A Study of Hachimijiogan for Diabetic Nephropathy

Takako Yokozawa, Noriko Yamabe

Division of Biofunctional Evaluation, Institute of Natural Medicine, University of Toyama

1. Background

Diabetic nephropathy has been the leading cause of patients requiring chronic hemodialysis therapy since 1998, and the number of patients has continually increased to approximately 43.5% (**Figure 1**).[1] This is a very serious problem because there are many risk factors for diabetic nephropathy in type 1 and 2 diabetes; for example, the duration of diabetes, familial and genetic factors, hyperglycemia, high blood pressure, dyslipidemia, proteinuria, and smoking. Several basic ways to prevent diabetic nephropathy involve the reduction of the last five risk factors, primarily through tight glycemic control. However, the International Diabetes Federation estimated that 285 million people globally have diabetes, and the total is expected to rise to 438 million within 20 years. This means that, each year, a further 7 million people develop diabetes. Therefore, it is important mentally, physically, and financially to develop an effective treatment to prevent or delay the progression of end-stage renal disease in people with diabetic nephropathy worldwide.

Diabetic nephropathy is one of the major diabetic microvascular complications, as well as retinopathy and neuropathy, and it is characterized by albuminuria, hypertension, a decline of the glomerular filtration rate (GFR), and glomerular sclerosis.[2] During its development, glucose exerts toxic actions as a result of processes that are activated within the diabetic kidney, that is, the accumulation of advanced glycation endproducts (AGEs), increase in oxidative stress, abnormal polyol metabolism, and synthesis of growth factors.[3] For a decade, clinical and experimental studies have provided evidence of various beneficial effects of glycemic control, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, and antihypertensive drugs against diabetic nephropathy.[4-7] However, large numbers of patients in many countries are still suffering from diabetic nephropathy.

Traditional medicines, including Chinese prescriptions, have attracted much attention due to their extensive and unique biological activities without toxicity and/or side effects. Formerly, we performed *in vitro* and *in vivo* studies using twelve Chinese prescriptions to investigate possible cures of diabetic nephropathy,[8] and then demonstrated the effects of a 5-week administration of four Kampo prescriptions, Wen-Pi-Tang (ompito), keishibukuryogan, saireito, and hachimijiogan, in an animal model of diabetic nephropathy by measuring biochemical parameters affected by persistent hyperglycemia.[9] In these prescriptions, hachimijiogan is used clinically to improve several disorders associated with diabetes,[10,11] and it has been employed widely for the treatment of renal dysfunction in human subjects.[12] Furthermore, hachimijiogan has long been used widely to treat several chronic diseases, including chronic nephritis, sterility, and vegetative ataxia,[13] although scientific evidence supporting a pharmacological basis for its therapeutic effects has yet to be published. Therefore, this study

focused on hachimijiogan, and evaluated its effect on diabetic renal damage using a type 1 diabetic nephropathy rat model which underwent subtotal nephrectomy plus streptozotocin (STZ) injection, and Otsuka Long-Evans Tokushima Fatty (OLETF) rats as a model of human type 2 diabetes. Moreover, in order to explore the active components of hachimijiogan, the antidiabetic effects of Corni Fructus, one of the constituents of hachimijiogan, and its iridoid glycosides and polyphenol fractions were investigated using STZ-induced diabetic rats, and we sought to elucidate the major active components of hachimijiogan.

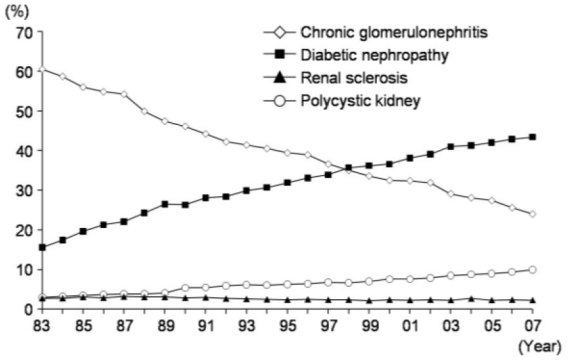

Figure 1 Changes in primary diseases in patients introduced to dialysis annually.

2. Hachimijiogan extract

The water extract of hachimijiogan used in this study was produced by Tsumura & Co. (Tokyo, Japan), and its composition is shown in **Table 1**. In brief, eight medicinal plants with their contents weighed were boiled gently in 10 times their volume of water for 60 min, filtered, and the filtrate was spray-dried to obtain the extract at a yield of about 10%, by weight, of the original preparation. For analysis of the components of hachimijiogan, an aqueous extract (0.5 g) was obtained using 20 ml of 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 (Ø 4.6 ×250 mm, Tosoh, Japan) with an LC $10AD_{vp}$ pump and an 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 and the effluent from the column was monitored and processed into three-dimensional data using an SPD-M10A array detector. All assigned peaks were identified by comparing their UV spectral data with those co-injected authentic samples using Class LC-10 version 1.62 software (Shimadzu, Japan). The three-dimensional HPLC profile of hachimijiogan extract is shown in **Figure 2**. Morroniside, loganin, and paeoniflorin were the major components of hachimijiogan; penta-*O*-galloylglucose, benzoylmesaconine, cinnamic acid, benzoylpaeoniflorin, cinnamaldehyde, and 16-ketoalisol A were also detected.

Table 1 Composition of hachimijiogan

Botanical name	Common name	Family name	Part used	Content (%)
Rehmannia glutinosa Libosch. var. *purpurea* Makino	Rehmanniae Radix	Scrophulariaceae	Root	27.27
Cornus officinalis Sieb. et Zucc.	Corni Fructus	Cornaceae	Fruit	13.64
Dioscorea japonica Thunb.	Dioscoreae Rhizoma	Dioscoreaceae	Rhizome	13.64
Alisma orientale Juzep.	Alismatis Rhizoma	Alismataceae	Rhizome	13.64
Poria cocos Wolf	Hoelen	Polyporaceae	Sclerotium	13.64
Paeonia suffruticosa Andrews	Moutan Cortex	Paeoniaceae	Bark	11.36
Cinnamomum cassia Blume	Cinnamomi Cortex	Lauraceae	Bark	4.54
Aconitium carmichaeli Debx	Aconiti Tuber	Ranunculaceae	Tuber	2.27

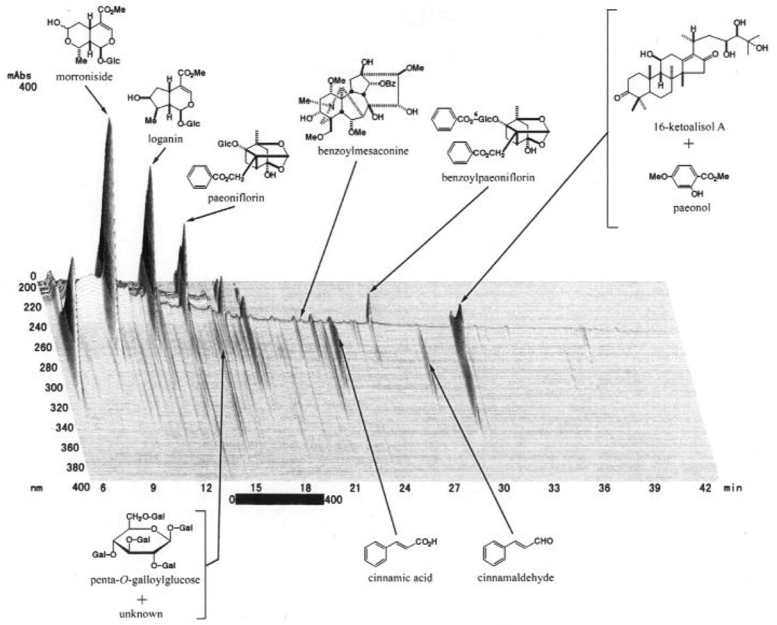

Figure 2 Three-dimensional HPLC profile of hachimijiogan extract.

