Evaluation of Keishi-bukuryo-gan in a diabetic nephropathy model by comparison with aminoguanidine, butylated hydroxytoluene and captopril

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(Received October 22, 2002. Accepted November 22, 2002.)

Abstract

A study was done to investigate whether Keishi-bukuryo-gan can delay the progression of diabetic nephropathy in an experimentally induced diabetic nephropathy model. The efficacy of Keishi-bukuryo-gan against renal functional and structural changes and its influence on accumulation of advanced glycation end-products (AGEs) and oxidative stress were also examined by comparison with aminoguanidine (an AGEs inhibitor), butylated hydroxytoluene (BHT; an antioxidant) and captopril (an angiotensin converting enzyme inhibitor). Treatment with Keishi-bukuryo-gan for 10 weeks preserved renal function, as assessed in terms of proteinuria and serum creatinine, and prevented the morphological changes peculiar to diabetic nephropathy. However, its renoprotective activity was inferior to that of captopril and comparable to that of aminoguanidine. BHT lacked any of these effects. On the other hand, renal AGEs accumulation and oxidative stress were significantly enhanced in rats with untreated diabetic nephropathy compared with normal rats. Keishi-bukuryo-gan, captopril and BHT showed significant reduction of AGEs levels, but not to the extent shown by aminoguanidine. Renal lipid peroxidation levels were significantly lowered in the groups given Keishi-bukuryo-gan and captopril, but not to the extent shown in the rats given BHT. The reduction of serum lipid peroxidation levels by captopril was stronger than that by BHT. The effects of Keishi-bukuryo-gan and aminoguanidine on serum lipid peroxidation levels were similar to those of BHT. These results suggest that the pharmaceutical characteristics of Keishi-bukuryo-gan may differ from those of the other three medicines examined.

Key words Keishi-bukuryo-gan, diabetic nephropathy, aminoguanidine, butylated hydroxytoluene, captopril.

Introduction

Diabetic nephropathy is the leading cause of end-stage renal failure in many countries and its prognosis is poor even after the introduction of dialysis therapy.[1] Therefore, it is a serious medical matter to prevent the occurrence and progression of diabetic nephropathy and to seek effective therapeutic interventions. Clinical trials have shown that control of hypertension, which is a concomitant risk factor, slows the decline of renal function, as well as control of hyperglycemia.[2-4] Among various antihypertensive therapies, angiotensin-converting enzyme (ACE) inhibitors are highly effective in retarding the progression of diabetic nephropathy and reducing albuminuria, as shown both experimentally and clinically.[5,6] However, it is extremely difficult to prevent the occurrence and progression of diabetic nephropathy, even when hypertension and hyperglycemia are well controlled. Therefore, it would be desirable to prove the pathogenic mechanisms of diabetic nephropathy, as well as to develop drugs that can ameliorate specific pathogenic factors.

Recent clinical and experimental studies have been proposed a number of pathogenic mechanisms in the development of diabetic nephropathy, including acceleration of the glycation reaction and oxidative stress,[7,8] and these metabolic abnormalities are thought to be closely associated with the progression of proteinuria and renal dysfunction. Therefore, correction of these metabolic

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abnormalities may be important therapeutic avenues, and agents such as advanced glycation end-products (AGEs) inhibitors and antioxidants have been the focus of considerable interest.

Traditional herbal medicines have been employed for thousands of years and have contributed to the prevention and treatment of various diseases, including diabetic nephropathy. 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 herbal materials and have low toxicity. However, there is little information about their usefulness for treatment of diabetic nephropathy or their mechanisms of action based on scientific evidence. With this in mind, we have been examining the effects of traditional herbal medicines on the metabolic abnormalities accompanying diabetes, and found that Keishi-bukuryo-gan attenuated the accumulation of AGEs and oxidative stress, suggesting renoprotective effects.[9] Therefore, we conducted the present study to determine whether prolonged treatment with Keishi-bukuryo-gan can delay the progression of diabetic nephropathy, and to elucidate its renoprotective activity and influence on AGEs accumulation and oxidative stress by comparing its efficacy with those of aminoguanidine (an AGEs inhibitor), butylated hydroxytoluene (BHT; an antioxidant) and captopril (an ACE inhibitor).

