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Therapeutic usefulness of Keishi-bukuryo-gan for diabetic nephropathy

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Abstract

Keishi-bukuryo-gan is a traditional herbal medicine, which is used clinically as a vascular system disorder-eliminating drug. In this study, its effect on the progression of diabetic nephropathy in experimental rats was investigated. The diabetic nephropathy model used in this study shows functional and morphological changes of the kidney resembling those seen in patients with diabetic nephropathy. Increased proteinuria and serum urea nitrogen and creatinine levels and decreased creatinine clearance, which are important parameters of renal function, were observed in rats with diabetic nephropathy. Pathological examination of the kidney revealed diffuse, nodular and exudative lesions and arteriolar hyalinosis. The deterioration of renal function was ameliorated in rats treated with Keishi-bukuryo-gan for 15 weeks and these results agreed with the renal histological findings. In addition, metabolic abnormalities mediated by persistent hyperglycaemia (the glycation reaction, excessive polyol pathway activity, oxidative stress and lipid metabolic abnormalities) were also observed. However, Keishi-bukuryo-gan reduced accumulation of advanced glycation end products, determined by measuring fluorescence, and serum lipid peroxidation, triglyceride and total cholesterol levels dose-dependently. Thus, this study indicates the potential therapeutic usefulness of Keishi-bukuryo-gan for retarding the progression of renal damage and suggests that its beneficial effects were due to its ability to improve metabolic abnormalities associated with diabetes.

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Introduction

Diabetes is associated with various complications, including nephropathy, which is one of the most life-threatening diseases. Indeed, diabetic nephropathy is the main cause of end-stage renal failure in many countries and the associated mortality is known to be very high compared with that of diabetic patients without nephropathy. Therefore, it is a matter of great urgency to prevent the occurrence and progression of diabetic nephropathy. However, effective treatments for diabetic nephropathy have not been established.

Traditional herbal medicines have been employed for thousands of years and have contributed greatly to the prevention and treatment of various diseases, including diabetes. They are still valuable for human health and have received much attention as potential sources of new therapeutic agents due to their varied biological activity and low toxicity. Therefore, in a previous study, we selected, on the basis of in-vitro screening test results, Ompi-to, Hachimi-jio-gan, Keishi-bukuryo-gan and Sairei-to from 12 traditional herbal medicines (Yokozawa et al 2001a) and investigated their effects in rats with diabetic nephropathy (Nakagawa et al 2001). We found that Keishi-bukuryo-gan improved the metabolic abnormalities that accompanied diabetic nephropathy, such as the formation of advanced glycation end products (AGEs), overenhancement of the polyol pathway, oxidative stress and lipid metabolism abnormalities (Brownlee et al 1988; Moorhead 1991; Yabe-Nishimura 1998; Ha & Kim 1999). These results indicated that Keishi-bukuryo-gan might be a therapeutic agent for diabetic nephropathy. More detailed studies, focused on renal function parameters and histological examination, are required to prove its therapeutic usefulness.

Thus, we conducted a long-term experiment in rats with diabetic nephropathy to establish whether Keishi-bukuryo-gan can slow the progression of diabetic nephropathy. The mechanisms of action of Keishi-bukuryo-gan against diabetic metabolic abnormalities were also examined.

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 crude drug mixture (20 g each) 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. For the analysis of the Keishi-bukuryo-gan components, the aqueous extract (2.5 g) was extracted with 20 mL of methanol under ultrasonication for 30 min. The solution was filtered with membrane filter (0.45 μm) and then submitted for HPLC analysis. HPLC equipment was controlled with a SLC-10A (Shimazu, Tokyo, Japan) using a TSK-GEL ODS-80TS column (4.6ϕ × 250 mm), eluting with solvents 0.05 M $AcONH_4$, pH 3.6 (A) and CH_3CN (B). A linear gradient of 100% A and 0% B, changing over 60 min to 0% A and 100% B, was used. The flow rate was controlled with LC 10AD pump as 1.0 mL min^{-1}. The eluate from the column was monitored and the three-dimensional data were processed by SPD-M10A diode array detector. All assigned peaks were identified by co-injection test with authentic samples and compared with the UV spectral data. The three-dimensional HPLC profile of Keishi-bukuryo-gan extract is shown in Figure 1.

