Beneficial Effect of Corni Fructus, a Constituent of Hachimi-jio-gan, on Advanced Glycation End-product-Mediated Renal Injury in Streptozotocin-Treated Diabetic Rats

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Previous investigations have demonstrated that Hachimi-jio-gan, a Chinese prescription consisting of eight crude drugs, has a therapeutic potential in diabetes and diabetic nephropathy, using these model rats. To add to these findings, we performed this study to assess whether one of the crude drugs, Corni Fructus (*Cornus officinalis* SIEB. *et* ZUCC.), had an effect on streptozotocin-induced diabetic rats as a major active constituent, compared with an inhibitor of advanced glycation end-product (AGE) formation, aminoguanidine. Diabetic rats were orally administrated Corni Fructus extract (50, 100, 200 mg/kg body weight/d) or aminoguanidine (100 mg/kg body weight/d). Treatment with Corni Fructus for 10 d suppressed hyperglycemia, proteinuria, renal AGE formation, and related protein expressions, *i.e.*, receptor for AGEs, nuclear factor-κB, transforming growth factor-β_1, and N^{ε}-(carboxymethyl)lysine, in the same way as with aminoguanidine. However, improvement of renal function, shown *via* serum creatinine (Cr) and Cr clearance, was superior to aminoguanidine treatment. In conclusion, the present study supported the hypothesis that Corni Fructus plays an important role against diabetic pathogenesis, *i.e.*, reducing glucose toxicities, up-regulating renal function, and consequently ameliorating glycation-associated renal damage; thus, this study may provide a new recognition of crude drugs to clarify the mechanisms of Chinese prescriptions.

Key words Corni Fructus; aminoguanidine; advanced glycation end-product; renal damage; streptozotocin

Diabetes is a disorder of excessive urine excretion and chronic hyperglycemia, and glucose participates in diabetic complications such as atherosclerosis, cardiac dysfunction, and nephropathy. Chronic hyperglycemia accelerates activation of the formation of advanced glycation end-products (AGEs), oxidative stress, the polyol pathway, and protein kinase C pathway. These metabolic factors are synergistically correlated with one another; therefore, an effective treatment with wide-spread effects continues to be required.

Up to now, there have been many experiments focusing on the treatment of diabetes and its complications with traditional medicines including Chinese prescriptions because of their absence of toxic and/or side-effects. In our previous study, we reported that the Chinese prescriptions Hachimi-jio-gan and Keishi-bukuryo-gan had potential therapeutic effects against diabetic nephropathy, and had different functions in terms of their effects on metabolic disorders, especially on AGE formation in Hachimi-jio-gan and oxidative stress in Keishi-bukuryo-gan.[1—4)] In addition, we also clarified that administration of dried Rehmanniae Radix (*Rehmannia glutinosa* LIBOSCH. var. *purpurea* MAKINO) extract, which is the main constituent of Hachimi-jio-gan, attenuates renal dysfunction in diabetic nephropathy mainly due to its suppression of oxidative stress[5)]; however, for the analysis of this prescription, further characterization of the other constituents is needed. According to the three-dimensional HPLC profile, as previously shown,[2,4)] morroniside, loganin, and paeoniflorin were detected as the major compounds in Hachimi-jio-gan. Morroniside and loganin are the components of Corni Fructus (*Cornus officinalis* SIEB. *et* ZUCC.) and paeoniflorin is the component of Moutan Cortex (*Paeonia suffruticosa* ANDREWS) common in Keishi-bukuryo-gan. Therefore, in order to clarify the source of a particular action of Hachimi-jio-gan, we chose to evaluate the usefulness of one of the crude drugs, Corni Fructus.

Corni Fructus has been used as a traditional medicine in Japan and China, and the components of this plant are iridoid total glycoside such as morroniside and loganin and also a few polyphenols such as cornusiin A, B, and C, monomeric and trimeric hydrolysable tannins, and so on.[6,7)] Recently, it has been reported that Corni Fructus has a plasma glucose-lowering action in normal rats, along with anti-neoplastic and anti-microbial effects.[8—10)] Moreover, Vareed *et al.*[11)] also reported that Corni Fructus has been used for improving liver and kidney functions, and iridoid total glycoside has the effect of preventing the overexpression of transforming growth factor (TGF)-β_1 and matrixes in glomeruli with a diabetic model.[12)] However, the mechanisms of Corni Fructus against glucose-associated metabolic disorders in diabetes have yet to be explored. To determine whether Corni Fructus possesses the principal role in Hachimi-jio-gan, which has a strong effect on AGE formation in diabetes and/or diabetic nephropathy, we examined the effect of Corni Fructus in streptozotocin (STZ)-induced diabetic rats, comparing it with the inhibitor of AGE formation, aminoguanidine.

