneously diabetic WBN/Kob rats at 25 weeks. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs Wistar rats; [d]$P < 0.05$, [e]$P < 0.001$ vs WBN/Kob control rats.

tissue injury (Baynes, 1991; Ha and 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 and Kim (1995) demonstrated that the concentrations of lipid peroxides in the blood 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 causes the development of diabetic nephropathy. Additionally, Nath *et al.* (1998) reported that a chronic prooxidant state induced the renal expressions 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 an 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, 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 exerts an antioxidative activity in the kidneys of diabetic rats. Additionally, we found that Hachimi-jio-gan treatment resulted in a significantly elevated superoxide dismutase (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 the renal SOD activity of rats with STZ-induced diabetes is lower than that of non-diabetic rats, indicating that reduced SOD activity is associated with enhanced oxidative stress in diabetes (Loven *et al.*, 1986; Wohaieb and Godin 1987). These results suggest that the increased SOD activity after Hachimi-jio-gan treatment contributed to the enhancement of the antioxidative defense system, thereby reducing lipid oxidative damage.

In summary, our present results from spontaneously diabetic rats provide evidence that Hachimi-jio-gan preserves renal function and suppresses the expressions of fibronectin and TGF-β_1 proteins in the kidney, resulting in the retardation of diabetic nephropathy. In addition, our results suggest that the antioxidative properties of Hachimi-jio-gan are involved in its renoprotective effects. These findings indicate that this herbal medicine may be a potential therapeutic agent for diabetic nephropathy.

Diabetic nephropathy

The animal model of diabetic nephropathy used in this study, sub-totally nephrectomized rats given an injection of STZ, shows metabolic abnormalities and renal lesions resembling diabetic nephropathy in humans (Yokozawa *et al.*, 2001). Therefore, we

employed this animal model to search for a novel therapeutic agent for the treatment of diabetic nephropathy. On the basis of our previous study that suggested that some Oriental herbal medicines, including Hachimi-jio-gan, are potential therapeutic agents for diabetic nephropathy, this study focused on the effects of Hachimi-jio-gan in rats with diabetic nephropathy.

We confirmed that sub-total nephrectomy plus STZ injection induced progressive diabetic nephropathy in rats. Over the experimental period, the blood glucose and urinary protein excretion levels were markedly higher in the rat model employed in this study than in normal rats, indicating that disorders of glucose metabolism and changes in the capillary filtration barrier result in the increased permeability of the glomerular basement membrane. In addition, this rat model showed a significant decrease in the creatinine clearance (Ccr) (Fig. 3). In patients with diabetes and/or renal failure, the Ccr, which is an effective index for expressing the glomerular filtration rate, decreases exponentially, and patients eventually develop nephritic syndrome (Bell, 1991). However, the present investigation demonstrated that the administration of Hachimi-jio-gan for 15 weeks reduced the blood glucose and urinary protein excretion levels, but increased Ccr (Fig. 3). In addition, the decreased serum albumin level observed in this animal model was reversed by the administration of Hachimi-jio-gan (Table 6). On the basis of these results, we found that STZ injection to sub-totally nephrectomized rats resulted in progressive diabetic nephropathy, which we expect Hachimi-jio-gan to prevent or delay.

Recent studies strongly support the concept that the primary cause of diabetic nephropathy rests with metabolic derangements (Brownlee *et al.*, 1984; Larkins and Dunlop, 1992; Alaveras *et al.*, 1997; Cooper *et al.*, 1998). In particular, the importance of hyperglycemia as a risk factor for diabetic nephropathy is supported by several observations