Animals and treatment

The Guiding Principles for the Care and Use of Laboratory Animals and Guidelines for Animal Experimentation, approved by the Japan Pharmacological Society and Japanese Association for Laboratory Animal Science, respectively, were followed in these experiments. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan), 160–170 g, were kept in an automatically controlled room (the room temperature was about 23 °C and humidity was about 60%) with a conventional lighting regimen with a dark night. According to the method reported previously (Yokozawa et al 2001b), 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^{-1} streptozotocin 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 four groups (one control and three treatment groups), avoiding any inter-group differences in these blood indices. A normal group of rats that underwent a sham operation and did not receive streptozotocin was also included. Each experimental group contained 10 rats. Over the 15-week experimental period, the normal and control groups received plain drinking water, while the other three groups were given an oral solution of Keishi-bukuryo-gan at a dose of 50, 100 or 200 mg kg^{-1} daily via a stomach tube. Every three weeks, blood samples were obtained from the tail veins and 24-h urine samples

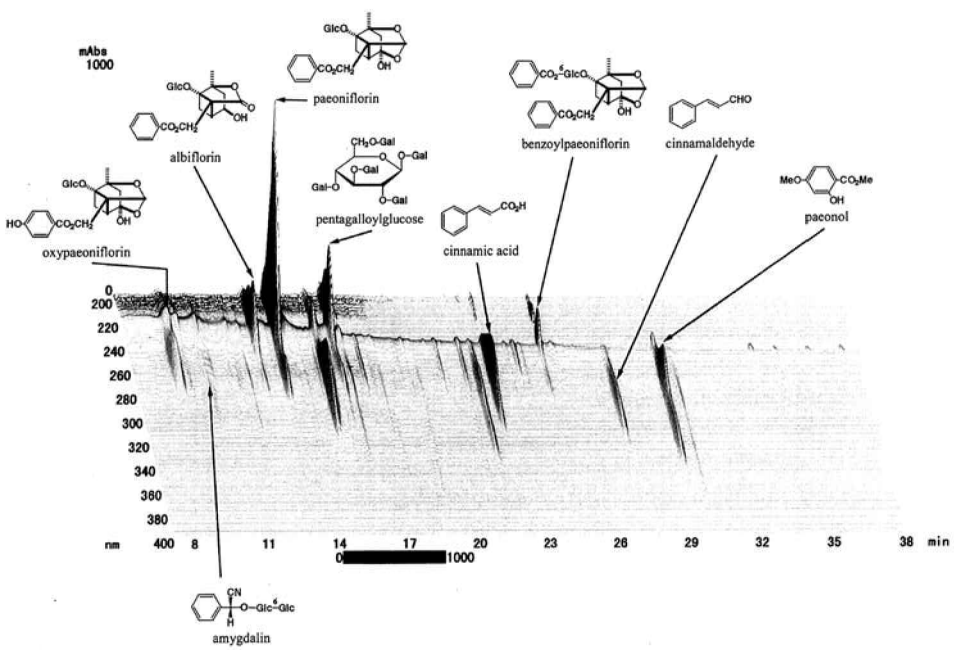

Figure 1 Three-dimensional HPLC profile of Keishi-bukuryo-gan extract.

were collected 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 $-80\,^{\circ}C$ until analysis.

Determination of blood and urine components

Serum levels of glucose, urea nitrogen, creatinine, total protein, albumin, triglyceride and total cholesterol were determined using commercial reagents (Glucose CII-Test Wako, A/G B-Test Wako, Triglyceride E-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 and malondialdehyde (MDA) levels were measured using the methods of McFarland et al (1979) and Naito & Yamanaka (1978), respectively. Urine component levels were determined as follows: creatinine using a commercial reagent (CRE-EN Kainos obtained from Kainos Laboratories, Inc.) and protein by the sulfosalicylic acid method (Sakagishi 1968). The creatinine clearance (Ccr) was calculated on the basis of urinary creatinine, serum creatinine, urine volume and body weight using equation 1.

$$\begin{aligned}\text{Ccr (mL min}^{-1}\text{ kg}^{-1}) = {} & [\text{urinary creatinine (mg dL}^{-1}) \\ & \times \text{urine volume (mL)} \\ & /\text{serum creatinine (mg dL}^{-1})] \\ & \times [1000/\text{body weight (g)}] \\ & \times [1/1440\ \text{(min)}] \qquad (1)\end{aligned}$$

Histological examination

Renal tissues were fixed in 10% neutral formalin solution, embedded in paraffin and cut into semi-thin sections ($2\,\mu$m thick). The sections were stained with hematoxylin and eosin and periodic acid–Schiff's reagent (PAS) and then examined by light microscopy. Two hundred or fewer glomeruli of each rat were examined and the severity of the histological lesions was evaluated and scored as follows: absent, 0; slight, 1; mild, 2; moderate, 3; severe, 4.