MATERIALS AND METHODS

Materials The following reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan: 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid (TBA)), 5-hydroxymethylfurfural (5-HMF), oxalic acid, NADPH, sulfanilamide, naphthylethylene diamine dihydrochloride, EDTA, nitro blue tetrazolium (NBT), NADH, phenazine methosulfate (PMS), sodium nitrite, bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris(hydroxymethyl)aminomethane), Tween 20, glycerol, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor mixture

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DMSO solution, and skim milk powder. The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Japan. Polyclonal anti nuclear factor-κB (NF-κB) p65 antibody (sc-109), polyclonal anti inhibitor binding protein κB-α (IκB-α) antibody (sc-371), polyclonal anti receptor for AGE (RAGE) antibody (sc-5563), polyclonal anti TGF-β_1 antibody (sc-146), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (sc-2004), and goat anti-mouse IgG HRP conjugated secondary antibody (sc-2005) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A. Polyclonal anti N^{ε}-(carboxymethyl)lysine (CML) antibody was kindly provided by Dr. R. Nagai (Kumamoto University, Japan). STZ, aminoguanidine hydrochloride, nitrate reductase, and anti-mouse β-actin antibody were purchased from Sigma-Aldrich, St. Louis, MO, U.S.A. ECL Western blotting detection reagents were purchased from Amersham Bioscience, Piscataway, NJ, U.S.A.

Corni Fructus Extract Corni Fructus extract used in this experiment was produced by Tsumura Juntendo, Inc., Tokyo, Japan. A voucher specimen is deposited in the Institute of Natural Medicine, University of Toyama, Japan. The extract gave a dark blue coloration with the $FeCl_3$ reagent suggesting presence of tannins. The 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 comparison of t_R and UV absorptions with those of authentic samples.

Experimental Design The Guidelines for Animal Experimentation approved by the University of Toyama were followed in all experimental studies. Five-week-old male Wistar rats (120—130 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), kept in wire-bottomed cages, and exposed to a 12-h light/dark cycle. The room temperature and humidity were maintained automatically at about 25°C and 60%, respectively. They were allowed free access to laboratory pellet chow (CLEA Japan, Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid, and 60.5% carbohydrate) and water. After several days of adaptation, the rats were injected intraperitoneally with STZ (50 mg/kg body weight) in 10 mM citrate buffer (pH 4.5). The blood glucose level was determined and the body weight was measured 10 d after the injection, and to avoid any inter-group differences in these indices, the rats were divided into five experimental groups: group 1, diabetic rats received water (diabetic control, $n=8$); groups 2, 3, and 4, diabetic rats received 50, 100, and 200 mg/kg body weight/d of Corni Fructus orally *via* gavage once a day (diabetic+CF50, 100, and 200, $n=8$, respectively); and group 5, diabetic rats received 100 mg/kg body weight/d of aminoguanidine orally *via* gavage once a day (diabetic+AG100, $n=5$). The rats which underwent a sham injection of citrate buffer without STZ were also used as controls ($n=5$). After 10 d of the administration period, 24-h urine samples were collected using metabolic cages and blood samples were obtained from the abdominal aorta under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally), and then the serum was immediately separated from the blood samples by centrifugation. After renal perfusion through the renal artery with ice-cold physiological saline, the kidneys were removed from each rat and frozen at −80°C until analysis.

Biochemical Measurements Serum levels of glucose, total protein, albumin, total cholesterol, urea nitrogen (urea-N), and creatinine (Cr) were examined using commercial reagents (Glucose CII-Test Wako, A/G B-Test Wako and Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan; BUN Kainos and CRE-EN Kainos obtained from Kainos Laboratories, Inc., Tokyo, Japan). Serum glycosylated protein was measured by the TBA assay of McFarland *et al.*,[13] in which non-enzymatically bound glucose is released as 5-HMF and quantitated colorimetrically. TBA-reactive substance levels were determined using the methods of Naito and Yamanaka.[14] Urine component levels were determined as follows: protein by the sulfosalicylic acid method[15] and Cr using a commercial reagent (CRE-EN Kainos). Cr clearance (Ccr) was calculated on the basis of urinary Cr, serum Cr, urine volume, and body weight using the following equation: Ccr (ml/min/kg body weight)=[urinary Cr (mg/dl)×urinary volume (ml)/serum Cr (mg/dl)]×[1000/body weight (g)]×[1/1440 (min)].

Antioxidative Capacity in Serum The serum nitrite/nitrate level was determined using the method of Mabley *et al.* [16] In brief, the serum was mixed with nitrate reductase and NADPH, which were both dissolved in 40 mM Tris, pH 7.6, and incubated at room temperature for 3 h. Following this period, Griess reagent was added and incubated for a further 10 min at room temperature. The absorbance of the samples was read at 550 nm. The concentration of nitrite/nitrate was determined from a standard curve of sodium nitrite.

Serum superoxide dismutase (SOD) activity was determined using a method based on that of Ewing and Janero.[17] The serum was mixed with 0.125 mM EDTA, 62 μM NBT, 98 μM NADPH, and 33 μM PMS in 50 mM phosphate buffer, pH 7.4, and then the reduced NBT level was measured at 550 nm.