3. Corni Fructus, a constituent of hachimijiogan

Corni Fructus extract used in this experiment was produced by Tsumura & Co. (Tokyo, Japan). HPLC-DAD analysis [250 × 4.6 mm i.d. Cosmosil 5C_{18}-AR II column (Nacalai Tesque Inc.) with gradient elutions of CH_3CN in 50 mM H_3PO_4 from 4-30% in 39 min and 30-75% in 15 min at a flow rate of 0.8 ml/min, and detection with a JASCO MD-910 photodiode array detector] showed major peaks arising from gallic acid (8.24 min), morroniside (18.24 min), and loganin (22.9 min), which were identified by comparing t_R and UV absorptioins with those of authentic samples. The Corni Fructus extract (100 g) was fractionated by Sephadex LH-20 column chromatography (32 × 5 cm) with water containing increasing proportions of methanol (0-100%, 10% stepwise gradient elution) and finally 60% acetone to give four fractions: S1 (94.52 g), S2 (1.20 g), S3 (2.15 g), and S4 (1.55 g). The fraction S1 was further separated by Diaion HP-20SS column chromatography (28 × 5 cm) with water-methanol (0-100%, 10% stepwise gradient elution) to give S1D1

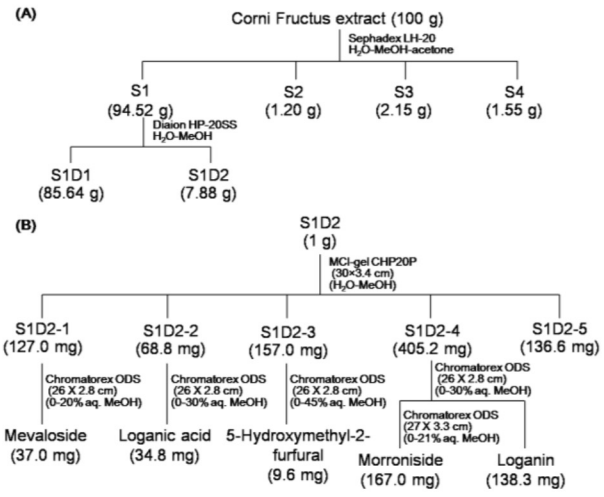

Figure 3 Fractionation (A) and isolation (B) of compounds from Corni Fructus.

(85.64 g) and S1D2 (7.88 g) (**Figure 3(A)**). A portion of S2 (150 mg) was further purified by MCI-gel CHP20P column chromatography (28 × 2 cm) with 0-10% MeOH to give 7-*O*-galloyl-D-sedoheptulose,[14] as shown in **Figure 4(A-C)**. Further separation of S1D2 (1 g) by MCI-gel CHP20P (30 × 3.4 cm) with water-MeOH (0-100%, 10% stepwise gradient elution) gave five fractions: S1D2-1 (127.0 mg), S1D2-2 (68.8 mg),

S1D2-3 (157.0 mg), S1D2-4 (405.2 mg), and S1D2-5 (136.6 mg). The purification of S1D2-1, S1D2-2, and S1D2-3 by Chromatorex ODS column chromatography (26 × 2.8 cm) using 0-20% MeOH, 0-30% MeOH, and 0-45% MeOH as elution solvents yielded mevaloside (37.0 mg),[15] loganic acid (34.8 mg),[16] and 5-hydroxymethyl-2-furfural (5-HMF) (9.6 mg),[17] respectively. A similar separation of S1D2-4 afforded morroniside (167.0 mg) and loganin (138.3 mg).[18-20] The structure of mevaloside was determined on the basis of COSY, HSQC, and HMBC spectral analysis, and other products were identified by ^{1}H- and ^{13}C-NMR comparison (**Figure 3(B)**). The chemical structures of substances purified from S1D2 are shown in **Figure 4(D)**.

The genus *Cornus* (dogwood) belongs to the family Cornaceae, which consists of about 55 species, and is widely distributed in the northern hemisphere, eastern Asia, and the eastern and northern parts of the United States,[21] and Corni Fructus belongs to the subgroup Cornelian cherries containing iridoid total glycosides such as morroniside and loganin and also a few polyphenols such as cornusiin A, B, and C, and monomeric and trimeric hydrolyzable tannin,[22,23] and it has been used as a traditional medicine in Japan and China. Furthermore, several reports of its use as a traditional medicine have been made, e.g., Corni Fructus has a plasma glucose-lowering action in normal rats, along with anti-neoplastic and anti-microbial effects.[24,25,17] Vareed *et al.* (2006) reported that Corni Fructus has been used for improving hepatic and renal functions,[26] and iridoid total glycosides have the effect of preventing the overexpression of transforming growth factor (TGF)-β_1 and matrixes in glomeruli with a diabetic model.[27] However, the action mechanisms of Corni Fructus against glucose-associated metabolic disorders in diabetes have not fully investigated.

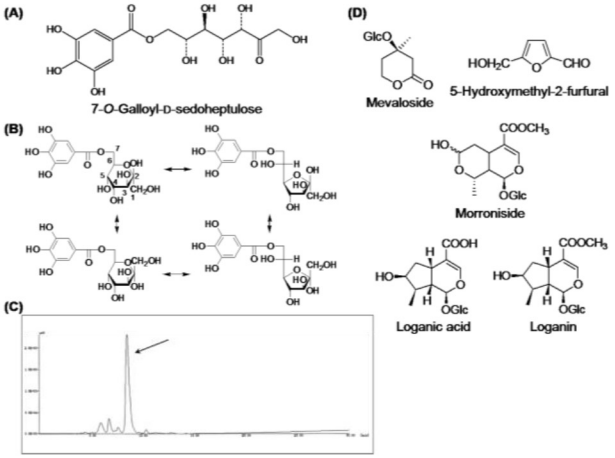

Figure 4 Chemical structures of isolated compounds and the HPLC profile of 7-*O*-galloyl-D-sedoheptulose. Chemical structure of 7-*O*-galloyl-D-sedoheptulose (A) and its isomers (B). (C) HPLC profile of 7-*O*-galloyl-D-sedoheptulose; the large peak delineated by the arrow is the main structure as described in (A), and the other peaks represent its four isomers, as described in (B).

4. Effect on early stage of type 1 diabetic kidney disease

4.1. Efficacy of hachimijiogan

This investigation was carried out to clarify the effect on oxidative stress related to diabetes and hyperglycemia with a diabetic rat model induced by STZ, which destroys pancreatic β-cells as a result of damage caused by radicals and impaired antioxidative defenses, thereby allowing reactive oxygen species to cause cellular and tissue damage. Hachimijiogan was orally administered at doses of 50, 100, and 200 mg/kg BW/day dissolved in distilled water *via* gavage,[28] and the doses were determined by a preliminary study that demonstrated biological activity without toxicity.[29] Alternatively, hachimijiogan successfully ameliorated diabetic oxidative stress. That is, rats with diabetes induced by STZ did not show body weight changes during the 10-day experimental period, suggesting that

these animals were undergoing growth retardation due to the obstruction of glucose uptake caused by the lack of insulin following STZ injection, but rats treated with hachimijiogan did not show any changes in body weight from the initial value (data not shown). However, hachimijiogan significantly reduced the serum levels of glucose and glycosylated protein which were markedly elevated in rats with STZ-induced diabetes (**Figure 5(A,B)**), while the serum creatinine (Cr) levels of groups given hachimijiogan were slightly lower than the control value (data not shown). Moreover, as shown in **Figure 5(C,D)**, the elevated thiobarbituric acid reactive substance (TBA-RS) levels in serum and renal mitochondria of diabetic rats were markedly reduced by the administration of hachimijiogan, and a notable reduction was observed in the serum level from the lowest dose, 50 mg/kg BW. According to these results, it was supposed that hachimijiogan played a role in ameliorating glucose metabolism and attenuating oxidative stress under diabetes through scavenging free radicals and inhibiting lipid peroxidation. Hence, hachimijiogan may be a beneficial therapy for pathological conditions associated with diabetic oxidative stress.

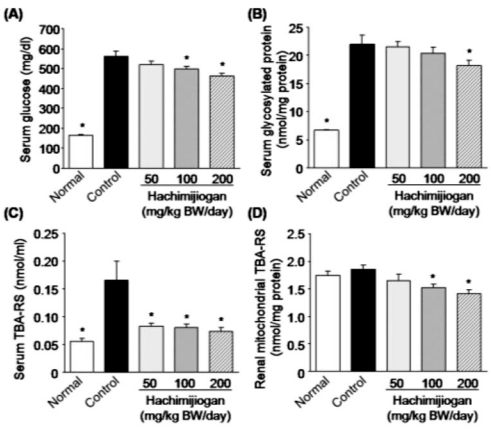

Figure 5 Effect of hachimijiogan on serum glucose (A), glycosylated protein (B), TBA-RS (C), and renal mitochondrial TBA-RS (D) levels at 10 days. *P<0.05 compared with diabetic control rats.

4.2. Which is the major contributor among the eight crude drugs comprising hachimijiogan?

There have been many experiments focusing on the treatment of diabetes and its complications with herbal medicines including traditional Chinese prescriptions. In our previous study, it was discovered that hachimijiogan had effects on metabolic disorders, especially on AGE formation and elevated oxidative stress in diabetic nephropathy.[9] We also demonstrated that keishibukuryogan showed potential therapeutic effects on diabetic nephropathy via reducing oxidative stress.[30] In addition, we clarified that the administration of dried Rehmanniae Radix extract, which is the main constituent of hachimijiogan, as described in **Table 1**, attenuates renal dysfunction in diabetic nephropathy mainly due to its suppression of oxidative stress;[31] however, for the analysis of this prescription, further characterization of the other constituents is needed. According to the three-dimensional HPLC profile, morroniside, loganin, and paeoniflorin were detected as the major compounds in hachimijiogan (**Figure 2**). Morroniside and loganin are components of Corni Fructus, and paeoniflorin is a component of Moutan Cortex which is common in keishibukuryogan.[30] Therefore, on the assumption that Corni Fructus would be a major contributor to the effects of hachimijiogan, the next study was carried out.