Determination of renal AGEs, MDA and sorbitol levels

According to the method of Nakayama et al (1993), 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 M 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 the assay of MDA, the kidney tissue was homogenized with a 9-fold volume of ice-cold 1.15% KCl. The MDA level of each homogenate was measured according to the method of Mihara & Uchiyama (1978), based on the reaction with thiobarbituric acid. The assay of sorbitol was carried out according to the method of Shinohara et al (1998): homogenized kidney tissue was deproteinized and the sorbitol concentration of a portion of the deproteinized supernatant was determined, based on the conversion of sorbitol to fructose by sorbitol dehydrogenase and nicotinamide adenine dinucleotide (NAD) and the formation of reduced NAD.

Statistics

Results are expressed as means $\pm$ s.d. One-way analysis of variance and Dunnett's test was employed to analyse the significant differences between normal group and diabetic nephropathy groups with or without Keishi-bukuryo-gan treatment, and between untreated diabetic nephropathy control group and Keishi-bukuryo-gan treated groups. A P value less than 0.05 was accepted as significant.

Results

Body weight and blood glucose levels

The body weight of the normal healthy rats increased during the 15-week experimental period (Figure 2A); that of the control rats with diabetic nephropathy was lower than that of normal rats. The difference between the normal and diabetic nephropathy groups became more distinct as time progressed. There were no significant differences at any time point between the body weights of the control and Keishi-bukuryo-gan treated groups of rats with diabetic nephropathy. At 0 weeks, the blood glucose levels of all the diabetic nephropathy groups were significantly higher (about 430 mg dL^{-1}) than the normal group value (about 120 mg dL^{-1}). From 6 weeks, the blood glucose levels of the groups treated with Keishi-bukuryo-gan at doses of 100 and 200 mg kg^{-1} daily were significantly lower ($P < 0.001$) than those of the untreated control group, but the glucose levels of the groups receiving Keishi-bukuryo-gan continued to be higher than the normal group levels for 15 weeks, as shown in Figure 2B. The blood glucose levels at the end of the experiment were 491, 444 and 439 mg dL^{-1} in the 50, 100 and 200 mg kg^{-1} daily Keishi-bukuryo-gan-treated groups and 571 mg dL^{-1} in the control group.

Urinary protein excretion

In normal rats, the daily urinary excretion of protein was less than 30 mg throughout the experimental period (Figure 3). However, urinary protein excretion by control rats with diabetic nephropathy increased markedly during the 15-week experimental period and reached 240.3 mg daily at 15 weeks, showing that progressive renal damage had occurred. In contrast, treatment with Keishi-bukuryo-gan