AGE Level in Kidney The renal AGE level was determined by the method of Nakayama *et al.*[18] Minced kidney tissue was delipidated with chloroform and methanol (2 : 1, v/v) overnight. After washing, the tissue was homogenized in 0.1 N NaOH, followed by centrifugation at 8000×$\boldsymbol{g}$ for 15 min at 4°C. The amounts of AGEs in these alkali-soluble samples were determined by measuring the fluorescence at an emission wavelength of 440 nm and an excitation wavelength at 370 nm. A native BSA preparation (1 mg/ml of 0.1 N NaOH) was used as a standard, and its fluorescence intensity was defined as one unit of fluorescence. The fluorescence values of samples were measured at a protein concentration of 1 mg/ml and expressed in AU compared with a native BSA preparation.

Mitochondrial TBA-Reactive Substance Level in Kidney Mitochondria were prepared from kidney homogenate by differential centrifugation (800×$\boldsymbol{g}$ and 12000×$\boldsymbol{g}$, respectively) at 4°C according to the methods of Johnson and Lardy[19] and Jung and Pergande,[20] respectively, with minor modifications. Each pellet was resuspended in preparation medium and the concentration of TBA-reactive substance was determined by the method of Uchiyama and Mihara.[21] The protein level was examined by the method of Itzhaki and Gill[22] with BSA as the standard.

Protein Extraction and Western Blot Analyses Renal cortical sections were homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris–HCl, 1% Tween 20, 10% glycerol, 1 mM PMSF, and protease inhibitor mixture DMSO solution. After centrifugation (2000×***g*** at 4°C) to ensure equal loading among lanes, the protein concentration of each tissue was determined using a Bio-Rad protein assay kit and BSA as a standard, and then immunoblotting was carried out.

For the determination of RAGE, NF-κBp65, IκB-α, TGF-β_1, and CML protein expressions, 30 μg protein of each sample was electrophoresed through 8, 12, and 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% skim milk solution for 1 h, and then incubated with primary antibodies to RAGE, NF-κBp65, IκB-α, TGF-β_1, CML, and β-actin, respectively, overnight at 4 °C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP conjugated secondary antibody for 90 min at room temperature. Each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Japan).

Band densities were determined by Scion image software (Scion Corporation, Frederick, MD) and quantified as the ratio to β-actin. The evaluation of these protein levels at mean values against control rats is represented as 1 and the corresponding values for the diabetic rats are expressed as the ratios of these values.

Statistical Analysis The results are presented as the mean±S.E. The effect of Corni Fructus on each parameter was examined using the one way Analysis of Variance. Individual differences among groups were analyzed by Dunnett's test and significance was accepted at $p<0.05$.

RESULTS

Body Weight Change and Serum Glucose Level A significant decrease in body weight during the 10 d was observed in the diabetic control rats compared with control rats, showing no difference between initial and final values; however, administration of Corni Fructus at doses of 100 and 200 mg to diabetic rats increased body weight gain significantly, similarly to aminoguanidine-treated diabetic rats (Fig. 1A).

Figure 1B shows serum glucose levels over the 10-d administration period. In contrast with body weight gain, diabetic control rats showed an increase from the initial value (from 524.1 to 577.2 mg/dl) and the final value was 5.6-fold higher than that of control rats. On the other hand, 50, 100, and 200 mg Corni Fructus-treated diabetic rats showed a significant decrease compared with diabetic control rats (498.3 mg/dl, 499.3 mg/dl, and 460.8 mg/dl, respectively), and oral administration of 100 mg aminoguanidine led to a reduction to 472.0 mg/dl.

Serum Constituents Table 1 shows the serum constituents except for the glucose level at the end of this experiment. The serum glycosylated protein and total cholesterol levels of diabetic control rats were significantly increased by about 1.81- and 1.88-fold, respectively, relative to the control levels, while Corni Fructus extract decreased these levels significantly, and the effects in the Corni Fructus 100 mg group were nearly the same as in the 100 mg aminoguanidine-treated group. Diabetes led to significant increases in TBA-reactive substance and decreases in albumin levels, and there was only a tendency of a decrease in the TBA-reactive substance level by treatment with Corni Fructus and aminoguanidine, and no effect on the albumin level in Corni Fructus-treated groups except for a slight increase without

