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Therapeutic potential of Keishi-bukuryo-gan on diabetic nephropathy

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Keishi-bukuryo-gan is a Chinese traditional medicine, which is used clinically as a vascular system disorder-eliminating drug. This review summarizes the effects of Keishi-bukuryo-gan on the occurrence and progression of diabetic nephropathy. To prove its usefulness, we employed two kinds of experimental model animals; diabetic nephropathy rats induced by subtotal nephrectomy plus streptozotocin injection and spontaneously diabetic WBN/Kob rats. In both animal models, Keishi-bukuryo-gan treatment suppressed increase of urinary protein excretion, which is a prognostically important clinical manifestation for renal functional deterioration in diabetic patients, and preserved renal morphological changes peculiar to diabetic glomerulosclerosis. On the other hand, disorders of the metabolic pathway mediated by persistent hyperglycemia (the glycation reaction, excessive polyol pathway and oxidative stress) were observed. However, oral administration of Keishi-bukuryo-gan normalized the accumulation of advanced glycation endproducts (AGEs), determined by measuring fluorescence, and lipid peroxidation levels in the kidney. Furthermore, by a comparison with aminoguanidine (an AGEs inhibitor), butylated hydroxytoluene (an antioxidant) and captopril (an angiotensin-converting enzyme inhibitor), we confirmed that Keishi-bukuryo-gan prevents diabetic kidney damage from developing and has inhibitory effects against AGEs accumulation and oxidative stress. From these results, we proposed the potential usefulness of Keishi-bukuryo-gan in the treatment of diabetic nephropathy.

Key words Keishi-bukuryo-gan, diabetic nephropathy, sorbitol, advanced glycation endproducts, oxidative stress.

1. Introduction

Diabetic nephropathy, a kidney disease associated with diabetes mellitus, is one of the most life-threatening complications. The number of patients started on dialysis therapy due to diabetic nephropathy is increasing every year. According to a report by the Japanese Society for Dialysis Therapy, diabetic nephropathy became the main reason for starting dialysis therapy in 1998, when 10,729 out of a total of 30,051 new dialysis patients (35.7%) required dialysis for this reason. Furthermore, this tendency continues. Many patients diagnosed as having diabetic nephropathy advance to end-stage renal failure within a few years, and their prognosis is poor even after the introduction of dialysis therapy. Therefore, it is a matter of great urgency to prevent the occurrence and progression of diabetic nephropathy, and so effective therapeutic interventions have been actively searched. Clinical trials have been shown that controlled hypertension, which is a concomitant risk factor, slowed the decline in renal function, as well as controlled hyperglycemia.[1-4] Among antihypertensive therapies, angiotensin-converting enzyme (ACE) inhibitors were highly estimated in retarding the progression of diabetic nephropathy and reduced albuminuria, as shown by experimental and human diabetic nephropathy.[1-3] However, it is extremely difficult to prevent the occurrence and progression of diabetic nephropathy, even when hypertension and hyperglycemia are well controlled. Therefore, it is desirable to prove the pathogenic mechanisms of diabetic nephropathy, as well as to develop drugs responsible for specific pathogenic factors.

From recent clinical and experimental studies, a number of pathogenic mechanisms have been implicated in the development of diabetic nephropathy, including the acceleration of polyol pathway, glycation reaction and oxidative stress,[5-7] and these abnormal biochemical processes are thought to be closely associated with the progression of proteinuria and renal dysfunction. Therefore, the improvement of these abnormal biochemical processes may prove to be important therapeutic avenues, and agents such as advanced glycation endproducts (AGEs) inhibitors, antioxidant and aldose reductase inhibitors receives much attention.

On the other hand, traditional herbal medicines have been employed for thousands of years and have greatly contributed to the prevention and treatment of various diseases, including diabetes mellitus and renal disease. They are still valuable for human health and have received much attention as potential sources of new therapeutic agents because they are composed of several crude drugs with low toxicity. Our research group has been investigating the effects of traditional herbal medicines, crude drugs and their components on renal diseases in various kinds of animal models such as acute and chronic renal failure, and diabetic nephropathy, indicating that they have potential for the treatment of kidney disease.[8-19] In this review based on our recent studies, we describe possible therapeutic approaches to diabetic nephropathy using Chinese prescription Keishi-bukuryo-gan.

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2. Potential activities of Keishi-bukuryo-gan on diabetic nephropathy

To examine the possibility of traditional herbal medicines in the treatment of diabetic nephropathy, we began our studies with a in vitro screening test to examine the inhibitory effect on AGEs formation among 12 traditional medicines. We found that a rhubarb-based preparation and a vascular system disorder-eliminating drug had strong activity compared to positive control aminoguanidine, while a rehmannia root preparation had moderate activity and a bupleurum root preparation had weak inhibitory activity.[20] Neverthless, the pathogenesis of diabetic nephropathy involves many factors stemming from persistent hyperglycemia. While the production of AGEs is an important pathogenic mechanism, other biochemical processes such as excessive activity of the polyol pathway or oxidative stress may also be closely involved. With this in mind, we selected Ompi-to (a rhubarb preparation), Hachimi-jio-gan (a rehmannia root preparation), Keishi-bukuryo-gan (a vascular system disorder-eliminating drug) and Sairei-to (a bupleurum root preparation) on the basis of screening test results, and we examined their possible therapeutic applications in rats with diabetic nephropathy.[16] We found that Keishi-bukuryo-gan had beneficial influence on the formation of AGEs, overenhancement of polyol pathway and oxidative stress, suggesting that this prescription would have therapeutic benefits. Furthermore, to prove its usefulness, we designed a long-term experiment focused on renal function parameters and histological examinations, using two kinds of animal models of diabetic nephropathy.