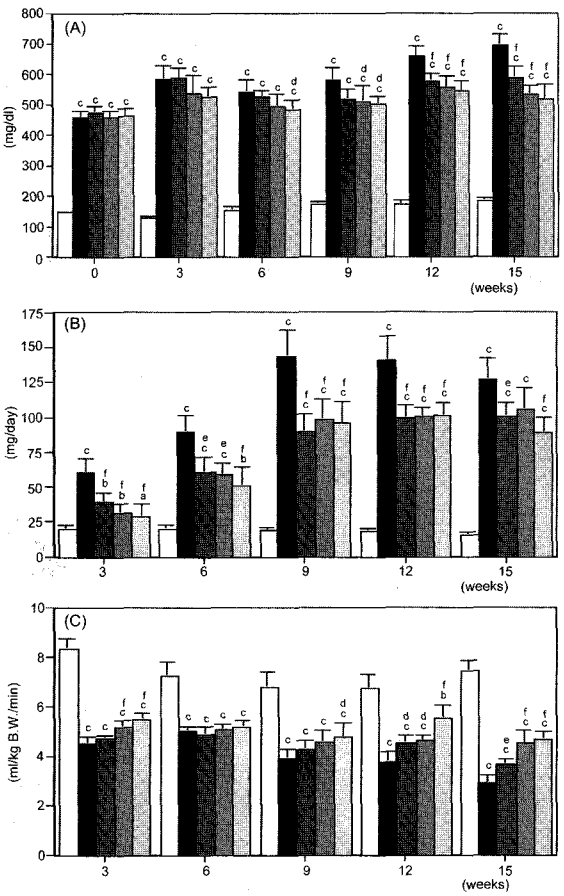

Fig. 3. Blood glucose (A), urinary protein (B) and creatinine clearance (C) in normal rats (□) and in diabetic nephropathy rats treated with either Hachimi-jio-gan (50 mg/kg B.W./day, ■; 100 mg/kg B.W./day, ■; 200 mg/kg B.W./day, ■) or control (■) for 15 weeks. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs normal rats; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ vs diabetic nephropathy control rats.

and pieces of experimental evidence (Larkins and Dunlop, 1992; Alaveras *et al.*, 1997). Hyperglycemia leads to irreversible tissue damage due to increased polyol pathway activity that results in several metabolic changes and the activation of protein kinase C (Derubertis and Craven, 1994; Fumo *et al.*, 1994). In addition, observed tissue damage has been attributed to the excessive nonenzymatic glycosylation of proteins and aberrant synthesis or actions of cytokines and growth factors (Cooper, 1998). Therefore, inhibitors

Table 6. Effect of the Hachimi-jio-gan on serum biochemical features of rats with diabetic nephropathy at 15 weeks

Item	Normal	Diabetic nephropathy			
		Control	Hachimi-jio-gan (50 mg/kg B.W./day)	Hachimi-jio-gan (100 mg/kg B.W./day)	Hachimi-jio-gan (200 mg/kg B.W./day)
Glycosylated protein (nmol/mg protein)	13.2 ± 0.7	25.8 ± 1.4[a]	24.1 ± 0.8[a,c]	20.6 ± 1.3[a,d]	20.3 ± 0.7[a,d]
Urea nitrogen (mg/dl)	20.7 ± 0.5	68.0 ± 6.9[a]	56.2 ± 3.1[a,c]	57.0 ± 2.5[a,c]	47.0 ± 5.0[a,d]
Albumin (g/dl)	3.43 ± 0.33	2.31 ± 0.08[a]	2.45 ± 0.10[a]	2.45 ± 0.09[a]	2.63 ± 0.04[a,c]
TG (mg/dl)	40.3 ± 5.0	340.9 ± 69.1[a]	244.0 ± 41.9[a,b]	235.9 ± 53.4[a,c]	150.6 ± 22.3[a,d]
Total cholesterol (mg/dl)	56.4 ± 1.4	285.4 ± 29.7[a]	240.5 ± 33.0[a,c]	267.9 ± 28.8[a]	186.6 ± 20.2[a,d]
MDA (nmol/ml)	2.44 ± 0.47	5.49 ± 0.29[a]	5.82 ± 0.28[a]	5.42 ± 0.31[a]	4.33 ± 0.14[a,d]

[a]$P < 0.001$ vs normal rats; [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$ vs diabetic nephropathy control rats.

of this pathway may provide new therapeutic approaches to the prevention and treatment of diabetic nephropathy. Actually, the available experimental data strongly support the importance of controlling the blood glucose level for primary prevention of diabetic nephropathy and possibly for slowing or even reversing some of the early abnormalities (Reddi and Camerini-Davalos, 1990). Beneficial effects of Hachimi-jio-gan on glucose metabolism in rats and rabbits with diabetes induced by alloxan or STZ (Nagoyashi *et al.*, 1966; Luo *et al.*, 1998), and human subjects with diabetic neuropathy (Yoshida, 1979) were reported. Our present study also showed that the increase in the blood glucose level associated with diabetic nephropathy was reversed significantly by the administration of Hachimi-jio-gan (Fig. 3), suggesting that the effective control of glucose metabolism plays an important role in the prevention of diabetic complications, including diabetic nephropathy.