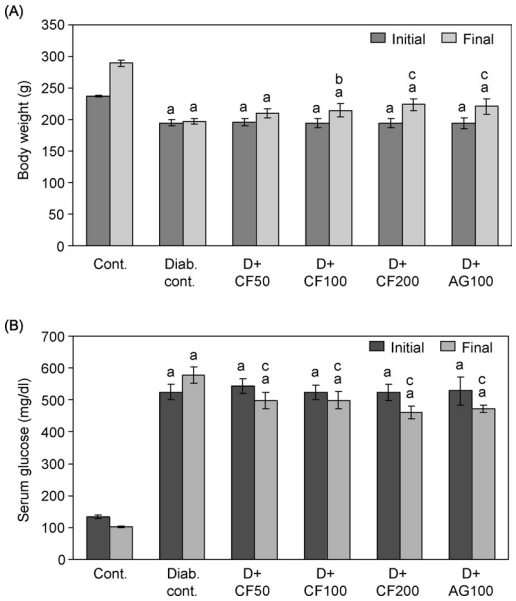

Fig. 1. Effect of Corni Fructus and Aminoguanidine on Body Weight Changes (A) and Serum Glucose Levels (B) in STZ-Induced Diabetic Rats

CF50, 100, or 200: Corni Fructus (50, 100, or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. ($n=5$ or 8). [a]$p<0.001$ *vs.* control rats; [b]$p<0.05$, [c]$p<0.001$ *vs.* diabetic control rats.

Table 1. Serum Parameters

Items	Control ($n=5$)	Diabetic control ($n=8$)	Diabetic +CF50 ($n=8$)	Diabetic +CF100 ($n=8$)	Diabetic +CF200 ($n=8$)	Diabetic +AG100 ($n=5$)
Glycosylated protein (nmol/mg protein)	12.2±0.2	22.1±0.8[a)]	21.1±0.7[a)]	19.4±0.8[a,c)]	18.5±1.0[a,c)]	20.2±1.1[a,b)]
Albumin (g/dl)	3.22±0.03	2.93±0.06[a)]	2.88±0.04[a)]	2.91±0.02[a)]	2.88±0.03[a)]	3.01±0.08[a)]
Total cholesterol (mg/dl)	42.8±1.4	80.5±5.4[a)]	67.9±5.1[a,c)]	65.4±3.8[a,c)]	62.6±3.7[a,c)]	64.2±4.7[a,c)]
TBA-reactive substance (nmol/ml)	2.47±0.26[a)]	5.57±0.45[a)]	5.20±0.51[a)]	5.03±0.40[a)]	4.96±0.54[a)]	4.71±0.60[a)]

CF50, 100 or 200: Corni Fructus (50, 100 or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. ($n=5$ or 8). *a*) $p<0.001$ *vs.* control rats; *b*) $p<0.05$, *c*) $p<0.001$ *vs.* diabetic control rats.

significance in the aminoguanidine-treated group.

Antioxidative Activity in Serum Serum nitrite/nitrate levels, indicative of nitric oxide formation and SOD activity, represented by the inhibition of NBT reduction, indicative of superoxide anion (O_2^-) generation, were increased in diabetic control rats; however, treatment with Corni Fructus affected these incidences dose-dependently, and especially, the Corni Fructus 200 mg group and aminoguanidine group showed strongly reduced nitrite/nitrate levels compared to the control group (Table 2).

Renal Function Table 3 shows the renal function parameters of the control and experimental groups. In the diabetic state, serum urea-N and Cr levels significantly increased 1.82- and 1.18-fold, respectively, while Corni Fructus treatment from the lowest dose of 50 mg caused a significant decrease in these levels, and these effects were superior to aminoguanidine treatment. Conversely, there was a significant decrease in the Ccr level in the diabetic control rats compared with the control rats; however, Corni Fructus-treated groups at all doses, particularly the highest dose group of 200 mg, improved further than the control level, while improvement was poor in the aminoguanidine-treated group. The urinary protein excretion level was increased in the diabetic control rats, without significance, relative to the control rats, but both Corni Fructus- and aminoguanidine-administered rats showed larger decreases in the level compared to control rats.

Renal Weight, AGE, and Mitochondrial TBA-Reactive Substance Levels The renal weights of diabetic rats treated with Corni Fructus and aminoguanidine decreased approximately to the same level, while diabetic control rats showed a significant 1.46-fold increase compared to the control rats (Table 4). The renal levels of AGEs and mitochondrial TBA-reactive substance were increased 1.85- and 1.55-fold, respectively, in diabetic control rats as compared to the control values (Table 4). These levels were equally decreased by the administration of Corni Fructus dose-dependently and of aminoguanidine; additionally, the effect observed in the 200 mg Corni Fructus-treated group was the same as in the aminoguanidine-treated group.

Western Blotting of Renal Cortex For further investigation, the protein levels of RAGE, NF-κB, IκB-α, TGF-β_1, and CML in the renal cortex were determined by Western blot analyses, and the results are presented in Fig. 2. Based on band densities, renal RAGE, NF-κB, TGF-β_1, and CML, which was estimated to be a 50 kDa molecule, CML-modified, and also anti-CML antibody-reactive protein, were significantly elevated in diabetic control rats compared with the control rats, but no significant changes were observed in IκB-α. However, down-regulation of these protein expressions was shown in the rats given high dose Corni Fructus but also aminoguanidine, and the levels of RAGE and NF-κB observed in the Corni Fructus 200 mg group were more prominent than those of the aminoguanidine-treated group.