4.3. Efficacy of Corni Fructus and its fractions

To determine whether Corni Fructus is the principal active agent in hachimijiogan, which has a strong effect on AGE formation in diabetes and/or diabetic nephropathy, the effect of Corni Fructus was examined using 50, 100, and 200 mg/kg BW/day, of which the doses were the same as in hachimijiogan, in order to compare the effect in STZ-induced diabetic rats,

comparing it with the inhibitor of AGE formation, aminoguanidine (100 mg/kg BW/day), for 10 days.[32] In addition, we also evaluated the effect of active fractions in Corni Fructus using diabetic rats, that is, one iridoid glycoside of S1D2 and three polyphenol fractions of S2, S3, and S4 (from low to high molecular weights) (**Figure 3(A)**), possessing greater activities compared with Corni Fructus regarding hydroxyl radical scavenging activity (data not shown).

As a result, Corni Fructus dose-dependently increased the body weight and ameliorated hyperglycemia (**Figure 6**), glucose-associated metabolic disorders in the serum and kidney, especially AGE levels (**Figure 7(A,C)**), as well as aminoguanidine, with these effects being similar to those of hachimijiogan. On the contrary, we found that S1D2 and S2 were the active fractions of Corni Fructus in diabetic rats, each having a different mechanism in diabetic metabolic disorders (**Figure 8(A-C)**).[33] However, the other polyphenol fractions of S3 and S4, which were more active concerning hydroxyl radical scavenging activity than S1D2 and S2 fractions (data not shown), led to a decrease in body weight gain compared with the diabetic control value (data not shown), although these fractions significantly inhibited the increase in renal TBA-RS levels (**Figure 8(E)**), suggesting that these two fractions may have radical scavenging activities but may include some toxic ingredients.

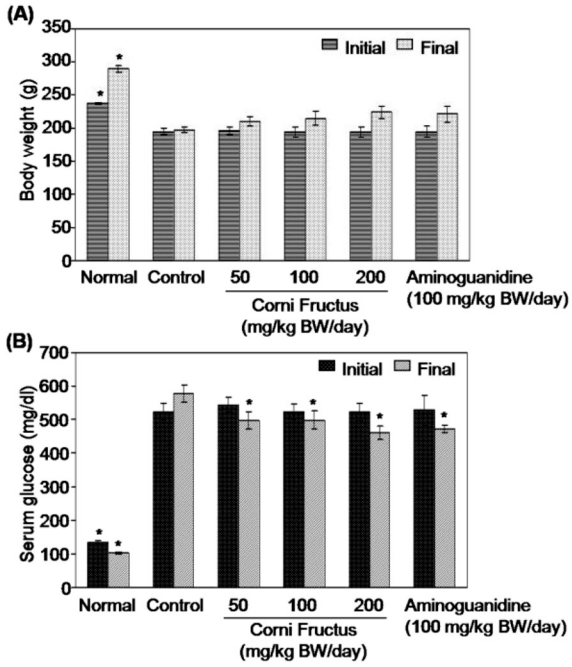

Figure 6 Effect of Corni Fructus and aminoguanidine on body weight changes (A) and serum glucose levels (B) in STZ-induced diabetic rats during the 10-day experimental period. *P<0.05 compared with each value of diabetic control rats.

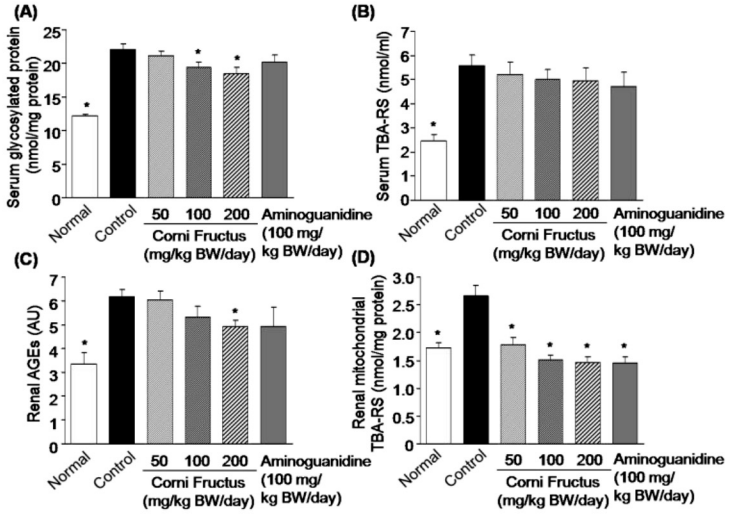

Figure 7 Effect of Corni Fructus and aminoguanidine on serum glycosylated protein (A), TBA-RS (B), renal AGEs (C), and mitochondrial TBA-RS (D) levels in STZ-induced diabetic rats at 10 days. *P<0.05 compared with diabetic control rats.

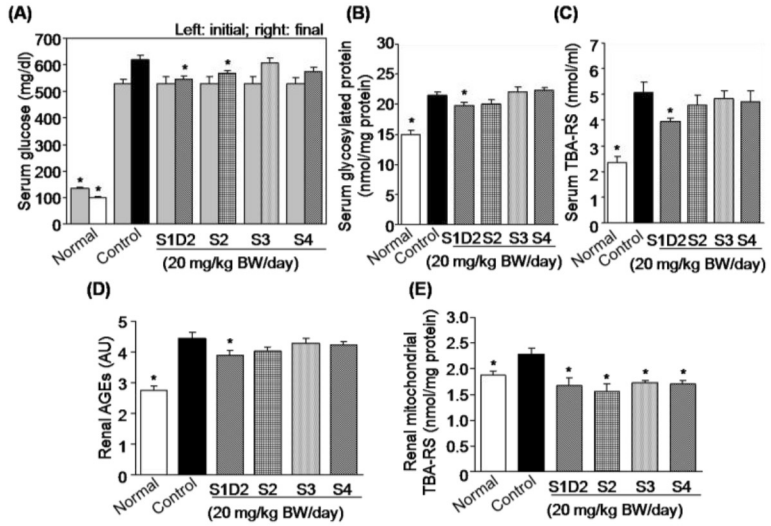

Figure 8 Effect of Corni Fructus fractions on serum glucose (A), glycosylated protein (B), and TBA-RS (C), and renal AGEs (D) and mitochondrial TBA-RS (E) levels in STZ-induced diabetic rats at 10 days. **P*<0.05 compared with diabetic control rats.

Table 2 Effect of Corni Fructus on renal function parameters in diabetes

Items	Normal	Diabetes				
		Control	Corni Fructus (50 kg/kg BW/day)	Corni Fructus (100 kg/kg BW/day)	Corni Fructus (200 kg/kg BW/day)	Aminoguanidine (100 kg/kg BW/day)
s-Urea nitrogen (mg/dl)	18.5 ± 0.7*	33.6 ± 2.1	27.8 ± 1.5*	23.1 ± 1.6*	20.9 ± 1.1*	24.7 ± 3.1*
s-Cr (mg/dl)	0.341 ± 0.010*	0.404 ± 0.021	0.348 ± 0.012*	0.351 ± 0.015*	0.338 ± 0.017*	0.364 ± 0.007
Ccr (ml/kg BW/min)	7.68 ± 0.14*	5.27 ± 0.59	6.82 ± 0.63*	7.47 ± 0.48*	8.92 ± 0.22*	5.73 ± 1.28
Urine protein (mg/day)	13.3 ± 0.9	16.8 ± 2.2	12.5 ± 3.1	12.1 ± 2.7	10.3 ± 0.9	9.7 ± 1.1*

**P*<0.05 compared with diabetic control rats.

Table 3 Antioxidative effects of Corni Fructus in diabetic serum

Groups	Nitrite/nitrate (μM)	Inhibition of NBT reduction (% of diabetic control)
Normal	15.7 ± 1.9*	84.7 ± 2.1
Diabetes		
Control	32.6 ± 5.7	100.0 ± 5.7
Corni Fructus (50 mg/kg BW/day)	26.4 ± 7.4	97.5 ± 2.1
Corni Fructus (100 mg/kg BW/day)	19.0 ± 3.4	93.7 ± 1.0
Corni Fructus (200 mg/kg BW/day)	10.5 ± 0.5*	91.5 ± 3.0
Aminoguanidine (100 mg/kg BW/day)	8.3 ± 0.3*	91.1 ± 5.5

**P*<0.05 compared with diabetic control rats.

Increased oxidative stress also participates in renal structural changes, i.e., oxidative substances affect glomerular endothelial cells directly, infiltrating into the mesangial area, tubulointerstitial area, and other parts of renal tissue, due to the abundant blood flow in the diabetic kidney. There is no doubt that microalbuminuria is an important indicator of the early stage of diabetic nephropathy. That is, glomerular damage increases the albumin filtration rate, but proximal tubular reabsorption of this increased albumin is decreased via a decline in its endocytosis due to a loss of megalin expression.[34] In this study, diabetic rats also showed renal dysfunction, i.e., increased serum Cr, urea nitrogen, and proteinuria and decreased Cr clearance (Ccr) levels, reflecting a decline in the glomerular filtration rate. However, the rats given Corni Fructus showed an up-regulated renal function and decreased uremic toxin levels (**Table 2**), with certain antioxidative activities like nitric oxide scavenging (**Table 3**). On the other hand, aminoguanidine had almost the same effects as those of Corni Fructus except concerning the renal function, shown by the serum Cr and Ccr levels, and these differences might reflect AGE clearance. That is, the reduction of serum glycosylated protein was contrary to the glycemic control shown in 100 mg/kg of Corni Fructus- and aminoguanidine-treated groups. Aminoguanidine was reported to inhibit nitric oxide production and to trap reactive dicarbonyls, impeding

conversion to AGEs, prevent cross-linking, and inhibit free radical formation. Therefore, it may be hypothesized that differences between Corni Fructus and aminoguanidine are that Corni Fructus can improve AGE clearance with an activated renal function, while aminoguanidine mainly inhibits AGE formation *via* its antioxidant properties. In addition, similar results were observed on treatment with S1D2 and S2 fractions in terms of renal function parameters (**Table 4**).