Materials and Methods

Preparation of Keishi-bukuryo-gan extract : Keishi-bukuryo-gan is composed of equal parts, by weight, of the following five crude drugs: Cinnamomi Cortex (*Cinnamomum cassia* BLUME), Hoelen (*Poria cocos* WOLF), Paeoniae Radix (*Paeonia lactiflora* PALLAS), Moutan Cortex (*Paeonia suffruticosa* ANDREWS) and Persicae Semen (*Prunus persica* BATSCH). These crude drugs were obtained from Tochimoto Tenkaidou Co. Ltd. (Osaka, Japan). The extract was obtained by boiling 100 g of the crude drug mixture (20 g each component) gently in 500 ml water for 50 min. The insoluble portion was removed by filtration, then the filtrate was concentrated under reduced pressure and lyophilized, yielding a brown residue, which represented 9.68%, by weight, of the original materials.

Animals and treatment : Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) weighing 160-170 g were kept in an automatically controlled room (temperature about 23℃ and humidity about 60%) under a conventional lighting regimen with a dark night. According to the method reported previously,[10] the rats underwent resection of half of the left kidney and total excision of the right kidney 7 days later. Thereafter, they were injected intraperitoneally with 25 mg/kg body weight streptozotocin (STZ) in citrate buffer (10 mM, pH 4.5). Their blood glucose and urea nitrogen levels were determined after recovery from the injection, and these diabetic rats were divided into five groups (one control and four treatment groups), avoiding any intergroup differences in these blood indices. A normal group of rats that underwent a sham operation and did not receive STZ was also included. Each experimental group contained eight rats. Over the 10-week experimental period, the normal and control groups received plain drinking water, while the other three groups were given Keishi-bukuryo-gan (150 mg/kg body weight/day via a stomach tube), aminoguanidine (1 g/l in drinking water), BHT (0.4% in the diet) and captopril (50 mg/kg body weight/day via a stomach tube). These doses were determined by reference to a dose that matched the efficacy of each medicine.[9,11-13] Five weeks after treatment, blood samples were obtained from the tail veins and 24-h urine samples were collected from the rats in metabolic cages. At the end of the experimental period, the urine was collected and blood samples were obtained by cardiac puncture. 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 the rats and one part of the tissue was immersed in formalin for histological examination and the other part was kept at -80℃ until analysis.

Determination of blood and urine components : Serum levels of glucose and creatinine (Cr) were determined using commercial reagents (Glucose CII-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan; CRE-EN Kainos obtained from Kainos Laboratories, Inc., Tokyo, Japan). Serum glycosylated protein and malondialdehyde (MDA) levels were measured using the methods of McFarland *et al.*[14] and Naito & Yamanaka,[15] respectively. Levels of urine components were determined as follows: Cr using a commercial reagent (CRE-EN Kainos) and protein by the

sulfosalicylic acid method.[16] Creatinine clearance (Ccr) was calculated on the basis of urinary Cr, serum Cr, urine volume and body weight using the equation: Ccr (ml/kg body weight /min) = {urinary Cr (mg/dl) x urine volume (ml) / serum Cr (mg/dl)} x {1,000/body weight (g) } x {1/1,440 (min)}.

Blood pressure determination : After 4 and 8 weeks of treatment, blood pressure was measured using the tail-cuff method with a MK-100 unit (Neuroscience, Tokyo, Japan). Four consecutive determinations were performed in each rat.

Determination of renal AGEs and MDA levels : According to the method of Nakayama *et al.*,[17] kidney tissue delipidated with chloroform and methanol (2:1, v/v) was used for determination of AGEs levels. After washing, the tissue was homogenized in 0.1 N NaOH and 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 of 370 nm. For assay of MDA, the kidney tissue was homogenized with a 9-fold volume of ice-cold 1.15% KCl. The MDA level in each homogenate was measured according to the method of Mihara & Uchiyama,[18] based on the reaction with thiobarbituric acid.

Histological examination : Renal tissues were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin. The tissues were then cut into 4-μm sections, mounted on silane-coated glass slides, and stained with hematoxylin-eosin (HE), periodic acid-Schiff reagent (PAS), periodic acid-methenamine silver (PAM) and phosphotungstic acid-hematoxylin (PTAH). Two hundred or fewer glomeruli in each sample were examined by light microscopy, and the severity of the histological lesions was evaluated and scored as follows: absent, 0; slight, 1; mild, 2; moderate, 3; severe, 4.

Statistics : Values are presented as means $\pm$ S.E. Differences among groups were analyzed by Dunnett's test and those at $p<0.05$ were accepted as significant.