DISCUSSION

The role of tight glycemic control, which was performed by The Diabetes Control and Complication Trial Research Group,[23] has been emphasized and is still supported as being important to reduce diabetic microvascular disease in type 1 (insulin-dependent) diabetes, and for more than two decades, many researchers have discussed the pathological features of diabetes and/or its complications which are caused by the hyperglycemia-accelerated formation of AGEs in tissue. On the other hand, aminoguanidine, the prototype AGE inhibitor, has been effective in retarding the full range of diabetic complications, such as nephropathy, neuropathy, retinopathy, and vasculopathy. According to studies in STZ diabetic rats, administration of aminoguanidine attenuates the accumulation

Table 2. Antioxidative Effects

Groups	Nitrite/nitrate (μM)	Inhibition of NBT reduction (% of diabetic control)
Control (n=5)	15.7±1.9	84.7±2.1
Diabetic control (n=8)	32.6±5.7[c)]	100.0±5.7[c)]
Diabetic+CF50 (n=8)	26.4±7.4[a)]	97.5±2.1[c)]
Diabetic+CF100 (n=8)	19.0±3.4[e)]	93.7±1.0[b)]
Diabetic+CF200 (n=8)	10.5±0.5[e)]	91.5±3.0[d)]
Diabetic+AG100 (n=5)	8.3±0.3[e)]	91.1±5.5[d)]

CF50, 100 or 200: Corni Fructus (50, 100 or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. (n=5 or 8). a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$ *vs.* control rats; *d*) $p<0.01$, *e*) $p<0.001$ *vs.* diabetic control rats.

Table 4. Renal Weight, AGE, and Mitochondrial TBA-Reactive Substance Levels

Groups	Weight (g/100 g B.W.)	AGE (AU)	Mitochondrial TBA-reactive substance (nmol/mg protein)
Control (n=5)	0.72±0.03	3.35±0.48	1.72±0.09
Diabetic control (n=8)	1.05±0.03[a)]	6.19±0.29[a)]	2.66±0.19[a)]
Diabetic+CF50 (n=8)	0.98±0.01[a,d)]	6.05±0.36[a)]	1.79±0.13[d)]
Diabetic+CF100 (n=8)	0.97±0.02[a,d)]	5.32±0.46[a,b)]	1.51±0.08[d)]
Diabetic+CF200 (n=8)	0.98±0.01[a,d)]	4.92±0.27[a,d)]	1.47±0.09[d)]
Diabetic+AG100 (n=5)	0.99±0.03[a,c)]	4.93±0.81[a,c)]	1.45±0.12[d)]

CF50, 100 or 200: Corni Fructus (50, 100 or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. (n=5 or 8). *a*) $p<0.001$ *vs.* control rats; *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$ *vs.* diabetic control rats.

Table 3 Renal Function Parameters

Items	Control (n=5)	Diabetic control (n=8)	Diabetic +CF50 (n=8)	Diabetic +CF100 (n=8)	Diabetic +CF200 (n=8)	Diabetic +AG100 (n=5)
Serum Urea-N (mg/dl)	18.5±0.7	33.6±2.1[c)]	27.8±1.5[c,f)]	23.1±1.6[c,f)]	20.9±1.1[f)]	24.7±3.1[c,f)]
Serum Cr (mg/dl)	0.341±0.010	0.404±0.021[c)]	0.348±0.012[f)]	0.351±0.015[f)]	0.338±0.017[f)]	0.364±0.007[e)]
Ccr (ml/kg B.W./min)	7.68±0.14	5.27±0.59[c)]	6.82±0.63[e)]	7.47±0.48[f)]	8.92±0.22[a,f)]	5.73±1.28[b)]
Urine protein (mg/d)	13.3±0.9	16.8±2.2	12.5±3.1[d)]	12.1±2.7[d)]	10.3±0.9[f)]	9.7±1.1[f)]

CF50, 100 or 200: Corni Fructus (50, 100 or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. (n=5 or 8). *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$ *vs.* control rats; *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$ *vs.* diabetic control rats.