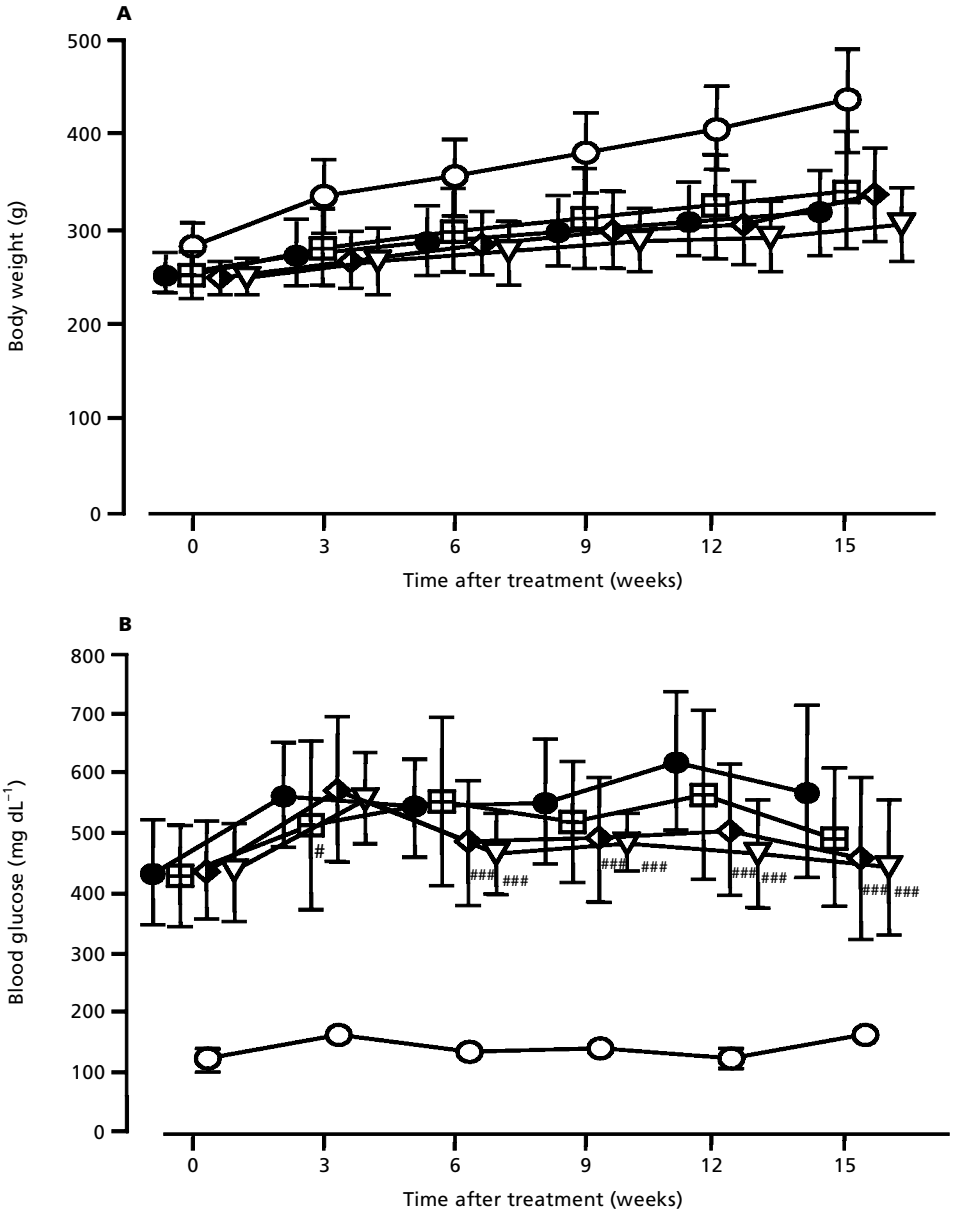

Figure 2 Body weight (A) and blood glucose levels (B) in normal rats (○) and in diabetic nephropathy rats treated with either Keishi-bukuryo-gan 50 mg kg^{-1} daily (⊞), 100 mg kg^{-1} daily (◆), 200 mg kg^{-1} daily (▽) or water (control, ●) for 15 weeks. Data are means ± s.d., n = 10. #$P < 0.05$, ###$P < 0.001$ vs control diabetic nephropathy group.

50, 100 and 200 mg kg^{-1} daily for 15 weeks significantly reduced it to 187, 177 and 174 mg day^{-1}, respectively.

General biochemical parameters

Table 1 shows the effects of Keishi-bukuryo-gan treatment for 15 weeks on serum biochemical parameters and Ccr in rats. The control rats with diabetic nephropathy had a significantly higher glycosylated protein level (21.2 nmol (mg protein)$^{-1}$) than the normal rats (11.8 nmol (mg protein)$^{-1}$) ($P < 0.001$). Following administration of Keishi-bukuryo-gan 100 and 200 mg kg^{-1} daily, the glycosylated protein levels (17.9 and 17.5 nmol (mg protein)$^{-1}$, respectively) were significantly lower than the control value ($P < 0.01$). The urea nitrogen and creatinine levels of the control rats (66.8 and 0.843 mg dL^{-1}, respectively) were significantly higher than the corresponding normal values (20.5 and 0.456 mg dL^{-1}) ($P < 0.001$). In comparison with the control values, Keishi-bukuryo-gan treatment (100 mg and 200 mg kg^{-1} daily) significantly reduced urea nitrogen levels to 52.8 and 51.7 mg dL^{-1}, respectively ($P < 0.01$), while the creatinine level was lowered to 0.726 mg dL^{-1} in the 200 mg Keishi-bukuryo-gan-treated group, but this failed to reach significance. In comparison with the respective normal group levels (5.04 and 3.41 g dL^{-1}), the serum total protein and albumin