Chronic hyperglycemia results in irreversible tissue damage caused by the protein glycation reaction that leads to the formation of glycosylated proteins and advanced glycation end-products (AGEs), and stimulation of the polyol pathway (Cooper *et al.*, 1998; Yabe-Nishimura, 1998). The glycosylated serum protein level increased in the animal model we used, which implies that sugar oxidation was stimulated, increasing damage to both sugars and proteins in the circulation and vascular walls, continuing and reinforcing the cycle of oxidative stress and damage. In addition, the accumulation of AGEs in the kidney was also observed (Tables 6 and 7). The excessive formation and accumulation of AGEs in tissues can alter the structure and function of tissue proteins. In people with diabetes and/or chronic renal failure, AGEs accumulate in the kidney and are responsible for pathological changes, including increased kidney weight, glomerular hypertrophy, glomerular basement membrane thickening and progressive albuminuria (Vlassara *et al.*, 1994). Moreover, AGEs stimulate free radical mechanisms and induce membrane peroxidation, which in turn increase membrane permeability. Therefore, AGEs accumulation in the kidney has been regarded as an index of progressive renal damage associated with diabetic nephropathy. In addition, it is recognized as playing a central role in various degenerative processes, such as aging, diabetes mellitus, dialysis-related amyloidosis and Alzheimer's disease (Niwa, 1997; Raj *et al.*, 2000). Hachimi-jio-gan reduced the levels of glycosylated serum proteins and AGEs significantly and dose-dependently (Tables 6 and 7), suggesting that it can inhibit oxidative damage and irreversible renal damage caused by protein glycation reactions.

In the polyol pathway, glucose is reduced to sorbitol by aldose reductase and this sorbitol is

Table 7. Effect of the Hachimi-jio-gan on renal advanced glycation end-products, sorbitol and malondialdehyde of rats with diabetic nephropathy at 15 weeks

Group	Dose (mg/kg B.W./day)	AGEs (AU)	Sorbitol (nmol/mg protein)	MDA (nmol/mg protein)
Normal	-	0.650 ± 0.015	0.605 ± 0.045	0.902 ± 0.075
Diabetic nephropathy				
Control	-	0.928 ± 0.047[a]	1.301 ± 0.087[a]	2.841 ± 0.450[a]
Hachimi-jio-gan	50	0.880 ± 0.045[a]	1.160 ± 0.031[a,b]	2.331 ± 0.148[a,b]
Hachimi-jio-gan	100	0.799 ± 0.024[a,d]	0.923 ± 0.081[a,d]	2.103 ± 0.314[a,c]
Hachimi-jio-gan	200	0.783 ± 0.022[a,d]	0.890 ± 0.062[a,d]	2.022 ± 0.209[a,d]

[a]$P < 0.001$ vs normal rats; [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$ vs diabetic nephropathy control rats.

subsequently converted to fructose by sorbitol dehydrogenase. Increased polyol pathway activity results in the depletion of myoinositol and changes in the cellular redox potential. In particular, this has been proposed to be a causative factor in the development of diabetic nephropathy. Moreover, it has been hypothesized that the polyol pathway encourages acceleration of the glycation reaction and the production of AGEs by increasing the supply of fructose, which is a reactive glycation agent with stronger reducing activity than glucose (Goldfarb *et al.*, 1991; Hamada *et al.*, 1996; Cooper *et al.*, 1998). As shown in Table 7, our present study showed that renal sorbitol levels were elevated markedly in rats with diabetic nephropathy compared with normal rats. The administration of Hachimi-jio-gan reduced the sorbitol level significantly, suggesting that the disturbance of the glucose-dependent metabolic pathway and irreversible tissue damage caused by such disturbance under conditions of diabetic nephropathy would be ameliorated by decreasing the activity of the polyol pathway and inhibiting the protein glycation reaction.