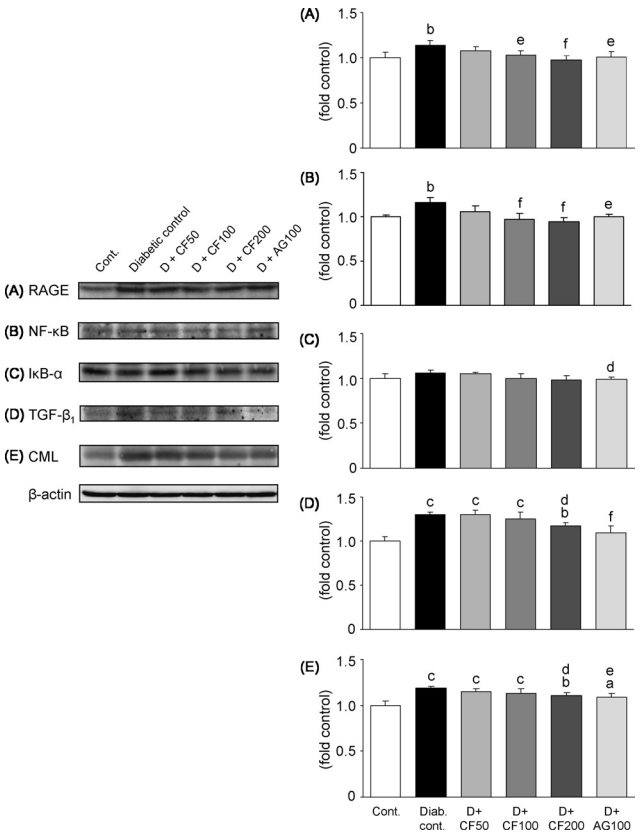

Fig. 2. Effect of Corni Fructus and Aminoguanidine on RAGE (A), NF-κB (B), IκB-α (C), TGF-β_1 (D), and CML (E) Protein Expression in Renal Cortex

CF50, 100, or 200: Corni Fructus (50, 100, or 200 mg/kg, *p.o.*); AG100, aminoguanidine (100 mg/kg, *p.o.*). Results are shown as the means±S.E. ($n=5$ or 8). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ *vs.* control rats; [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ *vs.* diabetic control rats.

of renal AGEs, as well as retarding the development of albuminuria and mesangial expansion,[24] and thus, we carried out this experiment with aminoguanidine to clarify whether Corni Fructus had an anti-diabetic effect through inhibiting AGE formation.

Our results indicated that diabetic rats induced by STZ did not show body weight changes during the 10-d 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 treatment with Corni Fructus increased the body weight from the initial value as well as that of aminoguanidine. On the contrary, the serum glucose level observed in diabetic control rats was increased beyond the initial level; however, Corni Fructus- and aminoguanidine-treated rats showed significant reductions in these levels compared with diabetic control rats, and these levels were all lower than the initial levels. Therefore, we supposed that Corni Fructus could show an anti-hyperglycemic effect, having a correlation with body weight gain.

In the present study, typical characteristics of diabetes were shown. First is the increase of serum glycosylated protein, which is a parameter caused by glucose and other reducing sugars such as ribose and fructose reacting with the amino residues of proteins to form Amadori products, for instance, glycosylated hemoglobin (HbA_{1c}), and the O_2^- is also generated in the process of AGE formation. The next is abnormal lipid metabolism, which can lead to lipid peroxidation with reactive oxygen species (ROS) and renal lipid accumulation, playing a role in the pathogenesis of diabetic nephropathy. In addition, diabetes also shows renal dysfunction, *i.e.*, increased serum Cr, urea-N and proteinuria and decreased Ccr levels, reflecting a decline in the glomerular filtration rate. However, the rats given Corni Fructus showed improved glycation and total cholesterol levels, and a strong renal function, with certain antioxidative activities, while there was only a tendency to reduce the TBA-reactive substance level. On the other hand, aminoguanidine had almost the same effects as those of Corni Fructus except concerning 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 of Corni Fructus and aminoguanidine treated groups. Aminoguanidine had been reported to inhibit nitric oxide production and to trap reactive di-carbonyls, impeding conversion to AGEs, prevent cross-linking, inhibit free radical formation, and lower total cholesterol and lipid peroxidation independent of glycemic control.[25—27] Hence, there are still uncertain mechanisms of these medicinal efficacies, but we may hypothesize that differences between Corni Fructus and aminoguanidine are that Corni Fructus can improve AGE clearance with activated renal function, while aminoguanidine mainly inhibits AGE formation *via* its antioxidant properties.

Several lines of studies have provided substantial evidence that multiple factors caused by hyperglycemia contribute to the development of diabetic kidney disease. Among them, the impacts of AGEs have been recognized over a wide range, resulting in the expression and activation of pathogenic mediators implicated in the development of diabetic nephropathy, such as extracellular matrix, oxidative stress, cytokines, and growth factors, *via* receptor-dependent and/or independent pathways. Therefore, we first demonstrated renal AGE accumulation and the mitochondrial lipid peroxidation level. As a result, diabetic control rats showed increased kidney weight and AGE accumulation significantly, indicating renal hypertrophy, and also showed an increased level of TBA-reactive substance. On the other hand, oral administration of Corni Fructus ameliorated these changes. Particularly, Corni Fructus successfully reduced the AGE and TBA-reactive substance levels at the dose of 100 mg, and the effect on the latter was strong compared with the control level, suggesting that Corni Fructus suppressed the state of oxidative stress. In contrast, the results of aminoguanidine resembled those of Corni Fructus 200 mg treatment.