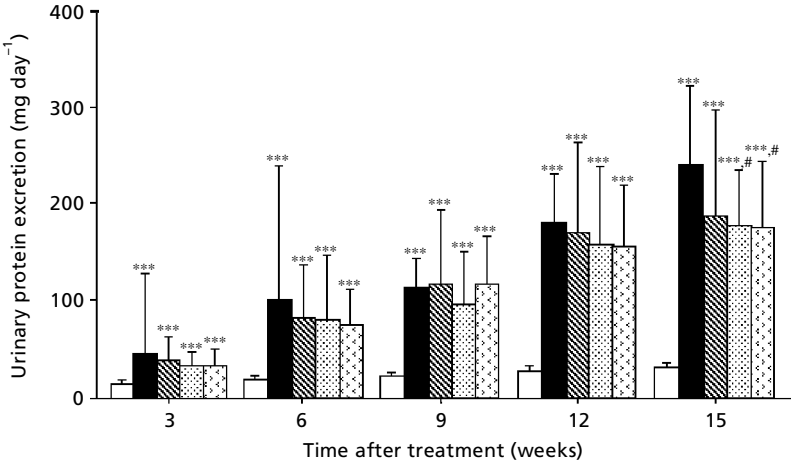

Figure 3 Urinary protein excretion in normal rats (□) and in diabetic nephropathy rats treated with either Keishi-bukuryo-gan 50 mg kg^{-1} daily (■), 100 mg kg^{-1} daily (▩), 200 mg kg^{-1} daily (▩) or water (control, ■) for 15 weeks. Data are means ± s.d., n = 10. ***$P < 0.001$ vs normal group; #$P < 0.05$ vs control diabetic nephropathy group.

levels were significantly reduced to 4.07 and 2.54 g dL^{-1} ($P < 0.001$), respectively, in control rats with diabetic nephropathy. After treatment with 200 mg kg^{-1} Keishi-bukuryo-gan daily, they increased to 4.35 and 2.74 g dL^{-1}, respectively ($P < 0.05$). The Ccr of the control group with diabetic nephropathy also decreased relative to the normal value (from 5.215 to 3.190 mL min^{-1} kg^{-1}) ($P < 0.001$), reflecting the renal dysfunction. No dose of Keishi-bukuryo-gan for 15 weeks changed the Ccr significantly. Hyperlipidaemia was observed in rats with diabetic nephropathy (Table 1): the serum triglyceride and total cholesterol levels were elevated markedly to 272.2 and 237.0 mg dL^{-1}, respectively, in control rats with diabetic nephropathy as compared with 37.3 and 63.6 mg dL^{-1} in normal rats ($P < 0.001$). Although both parameters were reduced dose-dependently by Keishi-bukuryo-gan, the triglyceride levels were reduced more than the total cholesterol levels (a reduction of approx. 38% vs 17%, respectively, for a daily dose of 200 mg) ($P < 0.01$ for triglyceride levels). Keishi-bukuryo-gan resulted in clearer and significant dose-dependent reduction of the serum MDA level: 2.90 and 2.68 nmol mL^{-1} in rats treated with 100 and 200 mg, respectively, of Keishi-bukuryo-gan compared with 3.82 nmol mL^{-1} in control rats with diabetic nephropathy ($P < 0.05$ and $P < 0.001$, respectively).

Histological findings

Table 2 summarizes the results of the histological examination of the kidney. The severity of renal damage was evaluated by assigning lesion scores, as described in Methods. The rats with diabetic nephropathy used in this study developed typical features of diabetic nephropathy

Table 1 General biochemical features of rats with diabetic nephropathy treated with Keishi-bukuryo-gan for 15 weeks.