2.1. Effects in an animal model of subtotal nephrectomy plus an injection of streptozotocin (STZ). The animal model of diabetic nephropathy, subtotal nephrectomy plus an injection of STZ, shows functional and morphological changes of the kidney resembling those seen in patients with diabetic nephropathy, as reported by Yokozawa *et al.*[21] Therefore, we employed this animal model to examine whether Keishi-bukuryo-gan can slow the progression of diabetic nephropathy. To determine the progression of diabetic nephropathy, blood glucose levels and the extent of proteinuria were monitored every three weeks. Throughout the 15-week experimental period, the blood glucose levels of diabetic nephropathy rats were over 430 mg/dl, as shown in Fig. 1. Among the diabetic nephropathy rats, the blood glucose levels of the Keishi-bukuryo-gan treated groups at a dose of 100 and 200 mg were significantly lower than those of the untreated control group from 6 weeks. Compared to normal rats, the urinary excretion of protein increased dramatically in untreated diabetic nephropathy rats throughout the experimental period, but Keishi-bukuryo-gan significantly suppressed this (Fig. 2). Furthermore, the serum urea nitrogen and creatinine levels (important therapeutic indices) of the diabetic nephropathy groups increased, but at the end of experiment they were lower in the Keishi-bukuryo-gan-treated groups than in the untreated control group (Table 1). The ameliorating effects of Keishi-bukuryo-gan on the blood glucose and renal function parameters corresponded with the severity of the morphological changes in the kidney, such as diffuse mesangial expansion, basement membrane thickening, nodular lesions, arteriolar hyalinosis and fibrin cap and capsular drop lesions (Table 2 and Fig. 3). These results show that Keishi-bukuryo-gan has the potential to retard the progression of diabetic nephropathy.

The pathogenesis of diabetic nephropathy involves many factors stemming from persistent hyperglycemia. It is well accepted that overenhancement of polyol pathway, glycation reaction and oxidative stress induced by persistent hyperglycemia is closely associated with the progression of proteinuria and renal dysfunction. Therefore, attenuation of these abnormal biochemical processes may prove to be important therapeutic avenues. In our previous study, treatment with Keishi-bukuryo-gan improved these metabolic abnormalities in rats. In this study, to examine the mechanisms of action of this traditional herbal medicine against these metabolic abnormalities and their contributions to attenuation of renal damage, we set up three groups which

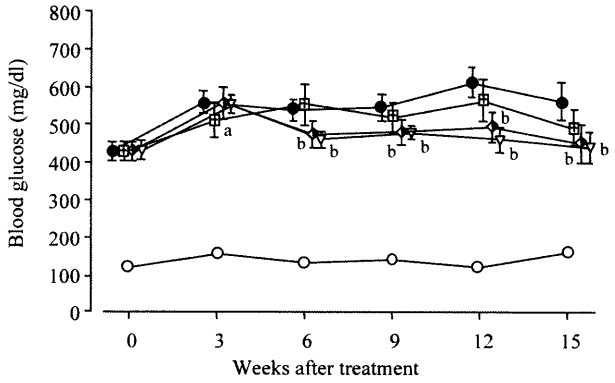

Fig. 1 Blood glucose levels in normal rats (○) and in diabetic nephropathy rats treated with either Keishi-bukuryo-gan (50 mg/kg B.W./day, ⊞; 100 mg/kg B.W./day, ◈ ; 200 mg/kg B.W./day, ▽) or control (water, ●) for 15 weeks. Statistical significance: [a]$p<0.05$, [b]$p<0.001$ vs. control rats with diabetic nephropathy.

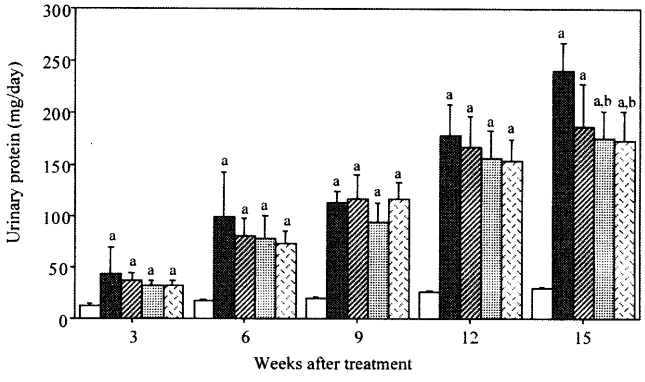

Fig. 2 Urinary protein excretion in normal rats (□) and in diabetic nephropathy rats treated with either Keishi-bukuryo-gan (50 mg/kg B.W./day, ▨ ; 100 mg/kg B.W./day, ▩ ; 200 mg/kg B.W./day, ▧) or control (water, ■) for 15 weeks. Statistical significance: [a]$p<0.001$ vs. normal rats, [b]$p<0.05$ vs. control rats with diabetic nephropathy.

Table 1 Serum biochemical features and Ccr.

Group	Dose (mg/kg B.W./day)	Glycosylated protein (nmol/mg protein)	Urea nitrogen (mg/dl)	Cr (mg/dl)	T. Protein (g/dl)	Albumin (g/dl)	Ccr (ml/min/ kg B.W.)
Normal rats	-	11.8 ± 0.3	20.5 ± 0.4	0.456 ± 0.017	5.04 ± 0.09	3.41 ± 0.08	5.215 ± 0.381
Diabetic nephropathy rats							
Control	-	21.2 ± 1.5[a]	66.8 ± 6.1[a]	0.843 ± 0.083[a]	4.07 ± 0.13[a]	2.54 ± 0.10[a]	3.190 ± 0.345[a]
Keishi-bukuryo-gan	50	19.3 ± 1.9[a]	58.3 ± 6.7[a]	0.851 ± 0.113[a]	4.23 ± 0.18[a]	2.61 ± 0.14[a]	3.145 ± 0.404[a]
Keishi-bukuryo-gan	100	17.9 ± 1.3[a,c]	52.8 ± 5.8[a,c]	0.774 ± 0.099[a]	4.36 ± 0.12[a,c]	2.68 ± 0.12[a]	3.473 ± 0.456[a]
Keishi-bukuryo-gan	200	17.5 ± 1.0[a,c]	51.7 ± 4.4[a,c]	0.726 ± 0.054[a]	4.35 ± 0.08[a,b]	2.74 ± 0.07[a,b]	3.570 ± 0.299[a]

Statistical significance: [a]$p<0.001$ vs. normal rats, [b]$p<0.05$, [c]$p<0.01$ vs. control rats with diabetic nephropathy.