To clarify the effect of Corni Fructus concerning AGE action with the receptor-dependent pathway, we performed Western blot analyses against AGE-related protein expressions in the renal cortex. It has been reported that AGEs trigger the activation of NF-κB by interaction with RAGE, leading to its translocation to the nucleus where it induces transcription,[28] and the promoter region of the RAGE gene contains NF-κB binding sites,[29] potentially producing a self-perpetuating pathway. Moreover, the AGE-RAGE interaction activates TGF-β-Smad signaling pathways and subsequently induces mesangial cell hypertrophy by the downstream mediator of p27 and glomerular sclerosis by fibronectin synthesis

through autocrine angiotensin II generation *via* ROS overproduction.[30] In this study, diabetic control rats showed slight but significant increases in RAGE, NF-κB, and TGF-β_1 but no change in IκB-α protein expressions which are not only degradated but also regulated by activated NF-κB and sequestered NF-κB in the cytoplasm to inactivate the NF-κB.[31] While the 10-d Corni Fructus administration dose-dependently normalized RAGE and NF-κB proteins, and these effects in the 200 mg-treated group were superior to aminoguanidine treatment, Corni Fructus decreased the expression of TGF-β_1 only at 200 mg but it was inferior to aminoguanidine.

When we consider AGEs from another viewpoint, over a dozen have been identified in tissues including CML, pentosidine, and pyraline, and the known AGEs are immunologically distinct and coexist on different carrier proteins with albumin, hemoglobin, collagens, lens crystalline, and low density lipoprotein cholesterol.[32] Particularly, CML is not only referred to as a glycoxidation product similar to pentosidine, but is also formed during the metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein.[33] Therefore, CML could serve as a general bio-marker of oxidative stress resulting from carbohydrate and lipid oxidation reactions; however, CML is characterized physicochemically by neither cross-linking nor fluorescence. Therefore, in this study, we measured renal AGEs using their characteristics of fluorescence, but also measured the level of CML by Western blot analysis in order to estimate another type of AGE. As a result, accumulation of CML in the renal cortex also increased in STZ-induced diabetic rats as well as the above-mentioned protein expressions, and the effects of Corni Fructus and aminoguanidine were similar to those of TGF-β_1. Taking these results into consideration, though these correlations were not clearly described due to the relatively short period of the examination, we could speculate that Corni Fructus influenced not only the AGE-RAGE pathway but also the TGF-β_1 signaling pathway and CML protein to some extent.

As discussed above, it was discovered that Corni Fructus showed beneficial influences on the hyperglycemic state and incidental renal damage and had a renoprotective effect, suggesting that Corni Fructus may ameliorate the course of diabetic renal complications. However, the mechanisms responsible for the effects of Corni Fructus on glucose-induced tissue damage still need to be discussed. Some researchers have emphasized the importance of hepatic function as a site of AGE metabolism.[34—36] In fact, tissue distribution was examined by orally absorbed AGEs and the increasing rate of AGEs was similar among kidney, liver, and other tissues, but the liver deposition was much higher,[37] and Schupp *et al.*[38] have also reported that the extent of AGE-induced DNA damage observed in different cell types is highest in liver cells, followed by kidney cells. According to our previous study, the oral administration of Hachimi-jio-gan for 10 d to STZ-induced diabetic rats could ameliorate the mitochondrial lipid peroxidation level in the liver as well as the kidney.[39] In line with this, to elucidate the functional role of Corni Fructus against hepatic AGEs in diabetes may provide novel evidence for an antidiabetic property.

In conclusion, this study was performed for the clarification of whether Corni Fructus could be an active component in Hachimi-jio-gan which showed an antidiabetic effect *via* reducing hyperglycemia and its related renal damage, as reported previously.[2,4] Consequently, Corni Fructus ameliorated glucose-associated metabolic disorders as well as aminoguanidine and a decline in renal function, these effects being similar to Hachimi-jio-gan. Thus, this study provides supporting evidence for the therapeutic potential of Hachimi-jio-gan, and Corni Fructus may also be helpful to prevent and/or delay the onset of diabetes-induced renal injury; based upon this, the investigation of major active constituents of Corni Fructus is in progress.