Item	Normal	Diabetic nephropathy			
		Control	Keishi-bukuryo-gan 50 mg kg^{-1} daily	Keishi-bukuryo-gan 100 mg kg^{-1} daily	Keishi-bukuryo-gan 200 mg kg^{-1} daily
Serum glycosylated protein (nmol (mg protein)$^{-1}$)	11.8 ± 0.6	21.2 ± 4.0***	19.3 ± 5.5***	17.9 ± 3.2***##	17.5 ± 3.1***##
Serum urea nitrogen (mg dL^{-1})	20.5 ± 1.8	66.8 ± 16.1***	58.3 ± 20.1***	52.8 ± 14.3***##	51.7 ± 11.7***##
Serum creatinine (mg dL^{-1})	0.456 ± 0.030	0.843 ± 0.235***	0.851 ± 0.339***	0.774 ± 0.263***	0.726 ± 0.144***
Serum total protein (g dL^{-1})	5.04 ± 0.20	4.07 ± 0.38***	4.23 ± 0.53***	4.36 ± 0.32***##	4.35 ± 0.22***#
Serum albumin (g dL^{-1})	3.41 ± 0.19	2.54 ± 0.27***	2.61 ± 0.42***	2.68 ± 0.31***	2.74 ± 0.20***#
Ccr (mL min^{-1} kg^{-1})	5.215 ± 0.852	3.190 ± 1.034***	3.145 ± 1.211***	3.473 ± 1.206***	3.570 ± 0.733***
Serum triglyceride (mg dL^{-1})	37.3 ± 8.9	272.2 ± 162.6***	224.5 ± 82.5***	202.5 ± 124.9***	167.9 ± 93.1***##
Serum total cholesterol (mg dL^{-1})	63.6 ± 12.3	237.0 ± 88.9***	218.5 ± 82.7***	211.8 ± 117.9***	197.3 ± 58.9***
Serum MDA (nmol mL^{-1})	1.49 ± 0.14	3.84 ± 1.38***	3.21 ± 1.70***	2.90 ± 1.68**#	2.68 ± 1.12*##

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs normal group; #$P < 0.05$, ##$P < 0.01$ vs control diabetic nephropathic group.

Table 2 Histopathological evaluation of the kidney in rats with diabetic nephropathy treated with Keishi-bukuryo-gan for 15 weeks.

Group	Dose (mg kg^{-1} daily)	Lesion score					
		Diffuse lesion	Nodular lesion	Fibrin cap	Capsular drop	Arteriolar hyalinosis	Total
Normal	–	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							
Control	–	2.38 ± 0.74	2.25 ± 1.04	2.88 ± 0.83	2.25 ± 0.46	1.25 ± 1.04	11.00 ± 3.66
Keishi-bukuryo-gan	50	2.17 ± 0.41	1.83 ± 0.75	2.83 ± 0.41	2.33 ± 0.52	1.00 ± 1.10	10.17 ± 2.48
Keishi-bukuryo-gan	100	1.83 ± 0.41###	1.67 ± 0.52#	2.50 ± 0.55	2.00 ± 0.00	0.67 ± 1.03	8.67 ± 1.63##
Keishi-bukuryo-gan	200	1.78 ± 0.44###	0.86 ± 0.69###	2.44 ± 0.53#	1.14 ± 0.69###	0.43 ± 0.79#	7.00 ± 1.63###

Lesion score was expressed as follows: absent, 0; slight, 1; mild, 2; moderate, 3; severe, 4. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs control diabetic nephropathy group.

(diffuse, nodular, fibrin cap and capsular drop lesions and arteriolar hyalinosis). In the untreated control rats, with the exception of arteriolar hyalinosis which was scored as slight to mild, all these lesions were scored as mild to moderate. Normal rats showed none of these morphological changes. These increased lesion scores in the diabetic nephropathy control group were dose-dependently lowered following oral administration of Keishi-bukuryo-gan for 15 weeks (Table 2). This was reflected by the reduction of the total score, which was expressed as the sum