Table 2 Histopathological evaluation of the kidney.

Group	Dose (mg/kg B.W./day)	Lesion score					
		Diffuse lesion	Nodular lesion	Fibrin cap	Capsular drop	Arteriolar hyalinosis	Total
Normal rats	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Diabetic nephropathy rats							
Control	-	2.38 ± 0.26	2.25 ± 0.37	2.88 ± 0.30	2.25 ± 0.16	1.25 ± 0.37	11.00 ± 1.30
Keishi-bukuryo-gan	50	2.17 ± 0.17	1.83 ± 0.31	2.83 ± 0.17	2.33 ± 0.21	1.00 ± 0.45	10.17 ± 1.01
Keishi-bukuryo-gan	100	1.83 ± 0.17[c]	1.67 ± 0.21[a]	2.50 ± 0.22	2.00 ± 0.00	0.67 ± 0.42	8.67 ± 0.67[b]
Keishi-bukuryo-gan	200	1.78 ± 0.15[c]	0.86 ± 0.26[c]	2.44 ± 0.20[a]	1.14 ± 0.26[c]	0.43 ± 0.30[a]	7.00 ± 0.62[c]

Lesion score was expressed as follows: absent, 0; slight, 1; mild, 2; moderate, 3; severe, 4. Statistical significance: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. control rats with diabetic nephropathy.

Table 3 AGEs, MDA and sorbitol levels in kidney

Group	Dose (mg/kg B.W./day)	AGEs (AU)	MDA (nmol/mg protein)	Sorbitol (nmol/mg protein)
Normal rats	-	0.556 ± 0.012	0.299 ± 0.018	0.575 ± 0.043
Diabetic nephropathy rats				
Control	-	0.841 ± 0.055[b]	0.394 ± 0.020[b]	1.915 ± 0.200[b]
Keishi-bukuryo-gan	50	0.776 ± 0.024[b,c]	0.391 ± 0.026[b]	1.691 ± 0.261[b]
Keishi-bukuryo-gan	100	0.767 ± 0.041[b,c]	0.375 ± 0.013[b]	1.521 ± 0.199[b,c]
Keishi-bukuryo-gan	200	0.654 ± 0.021[a,d]	0.360 ± 0.020[a]	1.648 ± 0.190[b]

Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. normal rats, [c]$p<0.05$, [d]$p<0.001$ vs. control rats with diabetic nephropathy.

were given different doses (50, 100 and 200 mg/kg body weight/day).

The protein glycation reaction can be broadly into the early phase (in which Amadori rearrangement products are produced) and the late phase (in which these products are converted to AGEs by various processes, including dehydration, cyclization and oxidation).[22,23] This reaction is accelerated in diabetes and causes excessive formation and accumulation of AGEs, resulting in glomerular basement membrane thickening and progressive albuminuria.[24] In this study, the serum glycosylated protein levels in the groups treated with 100 and 200 mg/kg body weight Keishi-bukuryo-gan were significantly lower than the control group levels (Table 1), and this difference was well reflected by serum glucose levels. While renal AGEs levels, determined by measuring fluorescence, were reduced significantly by even the lowest dose (50 mg/kg body weight/day) of Keishi-bukuryo-gan, treatment with 200 mg/kg body weight reduced it more than 100 mg/kg body weight (Table 3), although both the 100 and 200 mg/kg body weight Keishi-bukuryo-gan-treated groups showed similar glycemic conditions. Therefore, we speculated that Keishi-bukuryo-gan inhibited the late-phase reaction in which Amadori rearrangement products are converted to AGEs.

Recent studies have indicated that high glucose levels cause oxidative stress.[25] Glucose itself and the glycosylated proteins known as Amadori rearrangement products are susceptible to autoxidation and may be a source of reactive oxygen species (ROS).[26,27] Furthermore, enhanced oxidative stress due to diabetes may also result from a dysfunction in the defense system against ROS, such as the reduction of glutathione or inactivation of superoxide dismutase (SOD).[5,28] In this study, lipid peroxidation levels in the serum and kidney were 2.6 and 1.3 times higher in rats with diabetic nephropathy than in normal rats (Fig. 4 and Table 3), reflecting enhancement of oxidative stress. Oral administration of Keishi-bukuryo-gan for 15 weeks reduced these levels dose dependently.

The enhancement of the polyol pathway leads to the accumulation of sorbitol and fructose in the tissues, leading in turn to the alteration of the cytosolic ratio of NADPH: $NADH^{+}$[29] and the depletion myoinositol and changes in the

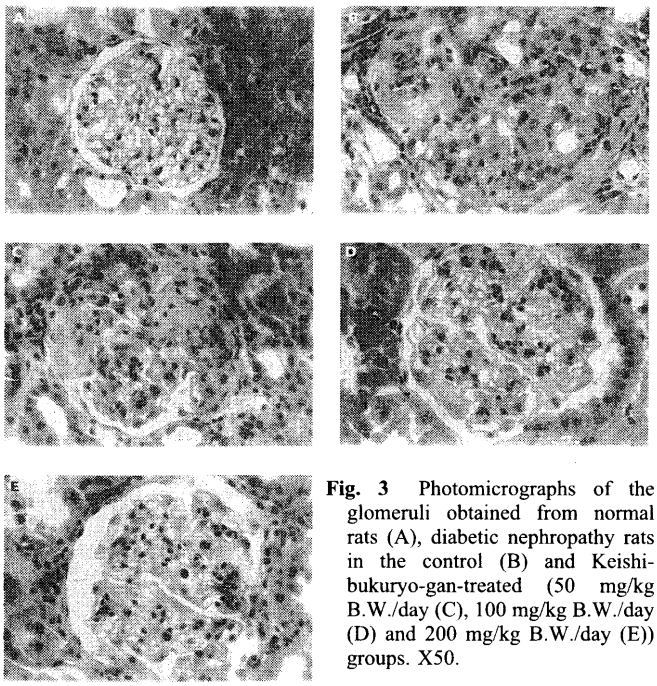

Fig. 3 Photomicrographs of the glomeruli obtained from normal rats (A), diabetic nephropathy rats in the control (B) and Keishi-bukuryo-gan-treated (50 mg/kg B.W./day (C), 100 mg/kg B.W./day (D) and 200 mg/kg B.W./day (E)) groups. X50.