Results

Body weight, kidney weight and urine volume

As shown in Table I, the body weight of the control rats with diabetic nephropathy was significantly lower than that of the normal rats, and there were no significant differences between untreated control rats and four treated rats. On the other hand, kidney weight and urine volume were significantly increased in the rats with diabetic nephropathy, reaching 0.939 g/100 g body weight and 126.8 ml/day, respectively. The increase in kidney weight was reduced by administration of Keishi-bukuryo-gan, aminoguanidine and captopril. Although urine volume was significantly lowered in rats treated with aminoguanidine and BHT, it was unchanged by administration of Keishi-bukuryo-gan and captopril.

General biochemical parameters

Table II shows the effect of each medicine on serum glucose and glycosylated protein levels. The rats with diabetic nephropathy showed higher glucose levels than the normal rats. The group that received BHT showed a significant decrease in serum glucose at 5 weeks, and a further reduction at 10 weeks. After 10 weeks of treatment, serum glucose was also lower in the rats given aminoguanidine and Keishi-bukuryo-gan than in the untreated controls. The level of glycosylated protein was also about 1.6 times higher in control rats than in normal rats. This parameter was significantly lower after administration of the four medicines, although a higher level also appeared in all four groups.

Table I Body weight, kidney weight and urine volume

Group	Body weight (g)	Kidney weight (g/100 g B.W.)	Urine volume (ml/day)
Normal rats	406.8 $\pm$ 11.6	0.568 $\pm$ 0.014	21.0 $\pm$ 3.0
Diabetic nephropathy rats			
Control	302.4 $\pm$ 20.4$^{\#\#}$	0.939 $\pm$ 0.101$^{\#\#}$	126.8 $\pm$ 16.5$^{\#\#}$
Keishi-bukuryo-gan	297.0 $\pm$ 13.3$^{\#\#}$	0.787 $\pm$ 0.039$^{\#,*}$	118.7 $\pm$ 20.6$^{\#\#}$
Aminoguanidine	283.4 $\pm$ 23.0$^{\#\#}$	0.787 $\pm$ 0.079$^{\#,*}$	66.3 $\pm$ 8.3$^{\#,***}$
BHT	303.3 $\pm$ 12.5$^{\#\#}$	0.840 $\pm$ 0.074$^{\#\#}$	83.9 $\pm$ 11.6$^{\#\#,**}$
Captopril	290.9 $\pm$ 17.8$^{\#\#}$	0.765 $\pm$ 0.066$^{\#,**}$	121.4 $\pm$ 17.4$^{\#\#}$

Statistical significance: $^{\#}p<0.01$, $^{\#\#}p<0.001$ vs. normal rats; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control rats with diabetic nephropathy.

Table II 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$^{\#\#}$	558.7 ± 59.2$^{\#\#}$	24.1 ± 1.6$^{\#\#}$
Keishi-bukuryo-gan	491.3 ± 48.4$^{\#\#}$	421.8 ± 71.5$^{\#\#,**}$	20.6 ± 1.3$^{\#\#,**}$
Aminoguanidine	497.4 ± 59.7$^{\#\#}$	405.5 ± 58.2$^{\#\#,**}$	19.4 ± 1.7$^{\#\#,***}$
BHT	471.7 ± 39.5$^{\#\#,*}$	365.1 ± 60.9$^{\#\#,***}$	19.1 ± 0.9$^{\#,***}$
Captopril	580.8 ± 31.2$^{\#\#}$	474.8 ± 26.4$^{\#\#}$	20.8 ± 1.4$^{\#\#,**}$

Statistical significance: $^{\#}p<0.01$, $^{\#\#}p<0.001$ vs. normal rats; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control rats with diabetic nephropathy.

Table III 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$^{\#}$	0.704 ± 0.087$^{\#}$	3.73 ± 0.28$^{\#}$	3.42 ± 0.37$^{\#}$
Keishi-bukuryo-gan	0.537 ± 0.059$^{\#}$	0.572 ± 0.028$^{\#,**}$	4.19 ± 0.43$^{\#}$	3.95 ± 0.33$^{\#}$
Aminoguanidine	0.614 ± 0.035$^{\#}$	0.603 ± 0.053$^{\#,*}$	3.46 ± 0.18$^{\#}$	3.29 ± 0.26$^{\#}$
BHT	0.550 ± 0.031$^{\#}$	0.613 ± 0.049$^{\#}$	3.32 ± 0.22$^{\#}$	3.03 ± 0.45$^{\#}$
Captopril	0.495 ± 0.011$^{\#,**}$	0.529 ± 0.026$^{\#,***}$	4.44 ± 0.29$^{\#,**}$	4.03 ± 0.47$^{\#}$

Statistical significance: $^{\#}p<0.001$ vs. normal rats; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control rats with diabetic nephropathy.