Table 4 Effect of Corni Fructus fractions on renal function parameters in diabetes

Groups	s-Urea nitrogen (mg/dl)	s-Cr (mg/dl)	Ccr (ml/kg BW/min)	Urinary protein (mg/day)
Normal	22.4 ± 1.0*	0.369 ± 0.004	7.75 ± 0.07*	9.9 ± 1.3
Diabetes				
Control	38.5 ± 2.4	0.370 ± 0.007	6.62 ± 0.20	11.2 ± 0.3
S1D2 (20 mg/kg BW/day)	33.1 ± 1.7	0.368 ± 0.012	6.78 ± 0.45	9.1 ± 0.9
S2 (20 mg/kg BW/day)	33.9 ± 1.2	0.368 ± 0.008	7.25 ± 0.10*	9.3 ± 0.6
S3 (20 mg/kg BW/day)	36.8 ± 2.1	0.369 ± 0.009	6.68 ± 0.25	11.8 ± 0.9
S4 (20 mg/kg BW/day)	34.9 ± 3.8	0.371 ± 0.018	6.68 ± 0.35	9.8 ± 0.5

*P<0.05 compared with diabetic control rats.

Furthermore, according to the identification of the two active fractions S1D2 and S2, the major components of morroniside or 7-*O*-galloyl-D-sedoheptulose are considered to be the most important contributors to prevent and/or delay the onset of diabetic renal damage. The biological activities of morroniside have recently been identified,[27,35,36] and these might be positively correlated with the activities of hachimijiogan. Moreover, 7-*O*-galloyl-D-sedoheptulose is only detected as a compound from Corni Fructus,[14] and its biological activity has been poorly understood until now except for our previous research. For these reasons, we further clarified the mechanisms of 7-*O*-galloyl-D-sedoheptulose acting against diabetic kidney disease in expectation of identifying it as the novel active contributor in Corni Fructus.[37] That is, 7-*O*-galloyl-D-sedoheptulose had marked effects on the suppression of AGE formation from glucose *via* the Maillard reaction and lipid peroxidation, and the effects were consistent with those of hachimijiogan and/or Corni Fructus (**Figure 9**).

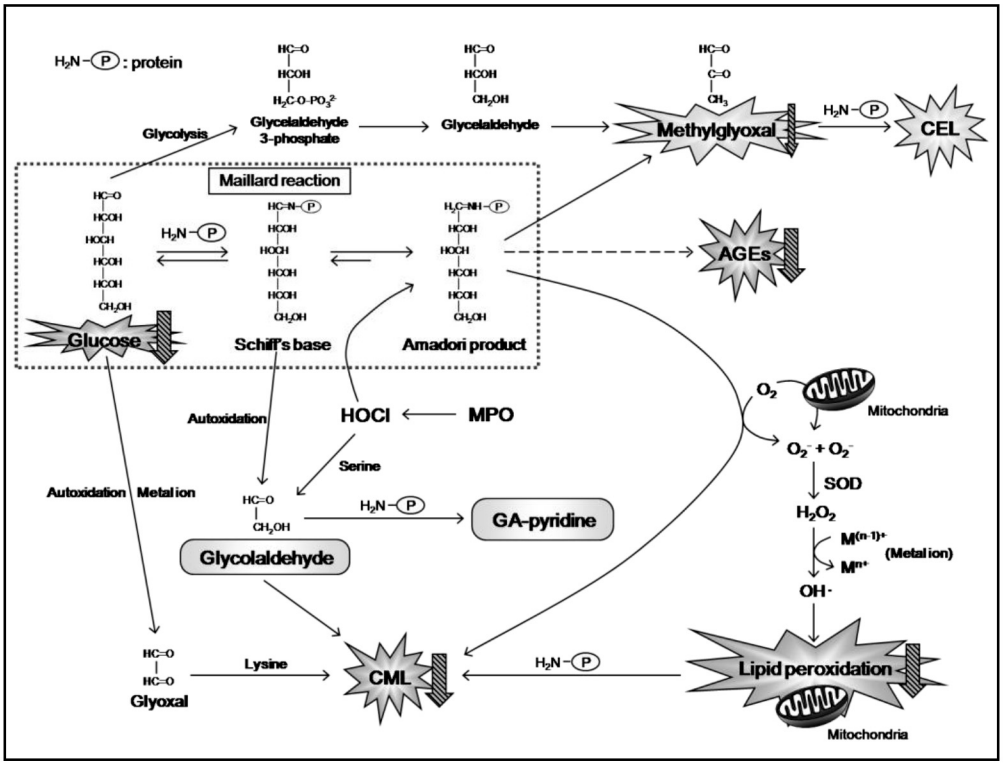

Figure 9 Effect of 7-*O*-galloyl-D-sedoheptulose on the possible mechanisms of AGE formation from glucose *via* the Maillard reaction, autoxidation, other metabolic pathways, lipid peroxidation, and the myeloperoxidase (MPO) system. CEL, N^{ε}-(carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; GA, glycolaldehyde.

5. Effect of hachimijiogan on type 1 diabetic nephropathy (advanced stage)

5.1. Typical characteristics

Diabetic nephropathy is characterized as advanced kidney disease caused by longitudinal hyperglycemia and its metabolic abnormalities. Thus, this study involved long-term administration for 15 weeks in order to show the effect of hachimijiogan on advanced kidney disease in type 1 diabetic nephropathy.[38] Rats which underwent sub-total nephrectomy and STZ injection showed metabolic abnormalities and renal lesions resembling diabetic nephropathy in humans.[39] In addition, the control rats showed a decrease in body weight gain compared with normal rats, but the 15-week administration of hachimijiogan led to a slight increase in body weight gain, suggesting that there might be no or exceedingly little toxicity derived from long-term treatment with hachimijiogan (data not shown). Conversely, over the experimental period, the serum glucose and urinary protein excretion levels were markedly higher in the rat model employed in this study than in normal rats (**Figure 10(A,B)**), indicating that disorders of glucose metabolism and changes in the capillary filtration barrier result in the increased permeability of the glomerular basement membrane (GBM). In addition, this rat model showed a significant decrease in Ccr (**Figure 10(C)**), an effective index for expressing the GFR, which decreases exponentially, and patients eventually develop nephritic syndrome.[40] However, the present investigation demonstrated that the administration of hachimijiogan for 15 weeks reduced the serum glucose and urinary protein excretion levels, but increased Ccr (**Figure 10**), suggesting that the favorable control of glucose metabolism plays an important role in the prevention of diabetic complications, including diabetic nephropathy. In addition, the decreased serum albumin level in this animal model was reversed by the administration of hachimijiogan (**Table 5**). On the basis of these results, it was found that STZ injection into sub-totally nephrectomized rats resulted in progressive diabetic nephropathy, with hachimijiogan preventing or delaying it.

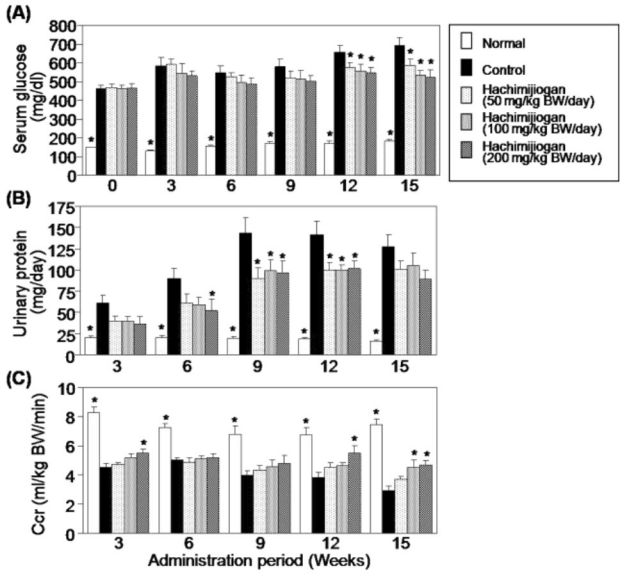

Figure 10 Effect of hachimijiogan on serum glucose (A), urinary protein (B), and Ccr (C) levels in experimental rats for 15 weeks. *P<0.05 compared with each value of diabetic nephropathy control rats.