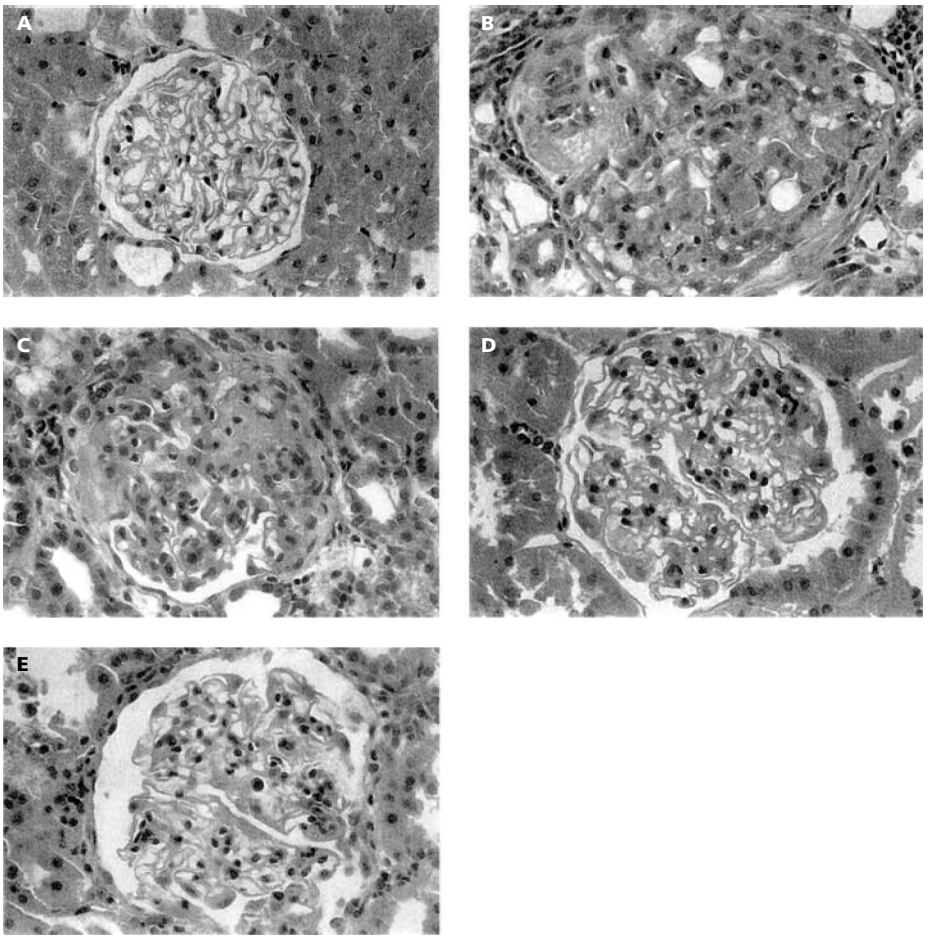

Figure 4 Photomicrographs of the glomeruli obtained from normal rats (A) and diabetic nephropathy rats in the control (B) and Keishi-bukuryo-gan-treated (50 mg kg^{-1} (C), 100 mg kg^{-1} (D) or 200 mg kg^{-1} daily (E)) groups. × 50.

Table 3 Renal AGEs, MDA and sorbitol levels in rats treated with Keishi-bukuryo-gan for 15 weeks.

Group	Dose (mg kg^{-1} daily)	AGEs (AU)	MDA (nmol (mg protein)$^{-1}$)	Sorbitol (nmol (mg protein)$^{-1}$)
Normal	–	0.556 ± 0.027	0.299 ± 0.041	0.575 ± 0.086
Diabetic nephropathy				
Control	–	0.841 ± 0.156***	0.394 ± 0.053***	1.915 ± 0.575***
Keishi-bukuryo-gan	50	0.776 ± 0.069***#	0.391 ± 0.069***	1.691 ± 0.690***
Keishi-bukuryo-gan	100	0.767 ± 0.102***#	0.375 ± 0.031***	1.521 ± 0.488***#
Keishi-bukuryo-gan	200	0.654 ± 0.059**###	0.360 ± 0.052**	1.648 ± 0.503***

**$P < 0.01$, ***$P < 0.001$ vs normal group; #$P < 0.05$, ###$P < 0.001$ vs control diabetic nephropathy group.

of the scores of the 5 lesions, showing that Keishi-bukuryo-gan inhibited the development of these glomerular lesions. Figure 4 shows typical photomicrographs of the glomeruli obtained from each group. The normal rats that underwent a sham operation had normal renal glomerular morphology (Figure 4A). In contrast, rats in the control and treatment groups with diabetic nephropathy had marked morphological changes associated with diabetes, including diffuse, nodular, fibrin cap and capsular drop lesions and arteriolar hyalinosis (Figure 4B–E). Among the rats with diabetic nephropathy, the untreated control group exhibited more severe lesions than the groups that received Keishi-bukuryo-gan treatment. It was also observed that Keishi-bukuryo-gan reduced the severity of the morphological changes in a dose-dependent manner.