Table IV 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$^{\#}$	162.4 ± 11.7$^{\#}$
Keishi-bukuryo-gan	158.6 ± 4.9$^{\#}$	158.8 ± 9.5$^{\#}$
Aminoguanidine	165.1 ± 14.3$^{\#}$	167.3 ± 13.7$^{\#}$
BHT	168.1 ± 3.8$^{\#}$	158.1 ± 5.9$^{\#}$
Captopril	151.1 ± 5.2$^{\#,*}$	133.2 ± 3.6**

Statistical significance: $^{\#}p<0.001$ vs. normal rats; $^{*}p<0.05$, $^{**}p<0.001$ vs. control rats with diabetic nephropathy.

Serum Cr levels were significantly increased in rats with diabetic nephropathy than in normal rats, as shown in Table III. After 10 weeks, the Cr level in control rats was further increased (from 0.593 mg/dl to 0.704 mg/dl), reflecting renal dysfunction. Of the four medicines, captopril reduced the Cr level to the greatest extent at 5 weeks, and this effect was observed even after 10 weeks. Although the other three medicines did not produce significant changes at 5 weeks, 10 weeks of treatment with Keishi-bukuryo-gan and aminoguanidine significantly lowered the Cr level to 0.572 mg/dl and 0.603 mg/dl, respectively. On the other hand, rats with diabetic nephropathy showed significant reduction of Ccr. As shown in Table III, the captopril-treated group showed a significant improvement in this value at 5 weeks, whereas at 10 weeks the captopril and Keishi-bukuryo-gan groups showed non-significantly higher values.

Blood pressure was measured at 4 and 8 weeks after treatment. The rats with diabetic nephropathy showed higher systolic blood pressure than normal rats, as shown in Table IV. Only captopril had a blood pressure-lowering effect, reaching close to the normal value at 8 weeks.

At 5 weeks, urinary protein excretion in control rats with diabetic nephropathy was about 77.9 mg/day, as shown in Fig. 1. Captopril showed the greatest lowering effect to 41.4 mg/day. Aminoguanidine and Keishi-bukuryo-gan also showed significant reduction to 57.7 mg/day and 61.3 mg/day, respectively, whereas BHT produced no change in urinary protein excretion. After 10 weeks, this parameter was further increased to 129.8 mg/day in untreated control rats, reflecting the progression of renal disorder. A trend similar to that observed at 5 weeks was evident even after 10 weeks.

Renal AGEs, MDA and serum MDA levels

In comparison with normal rats, an increase in

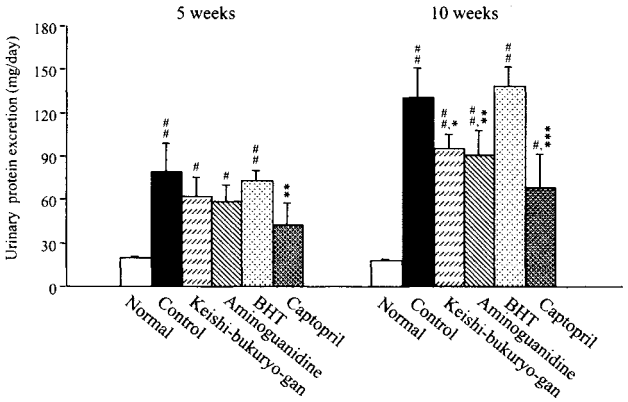

Fig. 1 Urinary protein excretion. Statistical significance: #$p<0.01$, ##$p<0.001$ vs. normal rats; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control rats with diabetic nephropathy.