5.2. Glycation reaction

Chronic hyperglycemia results in irreversible tissue damage caused by the protein glycation reaction that leads to the formation of glycosylated proteins and AGEs, and stimulation of the polyol pathway.[41,42] The glycosylated serum protein level increased in the animal model we used, which implies that the oxidation of sugars was stimulated (**Table 5**), enhancing 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 (**Figure 11(A)**). The excessive formation and accumulation of AGEs in tissue 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 an increased kidney weight, glomerular hypertrophy, GBM thickening, and progressive albuminuria.[43] Moreover, AGEs stimulate free-radical mechanisms and induce membrane peroxidation, which, in turn, increases membrane permeability. Therefore, AGE accumulation in the kidney has been regarded as an index of progressive renal damage associated with diabetic nephropathy. In addition, it has been recognized as playing a central role in various degenerative processes, such as aging, diabetes mellitus, dialysis-related amyloidosis, and Alzheimer's disease.[44,45] Hachimijiogan reduced the levels of glycosylated serum proteins and AGEs significantly and dose-dependently (**Table 5**, **Figure 11(A)**), suggesting that it can inhibit oxidative damage and irreversible renal damage caused by protein glycation reactions.

5.3. Polyol pathway

In the polyol pathway, glucose is reduced to sorbitol by aldose reductase and sorbitol dehydrogenase. Increased polyol pathway activity results in the depletion of myoinositol and changes in the cellular redox potential. Particularly, this has been proposed to be a causative factor in the development of diabetic nephropathy. Moreover, it has been hypothesized that the glycation reaction leads to the production of AGEs by increasing the supply of fructose, which is a reactive glycation agent with stronger reducing activity than glucose.[41,46,47] Our results showed that renal sorbitol levels were markedly elevated in rats with diabetic nephropathy compared with normal rats (**Figure 11(B)**). However, the administration of hachimijiogan significantly reduced the sorbitol level, 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.

5.4. Oxidative stress

The metabolic disorders associated with diabetic nephropathy, hyperlipidemia, and protein glycation reactions, induce lipid peroxidation caused by oxidative stress, which plays a potential role in diabetic glomerulosclerosis and renal fibrosis.[48,49] 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 the present study, the serum and renal TBA-RS levels were measured to determine the effects of hachimijiogan on oxidative stress in relation to the development of diabetic nephropathy, and the results are shown in **Table 5** and **Figure 11(C)**. Serum and renal lipid peroxidation levels were markedly elevated in rats with diabetic nephropathy compared with normal rats, while a 15-week course of hachimijiogan reduced these levels. These findings suggest that the administration of hachimijiogan 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.

Table 5 Effect of hachimijiogan on serum biochemical features in diabetic nephropathy

Items	Normal	Diabetic nephropathy			
		Control	Hachimijiogan (50 mg/kg BW/day)	Hachimijiogan (100 mg/kg BW/day)	Hachimijiogan (200 mg/kg BW/day)
Glycosylated protein (nmol/mg protein)	13.2 ± 0.7*	25.8 ± 1.4	24.1 ± 0.8	20.6 ± 1.3*	20.3 ± 0.7*
Urea nitrogen (mg/dl)	20.7 ± 0.5*	68.0 ± 6.9	56.2 ± 3.1	57.0 ± 2.5	47.0 ± 5.0*
Albumin (g/dl)	3.43 ± 0.33*	2.31 ± 0.08	2.45 ± 0.10	2.45 ± 0.09	2.63 ± 0.04
TBA-RS (nmol/ml)	2.44 ± 0.47*	5.49 ± 0.29	5.82 ± 0.28	5.42 ± 0.31	4.33 ± 0.14*

*P<0.05 compared with diabetic nephropathy control rats.

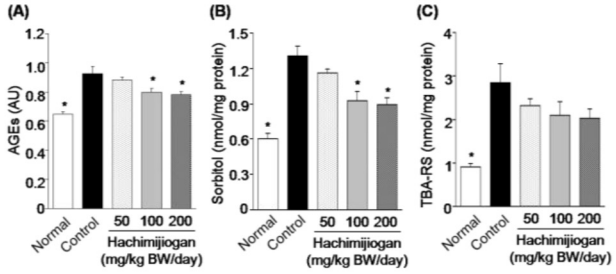

Figure 11 Effect of hachimijiogan on renal AGEs (A), sorbitol (B), and TBA-RS (C) levels at 15 weeks of the experimental period. *P<0.05 compared with diabetic nephropathy control rats.

5.5. Renal functional and structural changes

The clinical manifestations of diabetic nephropathy, notably proteinuria, hypertension, and renal insufficiency, are closely associated with the severity of renal lesions.[50,51] In the animal model 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 that are the most important morphologic characteristics of glomerular sclerosis and structural indicators of diabetic nephropathy were observed frequently. Recently, it was suggested that diffuse mesangial expansion in the glomerulus plays a critical role in the obliteration of the capillary lumen, ultimately leading to cessation of the 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.[48] This, in turn, would lead to the accumulation of urea nitrogen and Cr in the serum, and a subsequent decrease in Ccr. Therefore, the elevated serum urea nitrogen level in this animal model of diabetic nephropathy was considered to be related to the renal lesions of glomerulosclerosis, while the reduction of this level by hachimijiogan indicated the amelioration of renal lesions (**Table 6**, **Figure 12**). 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 of the glomerulus and tubulointerstitium.[50-52] Hachimijiogan had a significant protective effect against renal lesions, as demonstrated by the histopathological evaluations (**Table 6**), suggesting that it would improve renal dysfunction associated with renal lesions. Therefore, the results of the present study confirm that hachimijiogan has a protective effect in diabetic nephropathy rats through the amelioration of metabolic disorders, oxidative stress, and renal dysfunction associated with renal lesions.

Table 6 Histopathological evaluation of the kidney

Items	Normal	Diabetic nephropathy			
		Control	Hachimijiogan (50 mg/kg BW/day)	Hachimijiogan (100 mg/kg BW/day)	Hachimijiogan (200 mg/kg BW/day)
Glomerular sclerosis	0*	2.88 ± 0.87	1.38 ± 0.18*	0.79 ± 0.17*	0.43 ± 0.24*
Tubulointerstitial changes	0*	2.88 ± 0.13	2.25 ± 0.25	2.33 ± 0.21	2.29 ± 0.18
Mesangial matrix expansion	0*	2.38 ± 0.18	2.00 ± 0.01	1.83 ± 0.17*	1.43 ± 0.20*
Arteriolar sclerosis	0*	2.13 ± 0.23	2.00 ± 0.27	1.50 ± 0.22	1.43 ± 0.20
Total	0*	11.82 ± 2.66	7.38 ± 0.69*	6.45 ± 0.80*	5.58 ± 1.04*

*P<0.05 compared with diabetic nephropathy control rats.

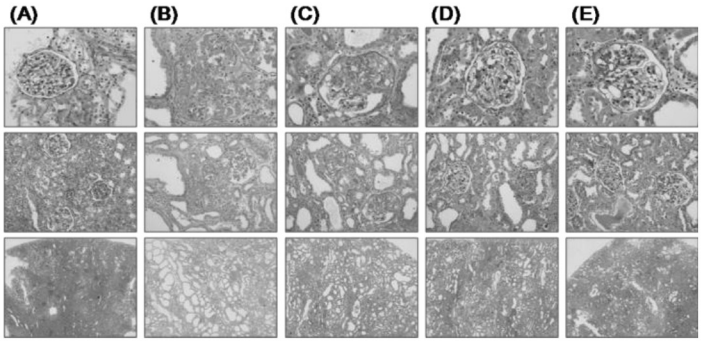

Figure 12 Photomicrographs of the glomeruli (upper panel, ×200), tubulus (middle panel, ×100), and interstitium (lower panel, ×20) obtained from normal rats (A), diabetic nephropathy rats in the control (B), and hachimijiogan-treated (50 mg/kg BW/day (C), 100 mg/kg BW/day (D), and 200 mg/kg BW/day (E)) groups.