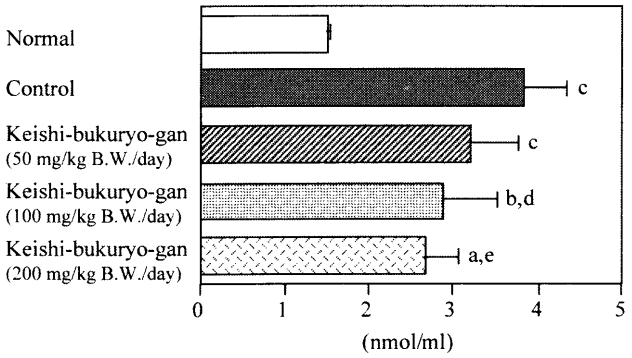

Fig. 4 Serum MDA levels. Statistical significance: $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. normal rats, $^{d}p<0.05$, $^{e}p<0.01$ vs. control rats with diabetic nephropathy.

cellular redox potential. Aldose reductase is a key enzyme for sorbitol synthesis and inhibition of this step by aldose reductase inhibitors has been reported to improve some diabetic complications in animal experiments and clinical trials.[30,31] As shown in Table 3, the renal sorbitol level was higher in diabetic nephropathy rats than in normal rats. Treatment with 100 mg Keishi-bukuryo-gan significantly lowered it, but the 200 mg dose did not reduce it further. Thus, it is likely that reduction of sorbitol accumulation is not the main effect of Keishi-bukuryo-gan.

From these results in which Keishi-bukuryo-gan effectively reversed many characteristic pathological manifestations in rats with diabetic nephropathy, we expect that Keishi-bukuryo-gan would be useful in the treatment of diabetic nephropathy.

2.2. Effects in spontaneously diabetic WBN/Kob rats. Proteinuria is a prognostically important clinical manifestation for the occurrence and progression of nephropathy in diabetic patients. Mori *et al.*[32] investigated the development of renal lesions in male WBN/Kob rats and reported that urinary total protein began to increase at about 13 months of age. In order to evaluate the effects of Keishi-bukuryo-gan on the occurrence of diabetic kidney damage, WBN/Kob rats received Keishi-bukuryo-gan treatment for 30 weeks from the age of 45 weeks (about 10.5 months) to 75 weeks (about 17.5 months). After treatment of Keishi-bukuryo-gan for 15 weeks (14 months of age), urinary protein excretion was about 10 mg/day and there were no significant differences in excretion between the control and Keishi-bukuryo-gan-treated groups (Fig. 5). After 30 weeks of treatment (17.5 months of age), urinary protein content increased to about 20 mg/day in the untreated control group, whereas the content of age matched male Wistar rats was about 10 mg/day. This showed that renal function had deteriorated with long-term diabetes mellitus. However, oral administration of Keishi-bukuryo-gan to diabetic WBN/Kob rats for 15 to 30 weeks inhibited the increase of proteinuria. The blood glucose level increased as time progressed, as shown in Fig. 6. Keishi-bukuryo-gan treatment did not show a blood glucose lowering effect. At the end of the experiment (75 weeks of age), glomerular sclerosis including the widening of mesangial areas and capillary wall thickness

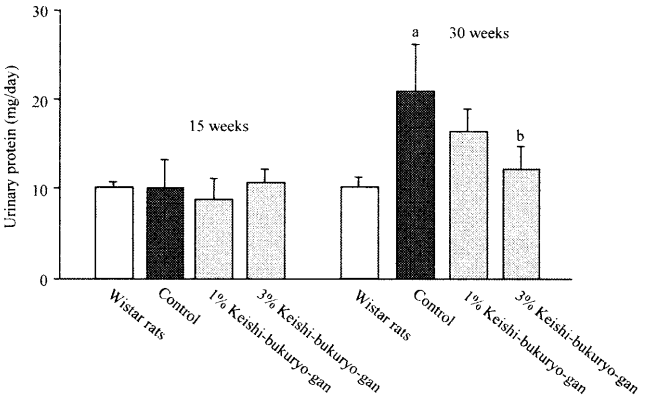

Fig. 5 Urinary protein excretion. Statistical significance: $^{a}p<0.01$ vs. Wistar rats; $^{b}p<0.01$ vs. WBN/Kob control rats.

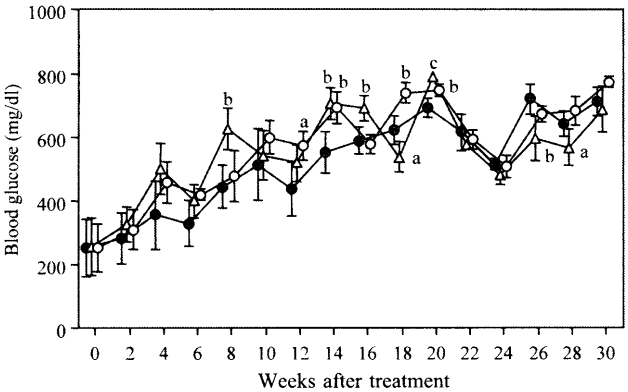

Fig. 6 Blood glucose levels (control, ● ; 1% Keishi-bukuryo-gan, △ ; 3% Keishi-bukuryo-gan, ○). Statistical significance: $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ vs. WBN/Kob control rats.

Table 4 Histopathological evaluation of the kidney.

Group	Lesion score			
	Glomerular sclerosis	Vascular changes	Tubulointerstitial damage	Total
Wistar rats	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
WBN/Kob rats				
Control	4.00 ± 0.00	2.50 ± 0.22	2.50 ± 0.22	9.00 ± 0.45
1% Keishi-bukuryo-gan	3.14 ± 0.26[a]	1.00 ± 0.00[b]	1.71 ± 0.18[b]	5.86 ± 0.40[b]
3% Keishi-bukuryo-gan	2.29 ± 0.18[b]	1.00 ± 0.00[b]	1.14 ± 0.14[b]	4.43 ± 0.30[b]

Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. WBN/Kob control rats.