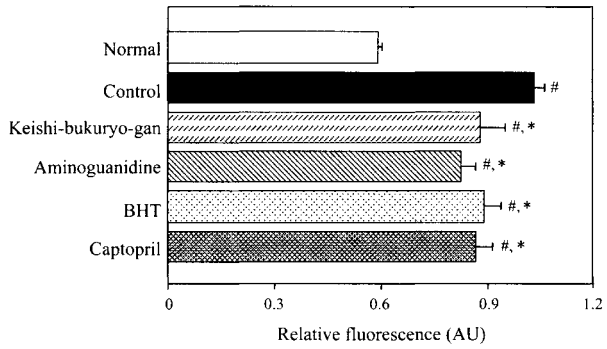

Fig. 2 AGEs levels in kidney. Statistical significance: #$p<0.001$ vs. normal rats; *$p<0.001$ vs. control rats with diabetic nephropathy.

related fluorescence (reflecting increased AGEs levels) was observed in control rats with diabetic nephropathy (from 0.592 to 1.031), as shown in Fig. 2. The aminoguanidine-treated group showed the lowest values, although the other three medicines also induced a significant reduction. As shown in Fig. 3, MDA levels in both serum and kidney were significantly higher in control rats with diabetic nephropathy than in normal rats. After administration of all four medicines, serum MDA levels were significantly lowered. The effect of captopril was particularly strong. In the kidney, BHT produced a significant ($p<0.001$) reduction, and the effects of Keishi-bukuryo-gan and captopril were also significant ($p<0.01$); aminoguanidine, however, did not change the renal MDA level.

Histological findings

Histopathological evaluation of the kidneys is summarized in Table V. The kidneys of rats treated with STZ plus subtotal nephrectomy showed the histopathological alterations typical of diabetic glomerulosclerosis.

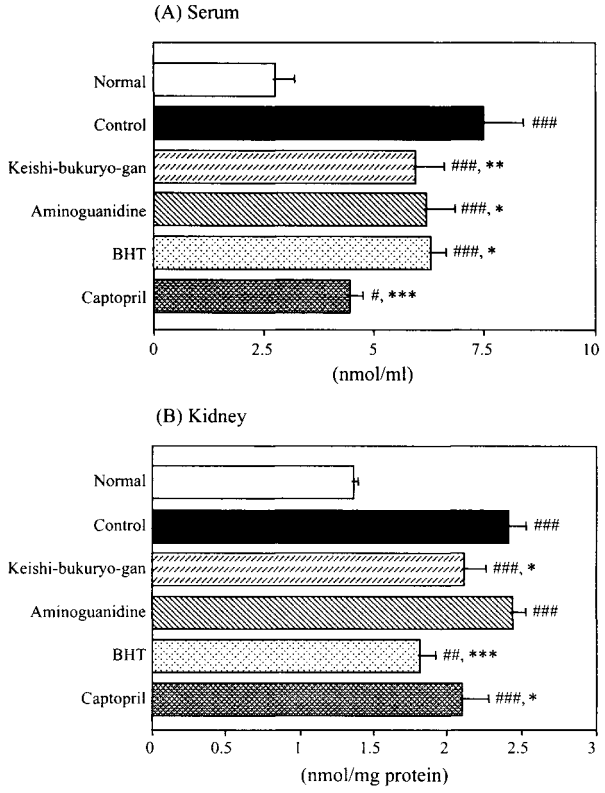

Fig. 3 MDA levels in serum and kidney. Statistical significance: #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs. normal rats; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control rats with diabetic nephropathy.

Normal rat kidneys lacked these morphological changes. In the untreated control rats, exudative lesions were scored as mild to severe, diffuse lesions as mild to moderate, nodular lesions as slight to moderate, and arteriolar hyalinosis as none to mild. In comparison, these scores for all four types of lesion were significantly lowered by captopril treatment. Keishi-bukuryo-gan and aminoguanidine significantly reduced the exudative lesion score to an extent similar to captopril. The scores for diffuse lesions, nodular lesions and arteriolar hyalinosis were non-significantly lower than the control ones. On the other hand, administration of BHT produced no significant changes in any of the four histopathological features of diabetic glomerulosclerosis in comparison with the control. Furthermore, tubulointerstitial lesions such as lymphocyte infiltration and interstitial fibrosis tended to be ameliorated by Keishi-bukuryo-gan, captopril, and aminoguanidine, but not by BHT (data not shown).