6. Effect of hachimijiogan on type 2 diabetic nephropathy (advanced stage)

6.1. Typical characteristics

OLETF rats were used as a model of human type 2 diabetes,[53] and hachimijiogan was orally administered for 32 weeks.[54] Male OLETF diabetic rats compared to Long-Evans Tokushima Otsuka (LETO) control rats over the time course maintained a higher body weight as well as food and water consumption levels, but hachimijiogan-treated groups did not show any differences (data not shown), while hachimijiogan reduced the increase of the serum glucose level in OLETF diabetic rats from the latter half of the administration period (**Figure 13(A)**). In addition, the urinary protein excretion rate of untreated OLETF rats was more than twice that of LETO rats and it gradually increased with age, reaching 130 mg/day by 24 weeks (**Figure 13(B)**). At 32 weeks, the value for untreated OLETF rats was significantly (about 17 times) higher (222 mg/day) than that for LETO rats (13 mg/day), indicating marked proteinuria in the former. However, hachimijiogan treatment markedly reduced the urinary protein expression rats from an early stage, as in type 1 diabetic nephropathy rats, and this was maintained up to week 32. The Ccr of each group fluctuated irregularly until 24 weeks (**Figure 13(C)**), but at 32 weeks, it was significantly lower in untreated OLETF than LETO rats (4.6 and 3.8 ml/kg BW/min, respectively). However, the Cr clearances of OLETF rats treated with 50, 100, and 200 mg/kg BW of hachimijiogan daily showed a tendency to improve compared with OLETF rats by 4.2, 4.2, and 4.6 ml/kg BW/min, respectively (**Figure 13(C)**).

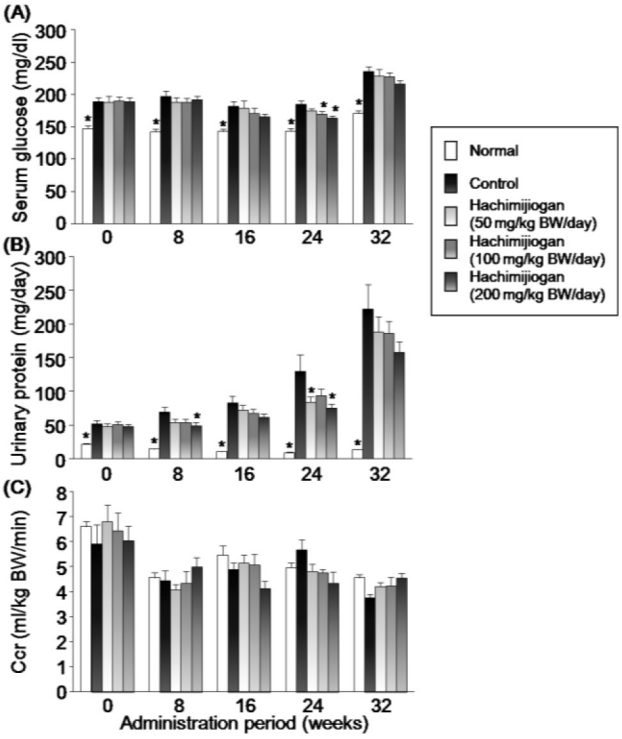

Figure 13 Effect of hachimijiogan on serum glucose (A), urinary protein (B), and Ccr (C) levels in LETO and OLETF rats. *P<0.05 compared with each value of untreated OLETF rats.

6.2. Glycation reaction and oxidative stress

In order to clarify the role of hachimijiogan in glucose toxicity, serum glycosylated protein and renal AGE levels were measured. OLETF rats had significantly elevated serum glycosylated protein and renal AGE levels, but hachimijiogan treatment significantly and dose-dependently reduced these, especially renal AGE levels, which were reduced below the level of non-diabetic LETO rats (**Tables 7,8**). Hence, hachimijiogan is applicable as a treatment against type 2 diabetes mellitus and diabetic complications, and, therefore, it may also have an effect against the process of aging. In addition, we investigated the TBA-RS levels in the serum and kidney. The long-term administration of hachimijiogan reduced renal TBA-RS levels significantly, despite only showing a non-significant tendency to reduce serum levels (**Tables 7,8**). These findings suggest that the effect of hachimijiogan in reducing oxidative stress caused by the binding activity of AGEs, plasma proteins, and RAGE in glomerular mesangial cells or macrophages is superior to its effect against markers of oxidative stress in serum.

Table 7 Serum glycosylated protein and TBA-RS levels in LETO and untreated and hachimijiogan-treated OLETF rats

Group	Dose (mg/kg BW/day))	Glycosylated protein (nmol/mg protein)	TBA-RS (nmol/ml)
LETO rats	–	11.4 ± 0.6*	2.26 ± 0.25*
OLETF rats			
Control	–	19.2 ± 1.1	3.58 ± 0.32
Hachimijiogan	50	16.0 ± 1.1*	3.47 ± 0.20
Hachimijiogan	100	15.3 ± 1.0*	3.39 ± 0.19
Hachimijiogan	200	13.4 ± 0.9*	3.27 ± 0.24

*P<0.05 vs. untreated OLETF rats.

Table 8 Renal AGEs and TBA-RS levels in LETO and untreated and hachimijiogan-treated OLETF rats

Group	Dose (mg/kg BW/day)	AGEs (AU)	TBA-RS (nmol/mg protein)
LETO rats	–	0.75 ± 0.02*	2.22 ± 0.24*
OLETF rats			
Control	–	0.89 ± 0.03	3.99 ± 0.19
Hachimijiogan	50	0.75 ± 0.02*	3.23 ± 0.19*
Hachimijiogan	100	0.73 ± 0.02*	3.04 ± 0.21*
Hachimijiogan	200	0.68 ± 0.03*	3.02 ± 0.15*

*P<0.05 vs. untreated OLETF rats.

6.3. Modulating effect of hachimijiogan on the development of diabetic nephropathy

In both diabetic rats and human glomeruli, the AGE-RAGE interaction in mesangial cells activates TGF-β-Smad signaling pathways and subsequently induces the synthesis of fibronectin and type IV collagen, which are individual matrix components, through the generation of angiotensin II via the overproduction of ROS, and then promotes adhesion to the extracellular matrix, leading to glomerular sclerosis.[55-57] Furthermore, protein kinase C (PKC), an important mediator of diabetes-induced vascular dysfunction, has been reported to modulate the function of glucose-inducing vascular endothelial growth factor (VEGF) and TGF-β_1 expression. The administration of a specific PKC β inhibitor to rats with STZ-induced diabetes attenuated glomerular hyperfiltration, reduced the urinary albumin excretion rate, and decreased the expression of TGF-β_1 and various extracellular matrix proteins, such as fibronectin and type IV collagen.[58,59] Lee *et al.* (2003) reported that the inhibition of PKC activity effectively blocked high glucose concentration- and hydrogen peroxide (without a high glucose concentration)-induced TGF-β_1 and fibronectin protein expression in mesangial cells,[60] indicating that there is a relationship between PKC and oxidative stress under conditions of hyperglycemia. In this study, TGF-β_1 and fibronectin protein expression in untreated OLETF rats significantly increased, but hachimijiogan down-regulated their expression to levels below those of non-diabetic LETO rats (**Figure 14**). These findings suggest that hachimijiogan may ameliorate functional abnormalities in association with the angiotensin II-TGF-β signaling pathway in mesangial cells, leading to glomerular sclerosis, and may ameliorate the expression of TGF-β_1 and fibronectin proteins induced by PKC activation related to oxidative stress.

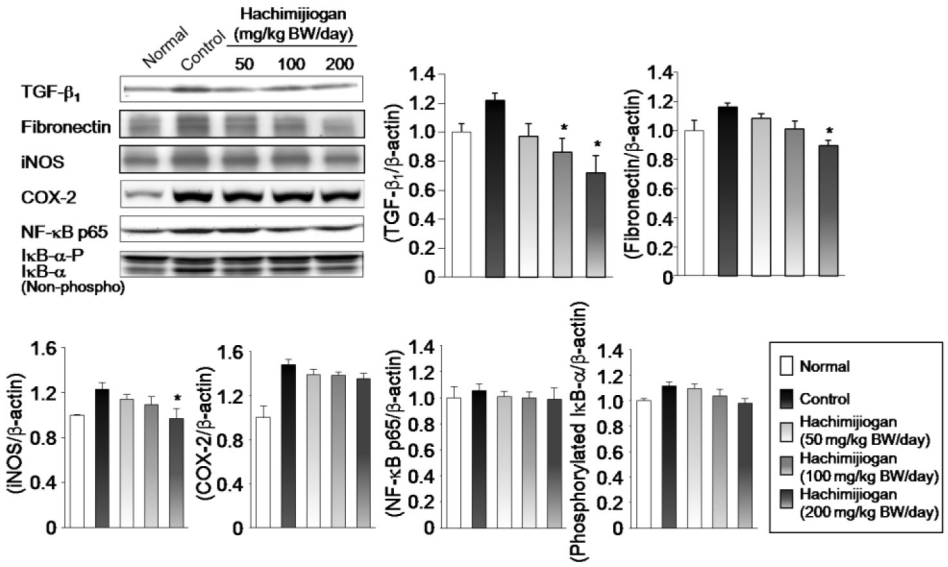

Figure 14 Western Blot analyses of TGF-β_1, fibronectin, iNOS, COX-2, NF-κB p65, and IκB-α (phosphorylated and non-phosphorylated) in the renal cortex of LETO and OLETF rats. *P<0.05 compared with each value of untreated OLETF rats.