Table 5 MDA and AGEs levels in serum.

Group	MDA (nM)	AGEs (mU/ml)
Wistar rats	2.18 ± 0.17	0.70 ± 0.01
WBN/Kob rats		
Control	2.82 ± 0.10[b]	1.26 ± 0.12[b]
1% Keishi-bukuryo-gan	2.80 ± 0.09[b]	1.26 ± 0.15[b]
3% Keishi-bukuryo-gan	2.54 ± 0.12[a,c]	1.22 ± 0.09[b]

Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. Wistar rats; [c]$p<0.01$ vs. WBN/Kob control rats.

Table 6 MDA and AGEs levels in kidney.

Group	MDA (nmol/mg protein)	AGEs (AU)
Wistar rats	0.571 ± 0.035	0.822 ± 0.025
WBN/Kob rats		
Control	0.713 ± 0.007[b]	1.166 ± 0.040[b]
1% Keishi-bukuryo-gan	0.649 ± 0.055[a,c]	1.041 ± 0.022[b,d]
3% Keishi-bukuryo-gan	0.534 ± 0.034[d]	0.939 ± 0.019[b,d]

Statistical significance: [a]$p<0.05$, [b]$p<0.001$ vs. Wistar rats; [c]$p<0.05$, [d]$p<0.001$ vs. WBN/Kob control rats.

Table 7 SOD activity in kidney.

Group	SOD (U/mg protein)
Wistar rats	40.8 ± 1.3
WBN/Kob rats	
Control	37.2 ± 0.6[b]
1% Keishi-bukuryo-gan	42.1 ± 1.6[c]
3% Keishi-bukuryo-gan	43.7 ± 1.1[a,c]

Statistical significance: [a]$p<0.05$, [b]$p<0.001$ vs. Wistar rats; [c]$p<0.001$ vs. WBN/Kob control rats.

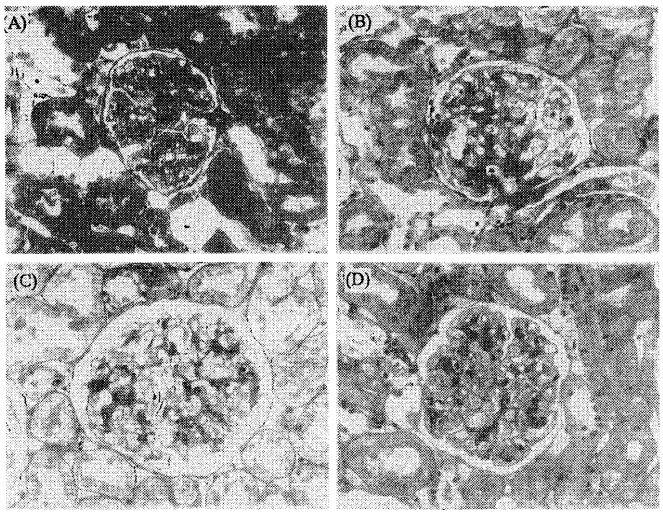

Fig. 7 Photomicrographs of the glomeruli. Wistar rats (A) and WBN/Kob rat (control, (B); 1% Keishi-bukuryo-gan, (C); 3% Keishi-bukuryo-gan, (D)). X200.

were observed in WBN/Kob rats. Keishi-bukuryo-gan treatment inhibited the development of glomerular sclerosis (Table 4 and Fig. 7). Although the kidneys of 75-week-old WBN/Kob rats had fewer vascular lesions and tubulointerstitial damage, Keishi-bukuryo-gan treatment significantly reduced the development of renal lesions (Table 4). These results show that long-term treatment of Keishi-bukuryo-gan could slow the deterioration of renal function in diabetic WBN/Kob rats.

In the section 2.1., we found that Keishi-bukuryo-gan had beneficial effects on oxidative stress and AGEs accumulation in rats receiving subtotal nephrectomy and an injection of STZ. Similar results were also observed in spontaneously diabetic WBN/Kob rats. Table 5 and 6 showed that lipid peroxidation levels in both serum and kidney decreased, following treatment with Keishi-bukuryo-gan. It was considered that alterations of the activities of antioxidant enzymes are involved in the pathological conditions associated with oxidative stress. SOD, a scavenger of O_2^-, plays a key role in the endogeneous defense system against ROS. Loven *et al.*[28] demonstrated that SOD activity decreased in the kidney of diabetic rats, and this phenomenon was confirmed by other authors.[33,34] Our results were in agreement, suggesting that decreased SOD activity is associated with enhanced oxidative stress in diabetes (Table 7). Thus, it is suggested that elevated SOD activity in Keishi-bukuryo-gan-treated groups causes a beneficial change to the diabetic kidney in which oxidative stress is involved.

On the other hand, Kawamura *et al.*[35] revealed an increased content of the glycated form of SOD in erythrocytes of diabetic patients. Arai *et al.*[36] demonstrated that glycation of SOD in lysine residues resulted in a loss of biological activity. Thus, it was considered that the enhanced glycation reaction which results in AGEs accumulation induces oxidative stress due to SOD inactivation. Keishi-bukuryo-gan treatment did not change AGEs levels in the circulation (Table 5), but effectively reduced renal AGEs levels (Table

6). In the diabetic body, many factors including SOD inactivation and AGEs accumulation interdependently contribute to the enhanced oxidative stress, and cause further development of nephropathy. We suggest that Keishi-bukuryo-gan exerts antioxidant effects in the kidney of WBN/Kob rats through increased SOD activity and reduces AGEs accumulation, and that these effects provide renoprotection under persistent hyperglycemic conditions.