Besides disorders of glucose-dependent metabolism, hyperlipidemia is also involved in the initiation and progression of diabetic nephropathy. Enhanced serum triglyceride (TG) and cholesterol concentrations have been described as characteristic alterations in patients with diabetic nephropathy (Mulec *et al.*, 1993; Saito, 1997). Therefore, such patients frequently develop severe atherosclerotic complications and vascular diseases (Kramer-Guth *et al.*, 1996; Saito, 1997). In addition, experimental evidence suggests that hyperlipidemia participates in the progression of glomerular injury, and a more rapid decline in renal function was observed in patients with diabetic nephropathy who had hyperlipidemia compared to those who did not. Therefore, lipid-lowering therapy may have the beneficial effect of retarding the progression of diabetic nephropathy. Several studies demonstrated that Hachimi-jio-gan has an impact on lipid metabolism in experimental animals (Yoshida, 1979; Haranaka *et al.*, 1982; Mochizuki *et al.*, 1982). Not only did Hachimi-jio-gan inhibit the elevations in serum TG, total cholesterol and lipid peroxide levels, but it also reduced the increase in the blood glucose level of rats fed a high-sugar diet (Kimura *et al.*, 1987), suggesting that Hachimi-jio-gan improves both carbohydrate and lipid metabolism. Our present study also showed that the administration of Hachimi-jio-gan reduced the magnitudes of the elevations of the TG and total cholesterol levels of rats with diabetic nephropathy (Table 6). From the above results, we conclude that Hachimi-jio-gan improved lipid metabolism as well as glucose-dependent metabolism, and thus we would expect it to exert protective activity against tissue damage induced by metabolic disorders associated with diabetic nephropathy.

The metabolic disorders associated with diabetic

nephropathy, hyperlipidemia and protein glycation reactions, induce lipid peroxidation, which is caused by oxidative stress, that plays a potential role in diabetic glomerulosclerosis and renal fibrosis (Giugliano *et al.*, 1996; Salahudeen *et al.*, 1997). Under this pathological condition, free radical production is thus exacerbated, leading to severe cytotoxic effects, such as lipid peroxidation and protein denaturation in cell membranes, followed by alterations of membrane receptors, fluidity and properties. In our present study, we measured the serum and renal MDA levels to determine the effects of Hachimi-jio-gan on oxidative stress in relation to the development of diabetic nephropathy. Lipid peroxidation levels in the serum and kidney were markedly elevated in rats with diabetic nephropathy compared with normal rats, while a 15-week course of Hachimi-jio-gan reduced these levels (Tables 6 and 7). These findings suggest that the administration of Hachimi-jio-gan would ameliorate the oxidative stress associated with diabetic nephropathy through the inhibition of lipid peroxidation, and thus it would result in the improvement of renal lesions caused by oxidative stress.

The clinical manifestations of diabetic nephropathy, notably proteinuria, hypertension and renal insufficiency, relate well to the severity of the renal lesions (Winetz *et al.*, 1982; Adler, 1997). In the animal model we used, the histopathological characteristics of diabetic nephropathy, glomerular sclerosis and tubulointerstitial lesions, were observed. In particular, the diffuse expansion of mesangial regions and basement membrane thickening of renal tubules, the most important morphologic characteristics of glomerular sclerosis and structural indicators of diabetic nephropathy, were observed frequently (Fig. 4). Recently, it was suggested that diffuse mesangial expansion in the glomerulus plays a critical role in the destruction of the capillary lumen, ultimately leading to the cessation of glomerular function in various forms of glomerulopathy, including diabetic glomerulosclerosis. Furthermore, the greatly expanded mesangial matrix results in a reduction of the surface area available for filtration (Giugliano *et al.*, 1996). This, in turn, would lead to the accumulation of urea nitrogen and Cr in the blood, and a subsequent decrease in Ccr. Therefore, the elevated blood urea nitrogen level in this animal model of diabetic nephropathy was considered to be related to the renal lesions of glomerulosclerosis, while the reduction in this level by Hachimi-jio-gan indicated the amelioration of the renal lesions. A substantial body of evidence from cell culture experiments and experimental models of diabetic nephropathy suggests that progressive renal insufficiency is the ultimate expression of the pathological consequences of accumulating abnormalities in the glomerulus and tubulointerstitium (Winetz

Table 8. Effect of the Hachimi-jio-gan on histopathological evaluation of the kidney obtained from diabetic nephropathy rats at 15 weeks