While hyperglycemia up-regulates TGF-β_1 and fibronectin protein synthesis, nuclear factor-κB (NF-κB) and activator protein-1 expression via ROS generation have important roles.[61] In contrast, activated NF-κB induces inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, the inducible isoforms that have been reported to contribute to cytotoxicity in some cell types. In general, NF-κB is known to be normally sequestered in the NF-κB/inhibitor binding protein κB (IκB) complex and resides in the cytoplasm of unstimulated cells, but the activation of NF-κB occurs *via* the phosphorylation of its inhibitory subunit, IκB-α. This phosphorylation precedes the rapid degradation of IκB, resulting in active NF-κB release and translocation into the nucleus, where it binds to specific κB-binding sites or interacts with other transcription factors, thereby promoting gene transcription.[62-64] The target genes regulated by NF-κB include several immune and inflammatory factors, such as iNOS and COX-2.[65,66] Of interest, in this study, hachimijiogan significantly reduced the renal cortical expression of iNOS protein and slightly reduced those of COX-2 and phosphorylated IκB-α proteins in OLETF rats (**Figure 14**). Therefore, chronic treatment with hachimijiogan may have an ameliorative effect on renal injury during the development of diabetic nephropathy through inhibition of the iNOS signaling pathway.

7. Conclusion

We have studied hachimijiogan in order to establish a therapeutic strategy for the prevention of diabetic nephropathy using rat models. These results indicate that hachimijiogan could have a protective role either in early or advanced stages of type 1 and 2 diabetic nephropathy *via* the amelioration of several metabolic disorders caused by hyperglycemia and also a renoprotective effect. To add to these findings, we identified that Corni Fructus has similar effects to hachimijiogan, and also successfully identified the most important contributors to the effect of hachimijiogan, i.e., morroniside and 7-*O*-galloyl-D-sedoheptulose, which were isolated from Corni Fructus. These components are expected to become novel therapeutic agents against the development of diabetic nephropathy.

References

1. Nakai, S., Masakane, I., Shigematsu, T., Hamano, T., Yamagata, K., Watanabe, Y., Itami, N., Ogata, S., Kimata, N., Shinoda, T., Syouji, T., Suzuki, K., Taniguti, M., Tsuchida, K., Nakamoto, H., Nishi, S., Nishi, H., Hashimoto, S., Hasegawa, T., Hanafusa, N., Fujii, N., Marubayashi, S., Morita, O., Wakai, K., Wada, A., Iseki, K. and Tsubakihara, Y. (2009) An overview of regular dialysis treatment in Japan (As of 31 December 2007). *Ther. Apher. Dial.* **13**: 457-504.
2. Remuzzi, G. and Bertani, T. (1998) Pathophysiology of progressive nephropathies. *N. Engl. J. Med.* **339**: 1448-1456.
3. Lehmann, R. and Schleicher, E.D. (2000) Molecular mechanism of diabetic nephropathy. *Clinica. Chemica. Acta* **297**: 135-144.
4. UK Prospective Diabetes Group. (1998) Intensive blood-glucoe control with sulphonylurea or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* **352**: 837-853.
5. Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. (2000) Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. *Lancet* **355**: 253-259.
6. Brenner, B.M., Cooper, M.E., de Zeeuw, D., Keane, W.F., Mitch, W.E., Parving, H.H., Remuzzi, G., Snapinn, S.M., Zhang, Z. and Shahinfar, S. (2001) Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N. Engl. J. Med.* **345**: 861-869.
7. Lewis, E.J., Hunsickern, L.G., Clarke, W.R., Berl, T., Pohl, M.A., Lewis, J.B., Ritz, E., Atkins, R.C., Rohde, R. and Raz, I. (2001) Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patiens with nephropathy due to type 2 diabetes. *N. Engl. J. Med.* **345**: 851-860.
8. Yokozawa, T., Nakagawa, T. and Terasawa, K. (2001) Effects of Oriental medicines on the production of advanced glycation end products. *J. Trad. Med.* **18**: 107-112.
9. Nakagawa, T., Yokozawa, T. and Terasawa, K. (2001) A study of Kampo medicines in a diabetic nephropathy model. *J. Trad. Med.* **18**: 161-168.
10. Goto, M., Inoue, H., Seyama, Y., Yamashita, S., Inoue, O. and Yumioka, E. (1989) Comparative effects of traditional Chinese medicines (dai-saiko-to, hatimi-zio-gan and byakko-ka-ninzin-to) on experimental diabetes and hyperlipidemia. *Nippon Yakugaku Zasshi* **93**: 179-186.
11. Furuya, Y., Kawakita, T. and Tajima, S. (1999) Effect of Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan) on insulin resistance in non-insulin dependent diabetes mellitus model mice. *J. Trad. Med.* **16**: 123-128.
12. Yamada, T. *Kinki Yoryaku*, 1992. Kyouwa-Kikaku, Tokyo, pp. 1-7.
13. Huang, T. *A Handbook of Traditional Chinese Prescriptions of Dubious and Complicated Cases*, 1997. China Medical and Pharmaceutical Science and Technology Publishing House, Beijing, pp. 527-538.
14. Zhang, Y., Chen, Y. and Zhao, S. (1999) A sedoheptulose gallate from the fruits of Cornus officinalis. *Acta Pharm. Sin.* **34**: 153-155.
15. Tschesche, R., Struckmeyer, K. and Wulff, G. (1971) Glycoside with lactone forming aglycones. IV. Mevaloside, the glucoside of (-) (R) mevalonic acid lactone from the leaves of the medlar and a simple preparation of (-) (R)-mevalonic acid lactone. *Chem. Ber.* **104**: 3567-3572.
16. El-Naggar, L.J. and Beal, J.L. (1980) Iridoids, a review. *J. Nat. Prod.* **43**: 649-707.
17. Liou, S.S., Liu, I.M., Hsu, S.F. and Cheng, J.T. (2004) Corni Fructus as the major herb of Die-Huang-Wan for lowering plasma glucose in Wistar rats. *J. Pharm. Pharmacol.* **56**: 1443-1447.
18. Inouye, H., Tobita, S., Akiyama, Y., Ito, K. and Shingu, T. (1973) The monoterpene glucosides and related natural substances. XXI: The stereochemistry of morroniside and its derivatives. *Chem. Pharm. Bull.* **21**: 846-854.
19. Otsuka, H. and Kijima, K. (2001) An iridoid gentiobioside, a benzophenone glucoside and acylated flavone C-glycosides from Triterospermum japonicum (Sieb. et Zucc.) Maxim. *Chem. Pharm. Bull.* **49**: 699-702.
20. Garcia, J. and Chulia, A.J. (1986) Loganin and new iridoid glucosides in Gentiana pedicellata. *Planta Medica* **4**: 327-329.
21. Fan, C. and Xiang, Q.Y. (2001) Phylogenetic relationships within Cornus (Cornaceae) based on 26S rDNA sequences. *Am. J. Bot.* **88**: 1131-1138.
22. Hatano, T., Ogawa, N., Kira, R., Tasuhara, T. and Okuda, T. (1989) Tannins of cornaceous plants. I. Cornusiins A, B and C, dimeric monomeric and trimeric hydrolyzable tannins from Cornus officinalis, and orientation of

valoneoyl group in related tannins. *Chem. Pharm. Bull.* **37**: 2083-2090.