3. Evaluation of Keishi-bukuryo-gan by comparison with aminoguanidine, butylated hydroxytoluene (BHT) and captopril

Based on the findings from section 2 using two kinds of diabetic nephropathy rats, we indicated that Keishi-bukuryo-gan has the potential to retard the occurrence and progression of diabetic nephropathy.[17,18] In addition, this traditional herbal medicine attenuated AGEs accumulation and oxidative stress, suggesting that these beneficial actions might lead to renoprotective effects. Based on this, we conducted an animal experiment used subtotal nephrectomy plus STZ injection, to elucidate its renoprotective activity and influence on AGEs accumulation and oxidative stress by comparing its efficacy with those of positive controls; aminoguanidine (an AGEs inhibitor), BHT (an antioxidant) and captopril (an ACE inhibitor).

ACE inhibitors are widely prescribed for patients with diabetes mellitus to retard the progression of renal failure and reduce the degree of proteinuria, and are now thought to be one of the most promising drugs for the treatment of diabetic nephropathy. We compared the efficacy of Keishi-bukuryo-gan with captopril. In this study, we confirmed that Keishi-bukuryo-gan preserved renal function, as assessed in terms of proteinuria and serum creatinine (Fig. 8 and Table 8), and prevented morphological changes peculiar to diabetic nephropathy (Table 9 and Fig. 9). However, captopril showed stronger renoprotective activity, indicating the importance of ACE inhibitors in preventing the progression of diabetic nephropathy. In addition, with respect to renal functional and structural outcomes, Keishi-bukuryo-gan was as equally effective as aminoguanidine, whereas BHT had

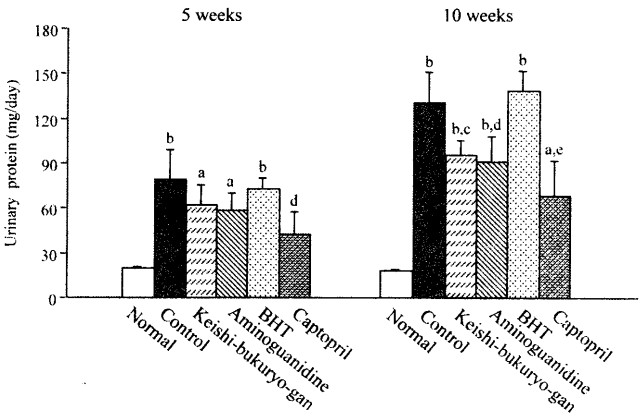

Fig. 8 Urinary protein excretion. Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. normal rats; [c]$p<0.05$, [d]$p<0.01$, [e]$p<0.001$ vs. control rats with diabetic nephropathy.

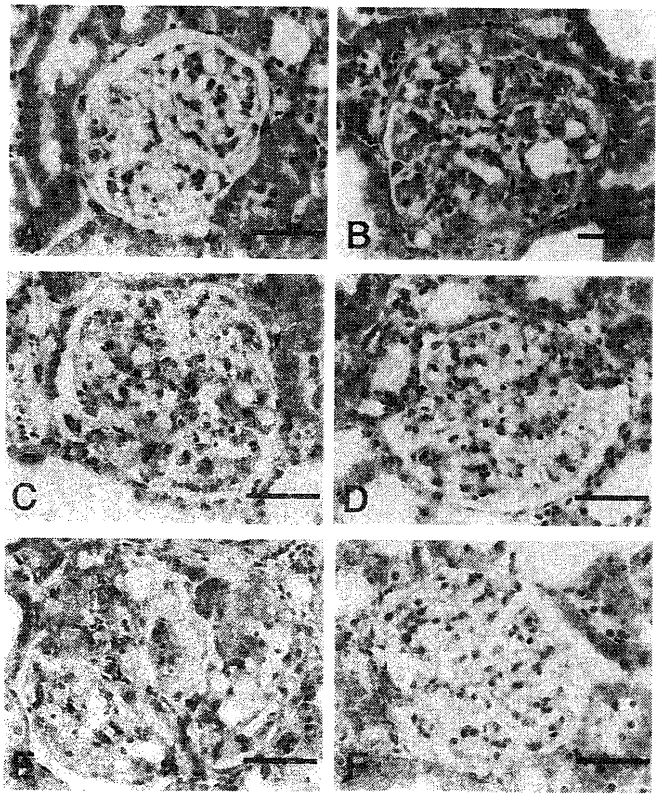

Fig. 9 Photomicrographs of the glomeruli obtained from normal rats (A), diabetic nephropathy rats in the control (B) and Keishi-bukuryo-gan (C), aminoguanidine (D), BHT (E) and captopril (F) treated groups. Scale bars = 50 μm.

Table 8 Serum Cr and Ccr.

Group	Cr (mg/dl)		Ccr (ml/kg B.W./min)	
	5 weeks	10 weeks	5 weeks	10 weeks
Normal rats	0.336 ± 0.022	0.341 ± 0.030	7.15 ± 0.31	6.91 ± 0.74
Diabetic nephropathy rats				
Control	0.593 ± 0.029[a]	0.704 ± 0.087[a]	3.73 ± 0.28[a]	3.42 ± 0.37[a]
Keishi-bukuryo-gan	0.537 ± 0.059[a]	0.572 ± 0.028[a,c]	4.19 ± 0.43[a]	3.95 ± 0.33[a]
Aminoguanidine	0.614 ± 0.035[a]	0.603 ± 0.053[a,b]	3.46 ± 0.18[a]	3.29 ± 0.26[a]
BHT	0.550 ± 0.031[a]	0.613 ± 0.049[a]	3.32 ± 0.22[a]	3.03 ± 0.45[a]
Captopril	0.495 ± 0.011[a,c]	0.529 ± 0.026[a,d]	4.44 ± 0.29[a,c]	4.03 ± 0.47[a]

Statistical significance: [a]$p<0.001$ vs. normal rats; [b]$p<0.05$, [c]$p<0.01$, [d]$p<0.001$ vs. control rats with diabetic nephropathy.

Table 9 Histopathological evaluation of the kidney.