Representative photomicrographs of the glomeruli obtained from each group are shown in Fig. 4. The glomeruli of untreated control rats were enlarged due to

Table V 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**	2.57 ± 0.20	0.86 ± 0.40	1.33 ± 0.42
Aminoguanidine	2.00 ± 0.33**	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**	2.25 ± 0.25*	0.38 ± 0.38**	0.50 ± 0.33***

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

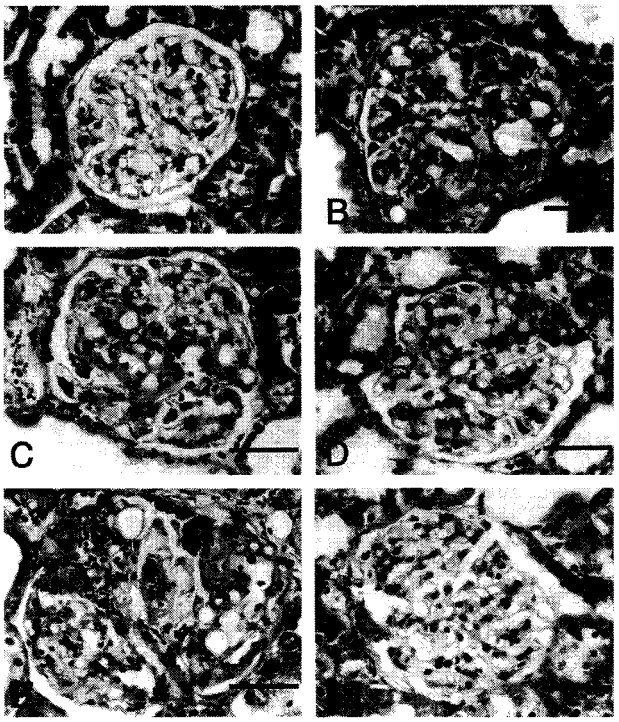

Fig. 4 Photomicrographs of the glomeruli obtained from normal rats (A), diabetic nephropathy rats in the control (B) and Keishi-bukuryo-gan (C), aminoguanidine (D), BHT (E) and captopril (F) treated groups. Scale bars = 50 μm.

diabetic exudative and diffuse lesions (Fig. 4-B). Widening of the mesangial areas, with increased staining of the mesangial matrix, was identified by PAS staining, and fibrinoid lesions in glomerular capillary loops or capsular drops were confirmed by PTAH staining (data not shown). Nodular lesions and arteriolar hyalinosis of afferent and efferent arterioles were also confirmed by PAS staining. Among the four tested medicines, rats given captopril (Fig. 4-F), Keishi-bukuryo-gan (Fig. 4-C) and aminoguanidine (Fig. 4-D) showed fewer diabetic glomerular lesions than the untreated control rats. In particular, captopril was effective for preservation of renal structures as well as lesion score.

Discussion

Proteinuria is an important parameter for assessing the progression of nephropathy in diabetic patients. It has been reported that captopril, an ACE inhibitor, reduces urinary albumin excretion in rats with STZ-induced diabetes and patients with non-insulin-dependent diabetes mellitus.[19,20] Indeed, 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 treatment of diabetic nephropathy. On the other hand, there has been a case report indicating that traditional herbal medicines, including Keishi-bukuryo-gan, improve the quality of life of patients with diabetic nephropathy as well as prolonging the predialysis stage.[21] Our previous experiment using rats with diabetic nephropathy suggested that Keishi-bukuryo-gan might be a useful therapeutic agent,[9] and therefore in the present study we compared Keishi-bukuryo-gan with captopril to elucidate and clarify its renoprotective efficacy using a methodological scientific approach.

In this study, rats with diabetic nephropathy induced by subtotal nephrectomy and injection of STZ had increased urinary excretion of protein and higher serum Cr levels as the experimental period was prolonged, indicating the progression of renal dysfunction. Together with deteriorations in renal function, morphological changes corresponding to diabetic nephropathy, such as diffuse lesions, exudative lesions and nodular lesions, were also

observed. Keishi-bukuryo-gan treatment effectively ameliorated increased kidney weight, serum Cr, proteinuria and histopathological changes, indicating the usefulness of this traditional herbal medicine for treatment of diabetic nephropathy. However, captopril showed stronger renoprotective activity, confirming 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 no effect on these parameters in this animal model.

Several clinical trials have shown that control of hyperglycemia and blood pressure can slow the progression of diabetic nephropathy.[2,3] In this study, administration of BHT significantly lowered serum glucose levels at both 5 and 10 weeks, 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. The renoprotective mechanism of captopril appears to involve not only a blood pressure-lowering effect but also other influences.[22-24] It is frequently noticed in a clinical setting that good control of hyperglycemia and hypertension cannot completely prevent the deterioration of renal function in patients with diabetic nephropathy. Therefore, the development of diabetic nephropathy is considered to be a multifactorial and complex process involving several mechanisms.