23. Wang, S.F., Chen, X.G., Hu, Z.D. and Ju, Y. (2003) Analysis of three effective components in Fructus corni and its preparations by micellar electrokinetis capillary chromatography. *Biomed. Chromatogr.* **17**: 306-311.
24. Mau, J.L., Chen, C.P., Hsieh, P.C. and Lin, C.C. (2001) Antimicrobial effect of extracts from Chinese Chive, Cinnamon, and Corni Fructus. *J. Agric. Food Chem.* **49**: 183-188.
25. Chang, J.S., Chiang, L.C., Hsu, F.F. and Lin, C.C. (2004) Chemoprevention against hepatocellular carcinoma of Cornus officinalis in vitro. *Am. J. Chin. Med.* **32**: 717-725.
26. Vareed, S.K., Reddy, M.K., Schutzki, R.E. and Nair, M.G. (2006) Anthocyanins in *Cornus alternifolia*, *Cornus controversa*, *Cornus kousa* and *Cornus florida* fruits with health benefits. *Life Sci.* **78**: 777-784.
27. Xu, H.Q. and Hao, H.P. (2004) Effects of iridoid total glycoside from Cornus officinalis on prevention of glomerular overexpression of transforming growth factor beta 1 and matrixes in an experimental diabetes model. *Biol. Pharm. Bill.* **27**: 1014-1018.
28. Kim, H.Y., Yokozawa, T., Cho, E.J. and Yamabe, N. (2004) Protective effects of the Chinese prescription Hachimi-jio-gan against diabetic oxidative stress. *J. Pharm. Pharmacol.* **56**: 1537-1644.
29. Yokozawa, T., Dong, E., Oura, H., Kashiwagi, H., Nonaka, G. and Nishioka, I. (1997) Magnesium lithospermate B suppresses the increase of active oxygen in rats after subtotal nephrectomy. *Nephron* **75**: 88-93.
30. Nakagawa, T., Yokozawa, T., Terasawa, K. and Nakanishi, K. (2003) Therapeutic usefulness of Keishi-bukuryo-gan for diabetic nephropathy. *J. Pharm. Pharmacol.* **55**: 219-227.
31. Yokozawa, T., Kim, H.Y. and Yamabe, N. (2004) Amelioration of diabetic nephropathy by dried Rehmanniae Radix (Di Huang) extract. *Am. J. Chin. Med.* **32**: 829-839.
32. Yamabe, N., Kang, K.S., Goto, E., Tanaka, T. and Yokozawa, T. (2007) Beneficial effect of Corni Fructus, a constituents of Hachimi-jio-gan, on advanced glycation endproduct-mediated renal injury in streptozotocin-treated diabetic rats. *Biol. Pharm. Bull.* **30**: 520-526.
33. Yamabe, N., Kang, K.S., Matsuo, Y., Tanaka, T. and Yokozawa, T. (2007) Identification of antidiabetic effect of iridoid glycosides and low molecular weight polyphenol fractions of Corni Fructus, a constitute of Hachimi-jio-gan, in streptozotocin-induced diabetic rats. *Biol. Pharm. Bull.* **30**: 1289-1296.
34. Cui, S., Verroust, P.J., Moestrup, S.K. and Christensen, E.I. (1996) Megalin/gp330 mediates uptake of albumin renal proximal tubule. *Am. J. Physiol.* **271**: F900-F907.
35. Xu, H.Q., Hao, H.P., Zhang, X. and Pan, Y. (2004) Morroniside protects cultured human umbilical vein endothelial cells from damage by high ambient glucose. *Acta Pharmacol. Sci.* **25**: 412-415.
36. Xu, H.Q., Shen, J., Liu, H., Shi, Y., Li, L. and Wei, M. (2006) Morroniside and liganin extracted from Cornus officinalis have protective effects on rat mesangial cell proliferation exposed to advanced glycation end products by preventing oxidative stress. *Can. J. Physiol. Pharmacol.* **84**: 1267-1273.
37. Yamabe, N., Kang, K.S., Park, C.H., Tanaka, T. and Yokozawa, T. (2009) 7-*O*-Galloyl-D-sedoheptuloze is a novel therapeutic agent against oxidative stress and advance glycation endproducts in the diabetic kidney. *Biol. Pharm. Bull.* **32**: 657-664.
38. Yokozawa, T., Yamabe, N., Cho, E.J., Nakagawa, T. and Oowada, S. (2004) A study on the effects to diabetic nephropathy of Hachimi-jio-gan in rats. *Nephron Exp. Nephrol.* **97**: e38-e48.
39. Yokozawa, T., Nakagawa, T., Wakaki, K. and Koizumi, F. (2001) Animal model of diabetic nephropathy. *Exp. Toxic. Pathol.* **53**: 359-363.
40. Bell, D.S. (1991) Diabetic nephropathy: Changing concepts of pathogenesis and treatment. *Am. J. Med. Sci.* **301**: 195-200.
41. Cooper, M.E., Gilbert, R.E. and Epstein, M. (1998) Pathophysiology of diabetic nephropathy. *Metabolism* **47** (suppl 1): 3-6.
42. Yabe-Nishimura, C. (1998) Aldose reductase in glucose toxicity: A potential target for the prevention of diabetic complications. *Pharmacol. Rev.* **50**: 21-33.
43. Vlassara, H., Striker, L.J., Teichberg, S., Fuh, H., Li, Y.M. and Steffes, M. (1994) Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats. *Proc. Natl. Acad. USA* **91**: 11704-11708.
44. Niwa, T. (1997) β_2-Microglobulin dialysis amyloid and its formation: Role of 3-deoxyglucosone and advanced glycation end products. *Nephron* **76**: 373-391.
45. Raj, D.S.C., Choudhury, D., Welbourne, T.C. and Levi, M. (2000) Advanced glycation end products: A neurologist's

perspective. *Am. J. Kidney Dis.* **35**: 365-380.

46. Goldfarb, S., Ziyadeh, F.N., Kern, E.F. and Simmons, D.A. (1991) Effects of polyol-pathway inhibition and dietary myo-inositol on glomerular hemodynamic function in experimental diabetes mellitus in rats. *Diabetes* **40**: 465-471.
47. Hamada, Y., Araki, N., Koh, N., Nakamura, J., Horiuchi, S. and Hotta, N. (1996) Rapid formation of advanced glycation end products by intermediate metabolites of glycolytic pathway and polyol pathway. *Biochem. Biophys. Res. Commun.* **228**: 539-543.
48. Giugliano, D., Ceriello, A. and Paolisso, G. (1996) Oxidative stress and diabetic vascular complications. *Diabetes Care* **19**: 257-267.
49. Salahudeen, A.K., Kanji, V., Reckelhoff, J.F. and Schmidt, A.M. (1997) Pathogenesis of diabetic nephropathy: A radical approach. *Nephrol. Dial. Transplant.* **12**: 664-668.
50. Winetz, J.A., Golbetz, H.V., Spencer, R.J., Lee, J.A. and Myers, B.D. (1982) Glomerular function in advanced human diabetic nephropathy. *Kidney Int.* **21**: 750-756.
51. Adler, S. (1997) Structure-function relationships in diabetic nephropathy: Lessons and limitations. *Kidney Int.* **60**: S42-S45.
52. Yaqoob, M., McClelland, P., Patrick, A.W., Stevenson, A., Mason, H., White, M.C. and Bell, G.M. (1994) Evidence of oxidative injury and tubular damage in early diabetic nephropathy. *QJM* **87**: 601-607.
53. Kawano, K., Hirashima, T., Mori, S., Saitoh, Y., Kurosumi, M. and Natori, T. (1992) Spontaneous long-term hyperglycemic rat with diabetic complications. *Diabetes* **41**: 1422-1428.
54. Yamabe, N. and Yokozawa, T. (2006) Activity of the Chinese prescription Hachimi-jio-gan against renal damage in the Otsuka Long-Evans Tokushima Fatty rat: a model of human type 2 diabetes mellitus. *J. Pharm. Pharmacol.* **58**: 535-545.
55. Ignotz, R.A. and Massagué, J. (1987) Cell adhesion protein receptors as targets for transforming growth factor-β action. *Cell* **51**: 189-197.
56. Yamamoto, T., Nakamura, T., Noble, N.A., Ruoslahti, E. and Border, W.A. (1993) Expression of transforming growth factor b is elevated in human and experimental diabetic nephropathy. *Proc. Natl. Acad. Sci. USA* **90**: 1814-1818.
57. Fukami, K., Ueda, S., Yamagishi, S., Kato, S., Inagaki, Y., Takeuchi, M., Motomiya, Y., Bucala, R., Iida, S., Tamaki, K., Imaizumi, T., Cooper, M.E. and Okuda, S. (2004) AGEs activate mesangial TGF-β-Smad signaling via an antiotensin II type I receptor interaction. *Kidney Int.* **66**: 2137-2147.
58. Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., Stramm, L.E., Feener, E.P. and King, G.L. (1996) Amelioration of vascular dysfunctions in diabetic rats by oral PKC β inhibitor. *Science* **272**: 728–731.
59. Koya, D., Jirousek, M.R., Lin, Y.W., Ishii, H., Kuboki, K. and King, G.L. (1997) Characterization of protein kinase C β isoform activation on the gene expression of transforming growth factor-β, extracellular matrix components, and prostanoid in the glomeruli of diabetic rats. *J. Clin. Invest.* **100**:115–126.
60 . Lee, G.T., Ha, H., Jung, M., Li, H., Hong, S.W., Cha, B.S., Lee, H.C. and Cho, Y.D. (2003) Delayed treatment with Lithospermate B attenuated experimental diabetic renal injury. *J. Am. Soc. Nephrol.* **14**: 709-720.
61. Chen, S., Khan, Z.A., Cukiernik, M. and Chakrabarti, S. (2003) Differential activation of NF-κB and AP-1 in increased fibronectin synthesis in target organs of diabetic complications. *Am. J. Physiol. Endocrinol. Metab.* **284**: E1089-E1097.
62. Baeuerle, P.A. and Baltimore, D. (1998) IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242**: 540-546.
63. Schmidt, K.N., Amstad, P., Cerutti, P. and Baeuerle, P.A. (1995) The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. *Chem. Biol.* **2**: 13-22.
64. DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. and Karin, M. Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell Biol.* **16**: 1295-1304.
65. Xie, Q.W., Kashiwabara, Y. and Nathan, C. (1994) Role of transcriptional factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**: 4705-47-8.
66. Harris, R.C. and Breyer, M.D. (2001) Physiological regulation of cyclooxygenase-2 in the kidney. *Am. J. Physiol. Renal Physiol.* **281**: F1-F11.