Group	Lesion score			
	Exudative lesion	Diffuse lesion	Nodular lesion	Arteriolar hyalinosis
Normal rats	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Diabetic nephropathy rats				
Control	2.75 ± 0.25	2.75 ± 0.16	1.38 ± 0.42	1.75 ± 0.25
Keishi-bukuryo-gan	2.00 ± 0.37[b]	2.57 ± 0.20	0.86 ± 0.40	1.33 ± 0.42
Aminoguanidine	2.00 ± 0.33[b]	2.63 ± 0.18	0.75 ± 0.37	1.25 ± 0.37
BHT	2.63 ± 0.26	3.00 ± 0.00	1.63 ± 0.38	1.50 ± 0.33
Captopril	2.00 ± 0.38[b]	2.25 ± 0.25[a]	0.38 ± 0.38[b]	0.50 ± 0.33[c]

Lesion score was expressed as follows: absent, 0; slight, 1; mild, 2; moderate, 3; severe, 4. Statistical significance: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. control rats with diabetic nephropathy.

Table 10 Serum glycemic condition.

Group	Glucose (mg/dl)		Glycosylated protein (nmol/mg protein)
	5 weeks	10 weeks	10 weeks
Normal rats	145.1 ± 5.9	165.5 ± 2.9	14.7 ± 0.6
Diabetic nephropathy rats			
Control	564.0 ± 41.8[b]	558.7 ± 59.2[b]	24.1 ± 1.6[b]
Keishi-bukuryo-gan	491.3 ± 48.4[b]	421.8 ± 71.5[b,d]	20.6 ± 1.3[b,d]
Aminoguanidine	497.4 ± 59.7[b]	405.5 ± 58.2[b,d]	19.4 ± 1.7[b,e]
BHT	471.7 ± 39.5[b,c]	365.1 ± 60.9[b,e]	19.1 ± 0.9[a,e]
Captopril	580.8 ± 31.2[b]	474.8 ± 26.4[b]	20.8 ± 1.4[b,d]

Statistical significance: [a]$p<0.01$, [b]$p<0.001$ vs. normal rats; [c]$p<0.05$, [d]$p<0.01$, [e]$p<0.001$ vs. control rats with diabetic nephropathy.

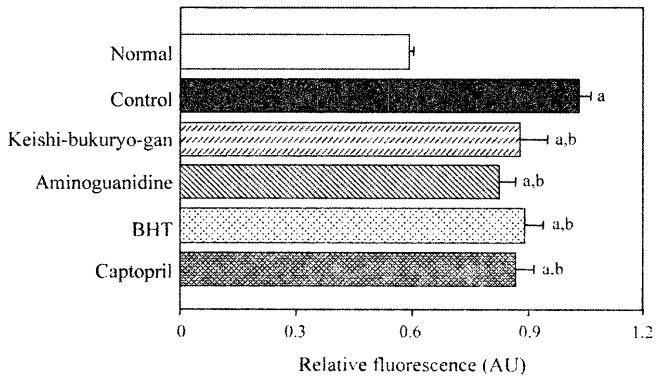

Fig. 10 AGEs levels in kidney. Statistical significance: [a]$p<0.001$ vs. normal rats; [b]$p<0.001$ vs. control rats with diabetic nephropathy.

Table 11 Systolic blood pressure.

Group	Systolic blood pressure (mmHg)	
	4 weeks	8 weeks
Normal rats	118.2 ± 5.9	126.1 ± 7.5
Diabetic nephropathy rats		
Control	167.0 ± 10.1[a]	162.4 ± 11.7[a]
Keishi-bukuryo-gan	158.6 ± 4.9[a]	158.8 ± 9.5[a]
Aminoguanidine	165.1 ± 14.3[a]	167.3 ± 13.7[a]
BHT	168.1 ± 3.8[a]	158.1 ± 5.9[a]
Captopril	151.1 ± 5.2[a,b]	133.2 ± 3.6[c]

Statistical significance: [a]$p<0.001$ vs. normal rats; [b]$p<0.05$, [c]$p<0.001$ vs. control rats with diabetic nephropathy.

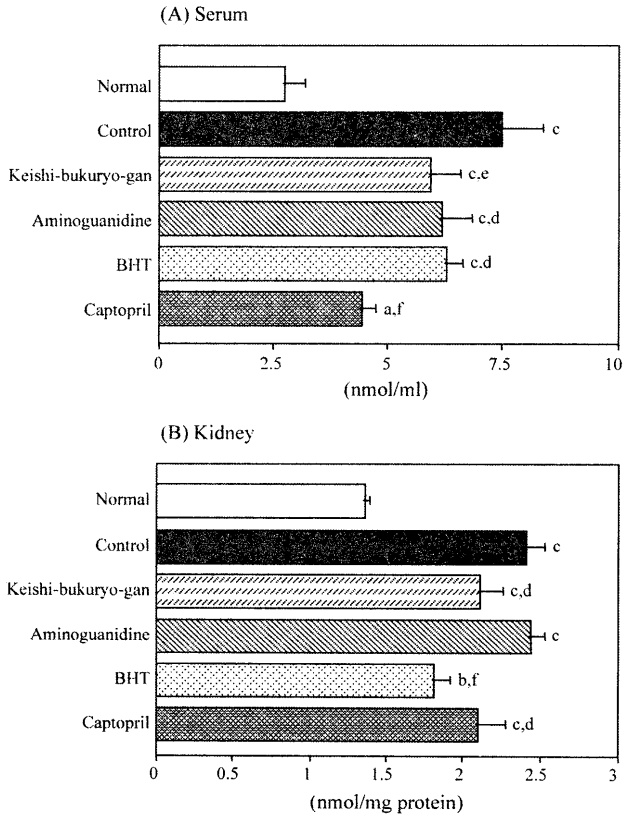

Fig. 11 MDA levels in serum and kidney. Statistical significance: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. normal rats; [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ vs. control rats with diabetic nephropathy.

no effect on these parameters in this animal model.