Item	Normal	Diabetic nephropathy			
		Control	Hachimi-jio-gan (50 mg/kg B.W./day)	Hachimi-jio-gan (100 mg/kg B.W./day)	Hachimi-jio-gan (200 mg/kg B.W./day)
Glomerular sclerosis	0	2.68 ± 0.87	1.38 ± 0.18[a]	0.79 ± 0.17[b]	0.43 ± 0.24[b]
Tubulointerstitial changes	0	2.88 ± 0.13	2.25 ± 0.25[b]	2.33 ± 0.21[a]	2.29 ± 0.18[b]
Mesangial matrix expansion	0	2.38 ± 0.18	2.00 ± 0.01[a]	1.83 ± 0.17[b]	1.43 ± 0.20[b]
Arteriolar sclerosis	0	2.13 ± 0.23	2.00 ± 0.27	1.50 ± 0.22[a]	1.43 ± 0.20[b]
Total	0	11.82 ± 2.66	7.38 ± 0.69[b]	6.45 ± 0.80[b]	5.58 ± 1.04[b]

[a]$P < 0.01$, [b]$P < 0.001$ vs diabetic nephropathy control rats.

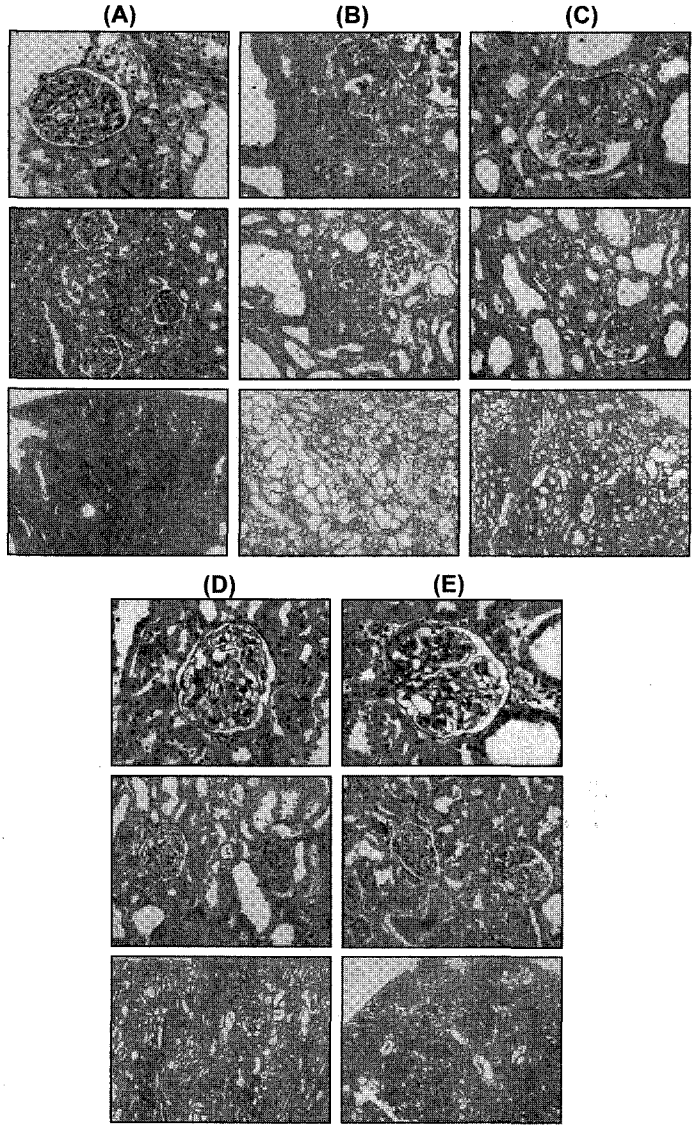

Fig. 4. Photomicrographs of the glomeruli (upper panel, ×200), tubulus (middle panel, ×100) and interstitium (lower panel, ×20) obtained from normal rats (A), diabetic nephropathy rats in the control (B) and Hachimi-jio-gan-treated (50 mg/kg B.W./day (C), 100 mg/kg B.W./day (D) and 200 mg/kg B.W./day (E) groups.

et al., 1982; Yaqoob *et al.*, 1994; Adler, 1997). Hachimi-jio-gan had a significant protective effect on the renal lesions, as demonstrated by the histopathological evaluations (Fig. 4, Table 8), suggesting that Hachimi-jio-gan would improve renal dysfunction associated with renal lesions.