AGEs are produced by the glycation reaction between glucose and biological proteins.[25] Excessive formation and accumulation of AGEs under hyperglycemic conditions leads to pathological changes in the kidney including increased kidney weight, glomerular basement thickening and progressive albuminuria.[26] Inhibition of AGEs formation by aminoguanidine has been shown to attenuate above pathological changes in STZ-induced diabetes.[27] 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.*[28] demonstrated that prevention of AGEs formation by ALT-946, an AGEs inhibitor without inhibition of NOS, reproduces the protective effects of aminoguanidine. Other agents which inhibit NOS without effects on AGEs formation such as N^G-nitro-L-arginine methyl ester and methylguanidine have failed to confer similar renal protection observed with aminoguanidine,[29] suggesting that the renoprotective effect of aminoguanidine is mediated predominantly by decreased AGEs formation rather than by NOS inhibition. In this study, aminoguanidine treatment did not affect systolic blood pressure and Ccr levels, and reduced the increased AGEs-related fluorescence in the kidney by 20% compared with untreated control rats, together with its renoprotective effects. These findings agree with previous reports[11,17,26] indicating the importance of AGEs formation in increased kidney weight, proteinuria and renal structural changes. In addition, the other three medicines also significantly reduced this parameter, although to a lesser extent.

Lipid peroxidation levels in the plasma, urine and kidneys of patients with diabetic nephropathy have been reported to be higher than those in normal subjects.[30,31] Each of the present four medicines was evaluated for its effects on serum and renal lipid peroxidation levels, which are well accepted parameters of oxidative stress. BHT, which is a lipophilic antioxidant, reduced the renal lipid peroxidation levels to the greatest extent, although it did not have renoprotective activity. Keishi-bukuryo-gan and captopril reduced renal lipid peroxidation to a similar degree, 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. 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. Therefore, agents such as ACE inhibitors, AGEs inhibitors and antioxidants which can act on distinct processes, will exert different degrees of renoprotective activity through their relative

contributions. From our comparison with the AGEs inhibitor and antioxidant, the present study proposed at least in part that Keishi-bukuryo-gan inhibits renal AGEs accumulation and oxidative stress, thus helping to preserve renal function and structure. In addition, we found that captopril had a beneficial influence on renal AGEs and lipid peroxidation levels, together with lowered blood pressure levels.

In conclusion, we have demonstrated in a model animal of experimentally induced diabetic nephropathy that Keishi-bukuryo-gan exerts a certain renoprotective action comparable to that afforded by captopril, as estimated in terms of functional and histopathological parameters. In addition, our data for renal AGEs accumulation and lipid peroxidation suggest that this renoprotective effect of Keishi-bukuryo-gan may be due to pharmaceutical characteristics that differ from those of the other three medicines examined.

Acknowledgement

We wish to thank Ms. T. Matsushima, Faculty of Medicine, Toyama Medical and Pharmaceutical University, for technical assistance.

和文抄録

桂枝茯苓丸の糖尿病性腎症に対する作用を，モデルラットを用い検討した。腎機能パラメーター，病理組織学的検討に加え，advanced glycation end products（AGEs）の蓄積，酸化ストレスに及ぼす影響を，アミノグアニジン（AGEs阻害薬），カプトプリル（アンジオテンシン変換酵素阻害薬），buthylated hydroxytoluene（BHT）（抗酸化剤）とで比較検討した。桂枝茯苓丸では腎機能（血清Cr，尿蛋白排泄量）と病理所見の有意な改善作用が認められ，糖尿病性腎症の進展を抑制することが実験的に明らかとなったが，このような腎保護作用はカプトプリルよりは弱く，アミノグアニジンと同程度であった。BHTには腎保護作用は認められなかった。腎組織中のAGEsの蓄積に対しては，桂枝茯苓丸，カプトプリル，BHTがいずれも有意に低下していたが，アミノグアニジンの作用よりは弱かった。腎組織中の脂質過酸化量はBHTで最も低下し，桂枝茯苓丸，カプトプリルでも有意に低下していた。一方，血中脂質過酸化に対しては，すべてにおいて有意な低下作用が認められたが，カプトプリルで最も強かった。このことから，桂枝茯苓丸はカプトプリルやアミノグアニジンとは異なった機序で糖尿病性腎症の進展を抑制している可能性が示された。

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