Several clinical trials have shown that the control of hyperglycemia and blood pressure can slow the progression of diabetic nephropathy.[1,4,37] Administration of BHT significantly lowered serum glucose levels at 5 and 10 weeks (Table 10), although BHT did not show renoprotective activity. On the other hand, captopril, which exerts renoprotective activity, did not affect blood glucose levels and significantly lowered blood pressure (Table 10 and 11). In addition, it was reported that the mechanisms of captopril appear to involve not only a blood pressure-lowering effect but also other influences.[3,38] It is frequently noticed in a clinical setting that the control of hyperglycemia and hypertension cannot completely prevent the deterioration of renal function in patients with diabetic nephropathy, suggesting that the development of diabetic nephropathy is a complex process involving several mechanisms.

Inhibition of AGEs formation by aminoguanidine has been shown to attenuate pathological changes such as increased kidney weight, glomerular basement membrane thickening and progressive albuminuria in STZ-induced diabetes.[39] It is well known that aminoguanidine also functions as an inhibitor of inducible nitric oxide synthase (NOS), which may participate in the regulation of blood pressure and renal hemodynamics such as renal blood flow and glomerular filtration rate in diabetes. However, Forbes *et al.*[40] demonstrated that the prevention of AGEs formation by ALT-946, an AGEs inhibitor without inhibition of NOS, reproduces the protective effects of aminoguanidine. Other NOS inhibitors without inhibitory effects on AGEs formation have failed to confer similar renal protection observed with aminoguanidine, suggesting that the renoprotective effect of aminoguanidine is mediated predominantly by decreased AGEs formation rather than NOS inhibition. In this study, aminoguanidine treatment did not affect systolic blood pressure and Ccr levels (Table 11 and 8), and reduced AGEs-related fluorescence in the kidney by 20 % compared with untreated control rats, together with its renoprotective effects (Fig. 10). In addition, the other three medicines also significantly reduced this parameter, although to a lesser extent (Fig. 10).

BHT, a lipophilic antioxidant, reduced the renal lipid peroxidation levels to the greatest extent (Fig. 11(B)), although it did not have renoprotective activity. Keishi-bukuryo-gan and captopril reduced renal lipid peroxidation to a similar degree (Fig. 11(B)), whereas aminoguanidine, which has renoprotective activity, did not affect this parameter. Captopril reduced the serum lipid peroxidation levels to the greatest extent, and Keishi-bukuryo-gan and aminoguanidine also produced significant reduction comparable in degree to that of BHT (Fig. 11(A)). In this study, administration of BHT had no effect on renal functional and structural changes. Although oxidative stress is widely recognized to be closely associated with the etiology of diabetic nephropathy, further studies on the relationship between attenuation of oxidative stress by antioxidants with hydrophilic or lipophilic characteristics and renoprotective activity are needed.

The fact that the effects of these medicines on renal AGEs and lipid peroxidation levels did not directly relate to their renoprotective activity reflects the multifactorial etiology of diabetic nephropathy. In the diabetic body, many factors stemming from persistent hyperglycemia and hypertension interdependently contribute to the progression of diabetic nephropathy. Therefore, agents such as ACE inhibitors, AGEs inhibitors and antioxidants which can act on distinct processes, will exert different degrees of renoprotective activity by their relative contributions. From our comparison with the AGEs inhibitor and antioxidant, these results provide evidence at least in part that Keishi-bukuryo-gan inhibits renal AGEs accumulation and oxidative stress, thus helping to preserve renal function and structure.

4. Conclusion

Traditional herbal medicines have often been used for diabetic mellitus to cure several symptoms. It was suggested that the therapy combined with such medicines was effective for diabetic microangiopathy, from the results of 141 patients with non-insulin dependent diabetes mellitus who were prescribed them.[41] In addition, case reports indicating that traditional herbal medicines including Keishi-bukuryo-gan improved the quality of life of patients with diabetic nephropathy have been published, suggesting the prolongation of the pre-dialysis stage of diabetic nephropathy.[42] Thus, it is believed that traditional herbal medicines have great potential for the management of diabetic nephropathy. However, it is not clear how the bioactivity of these medicines contribute towards these effects.

Recently, we found that Keishi-bukuryo-gan had a beneficial influence on metabolic abnormalities accompanied with diabetes such as AGEs accumulation and oxidative stress in rats receiving subtotal nephrectomy plus STZ injection and spontaneously diabetic WBN/Kob rats. Furthermore, Keishi-bukuryo-gan prevented proteinuria, serum Cr and morphological changes peculiar to diabetic nephropathy, indicating the potential therapeutic activities of Keishi-bukuryo-gan against the development of diabetic kidney disease. On the basis of these results, we proposed that Keishi-bukuryo-gan exerts antioxidant activity and has an inhibitory effect on AGEs accumulation in diabetic kidneys and that these effects might be the mechanisms responsible for the beneficial action of this traditional herbal medicine. Experimental evidence supporting the therapeutic usefulness of Keishi-bukuryo-gan for diabetic nephropathy is accumulating.

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Japanese abstract

桂枝茯苓丸の糖尿病性腎症に対する作用を，2種類のモデルラット（腎摘＋STZ，自然発症糖尿病 WBN/Kob ラット）を用い検討した。いずれのモデルにおいても桂枝茯苓丸投与により，腎機能（尿蛋白排泄量）と病理所見の改善作用が認められ，桂枝茯苓丸が糖尿病性腎症の発症・進展を遅延することが実験的に明らかとなった。一方，慢性的な高血糖状態では酸化ストレス，ポリオール経路，糖化反応の亢進をひき起こし，腎症の進展に関与しているが，これら指標の脂質過酸化，ソルビトール，advanced glycation endproducts（AGEs）を測定した結果，桂枝茯苓丸は脂質過酸化と AGEs に好影響を及ぼしていた。さらに，桂枝茯苓丸の作用を，aminoguanidine（AGEs 阻害薬），butylated hydroxytoluene（抗酸化剤），captopril（ACE阻害剤）と比較検討した結果，脂質過酸化と AGEs に対する抑制作用が認められた。また，桂枝茯苓丸の腎保護作用は captopril よりは弱く，aminoguanidine と同程度であった。これらの結果より，桂枝茯苓丸による糖尿病性腎症治療の可能性が示唆された。

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