The results of our present study confirm that Hachimi-jio-gan has a protective effect in rats with diabetic nephropathy through the amelioration of metabolic disorders, oxidative stress and renal dysfunction associated with renal lesions. The composition of Hachimi-jio-gan used in the experiment was as follows (figures indicate proportions of each ingredient): Rehmanniae Radix (*Rehmannia glutinosa* Libosch. var. *purpurea* Makino) 6, Corni Fructus (*Cornus officinalis* Sieb. et Zucc.) 3,

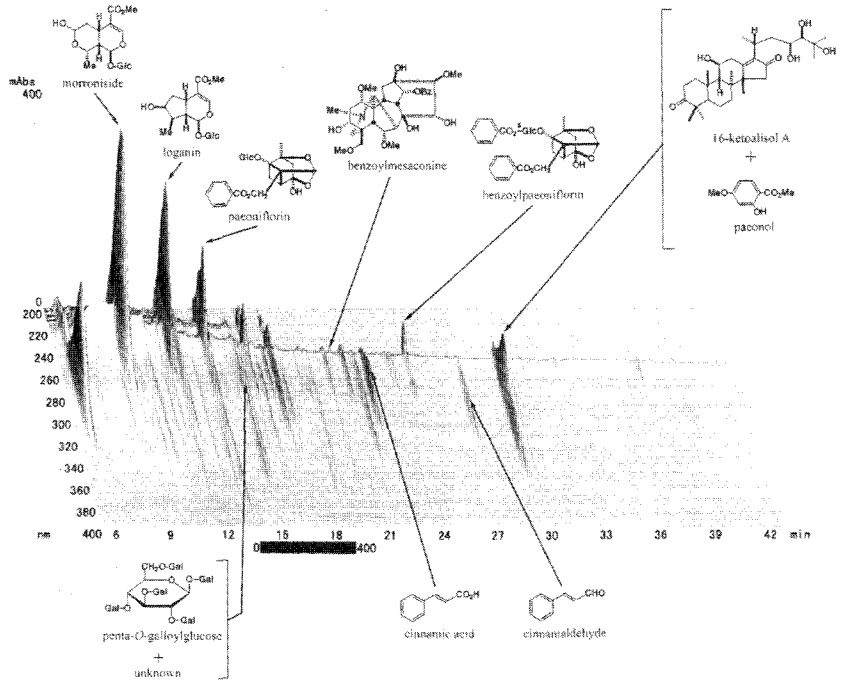

Fig. 5. Three-dimensional HPLC profile of Hachimi-jio-gan extract.

Dioscoreae Rhizoma (*Dioscorea japonica* Thunb.) 3, Alismatis Rhizoma (*Alisma orientale* Juzep.) 3, Hoelen (*Poria cocos* Wolf) 3, Moutan Cortex (*Paeonia suffruticosa* Andrews) 2.5, Cinnamomi Cortex (*Cinnamomum cassia* Blume) 1 and Aconiti Tuber (*Aconitum carmichaeli* Debx.) 0.5, and this extract includes many components, as shown in Fig. 5. We assume that the above-mentioned 8 ingredients may possess complex interactions, which therefore lead to the versatile biological activities observed in this study. To examine which of the ingredients or components is responsible for its protective effects on diabetic nephropathy will provide us with further important knowledge of this traditional medicine.

Until now, Hachimi-jio-gan has long been prescribed to patients, without serious adverse effects, for treating various diseases associated with aging including diabetes mellitus. Additionally, it was not observed, in this study, the reduction of body weight and survival rate by the administration of Hachimi-jio-gan. However, for the safe use of this medicine, further examinations of any adverse effects are needed. The prevention of diabetic nephropathy in clinical practice may be categorized into several stages. Primary prevention begins when diabetes is diagnosed and its aim is to prevent the development of any signs of nephropathy. The aim of secondary preventive measures is to halt or reverse the progression of nephropathy. Tertiary preventive efforts are directed at persons with clinical nephropathy and are intended to prevent or delay the onset of end-stage renal disease. A prospective study to determine at which stages Hachimi-jio-gan is involved will provide reliable evidence as to whether this Kampo prescription is a valid therapeutic agent for diabetic nephropathy.

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