REFERENCES

1) Nakagawa T., Yokozawa T., Terasawa K., Nakanishi K., *J. Pharm. Pharmacol.*, **55**, 219—227 (2003).
2) Yokozawa T., Yamabe N., Cho E. J., Nakagawa T., Oowada S., *Nephron Exp. Nephrol.*, **97**, e38—e48 (2004).
3) Nakagawa T., Yokozawa T., Yamabe N., Rhyu D. Y., Goto H., Shimada Y., Shibahara N., *J. Pharm. Pharmacol.*, **57**, 1205—1212 (2005).
4) Yamabe N., Yokozawa T., *J. Pharm. Pharmacol.*, **58**, 535—545 (2006).
5) Yokozawa T., Kim H. Y., Yamabe N., *Am. J. Chin. Med.*, **32**, 829—839 (2004).
6) Hatano T., Ogawa N., Kira R., Tasuhara T., Okuda T., *Chem. Pharm. Bull.*, **37**, 2083—2090 (1989).
7) Wang S. F., Chen X. G., Hu Z. D., Ju Y., *Biomed. Chromatogr.*, **17**, 306—311 (2003).
8) Mau J. L., Chen C. P., Hsieh P. C., *J. Agric. Food Chem.*, **49**, 183—188 (2001).
9) Chang J. S., Chiang L. C., Hsu F. F., Lin C. C., *Am. J. Chin. Med.*, **32**, 717—725 (2004).
10) Liou S. S., Liu I. M., Hsu S. F., Cheng J. T., *J. Pharm. Pharmacol.*, **56**, 1443—1447 (2004).
11) Vareed S. K., Reddy M. K., Schutzki R. E., Nair M. G., *Life Sci.*, **78**, 777—784 (2006).
12) Xu H. Q., Hao H. P., *Biol. Pharm. Bull.*, **27**, 1014—1018 (2004).
13) McFarland K. F., Catalano E. W., Day J. F., Thorpe S. R., Baynes J. W., *Diabetes*, **28**, 1011—1014 (1979).
14) Naito C., Yamanaka T., *Jpn. J. Geriat.*, **15**, 187—191 (1978).
15) Sakagishi Y., "Rinsho Kagaku Bunseki II," eds. by Saito M., Kitamura M., Niwa M., Tokyo Kagaku Dojin, Tokyo, 1968, pp. 115—142.
16) Mabley J. G., Pacher P., Southan G. J., Salzman A. L., Szabó C., *J. Pharmacol. Exp. Ther.*, **300**, 876—881 (2002).
17) Ewing J. F., Janero D. R., *Anal. Biochem.*, **232**, 243—248 (1995).
18) Nakayama H., Mitsuhashi T., Kuwajima S., Aoki S., Kuroda Y., Itoh T., Nakagawa S., *Diabetes*, **42**, 345—350 (1993).
19) Johnson D., Lardy H., *Methods Enzymol.*, **10**, 94—96 (1967).
20) Jung K., Pergande M., *FEBS Lett.*, **183**, 167—169 (1985).
21) Uchiyama M., Mihara M., *Anal. Biochem.*, **86**, 271—278 (1978).
22) Itzhaki R. F., Gill D. M., *Anal. Biochem.*, **9**, 401—410 (1964).
23) The Diabetes Control and Complications Trial Research Group, *N. Engl. J. Med.*, **329**, 977—986 (1993).
24) Soulis-Liparota T., Cooper M., Papazoglou D., Clarke B., Jerums G., *Diabetes*, **40**, 1328—1334 (1991).
25) Parlakpinar H., Ozer M. K., Cicek E., Cigremis Y., Vardi N., Acet A., *Int. J. Urol.*, **13**, 1327—1332 (2006).
26) Bucala R., *Exp. Physiol.*, **82**, 327—337 (1997).
27) Giardino I., Fard A. K., Hatchell D. L., Brownlee M., *Diabetes*, **47**, 1114—1120 (1998).
28) Yan S. D., Schmidt A. M., Anderson M., Zhang J., Brett J., Zou Y. S., Pinsky D., Stern D., *J. Biol. Chem.*, **269**, 9889—9897 (1994).
29) Li J., Schmidt A. M., *J. Biol. Chem.*, **272**, 16498—16506 (1997).
30) 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., Okuda S., *Kidney Int.*, **66**, 2137—2147 (2004).
31) Kumar A., Takada Y., Boriek A. M., Aggarwal B. B., *J. Mol. Med.*, **82**, 434—448 (2004).
32) Chappey O., Dosquet C., Wautier M. P., Wautier J. L., *Eur. J. Clin. Invest.*, **27**, 97—108 (1997).
33) Fu M. X., Requena J. R., Jenkins A. J., Lyons T. J., Baynes J. W., Thorpe S. R., *J. Biol. Chem.*, **271**, 9982—9986 (1996).

34) Takata K., Horiuchi S., Araki N., Shiga M., Saitoh M., Morino Y., *J. Biol. Chem.*, **263**, 14819—14825 (1988).
35) Yang Z., Makita Z., Horii Y., Brunelle S., Cerami A., Sehajpal P., Suthanthiran M., Vlassara H., *J. Exp. Med.*, **174**, 515—524 (1991).
36) Smedsrød B., Melkko J., Araki N., Sano H., Horiuchi S., *Biochem. J.*, **322**, 567—573 (1997).
37) He C., Sabol J., Mitsuhashi T., Vlassara H., *Diabetes*, **48**, 1308—1315 (1999).
38) Schupp N., Schinzel R., Heidland A., Stopper H., *Ann. N.Y. Acad. Sci.*, **1043**, 685—695 (2005).
39) Kim H. Y., Yokozawa T., Cho E. J., Yamabe N., *J. Pharm. Pharmacol.*, **56**, 1299—1305 (2004).