Renal AGEs, MDA and sorbitol levels

In comparison with normal rats, the renal AGEs, MDA and sorbitol levels of the control rats with diabetic nephropathy increased significantly (Table 3). The AGEs levels, determined from the relative fluorescence, were significantly lower in the Keishi-bukuryo-gan-treated groups, even at the lowest dose (50 mg kg^{-1} daily), than the control group. A further reduction was observed in the group treated with 200 mg kg^{-1} daily. The MDA level of control rats was 1.3 times higher than the corresponding normal group value (Table 3). Keishi-bukuryo-gan slightly reduced the MDA levels in a dose-dependent manner, although the reduction was not significant. The increased sorbitol level of the control group (relative to the normal group) was reduced significantly in the group treated with 100 mg Keishi-bukuryo-gan daily, but no further decrease occurred in the 200 mg kg^{-1} group (Table 3).

Discussion

The clinical features of diabetic nephropathy were characterized by hyperglycaemia, glycosuria, proteinuria, renal dysfunction, hypertension and oedema. In this study, to determine the progression and severity 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 the rats with diabetic nephropathy were over 430 mg dL^{-1}. Among the rats with diabetic nephropathy, the blood glucose levels of the groups treated with Keishi-bukuryo-gan at doses of 100 and 200 mg kg^{-1} daily were significantly lower from 6 weeks than those of the untreated control group. In comparison with normal rats, the urinary excretion of protein increased dramatically in untreated rats with diabetic nephropathy throughout the experimental period, but oral administration of Keishi-bukuryo-gan significantly suppressed this. Furthermore, the serum urea nitrogen and creatinine levels (important therapeutic indices) of the groups with diabetic nephropathy increased, but at the end of experiment they were lower in the Keishi-bukuryo-gan-treated groups than the untreated controls. These 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. Based on these findings that Keishi-bukuryo-gan effectively reversed many characteristic pathological manifestations in rats with diabetic nephropathy, we concluded that this traditional herbal medicine has the potential to retard the progression of diabetic nephropathy.

The pathogenesis of diabetic nephropathy involves many factors stemming from persistent hyperglycaemia. It is well accepted that persistent hyperglycaemia induces acceleration of the glycation reaction, overenhancement of the polyol pathway, oxidative stress and lipid metabolism abnormalities in the body (Brownlee et al 1988; Moorhead 1991; Yabe-Nishimura 1998; Ha & Kim 1999). These abnormal biochemical processes are thought to be 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 at a dose of 150 mg kg^{-1} daily for 5 weeks improved these metabolic abnormalities in rats (Nakagawa et al 2001). 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 given different doses (50, 100 and 200 mg kg^{-1} daily) and prolonged the

administration period to 15 weeks to evaluate the therapeutic usefulness at later stages of diabetic nephropathy.

The protein glycation reaction can be divided 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) (Bucala & Cerami 1992; Vlassara et al 1994a). This reaction is accelerated in diabetes and excessive formation and accumulation of AGEs in the kidney causes glomerular basement membrane thickening and progressive albuminuria (Vlassara et al 1994b). In this study, the serum glycosylated protein levels in the groups treated with 100 and 200 mg kg^{-1} Keishi-bukuryo-gan daily were significantly lower than the control group levels, and this difference was well reflected by the serum glucose levels. While renal AGEs levels, determined by measuring fluorescence, were reduced significantly by even the lowest dose (50 mg kg^{-1} daily) of Keishi-bukuryo-gan, treatment with 200 mg kg^{-1} daily reduced it more than 100 mg kg^{-1} daily, although both the 100 and 200 mg kg^{-1} Keishi-bukuryo-gan-treated groups showed similar glycaemic 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 (Sato et al 1979; Giugliano et al 1996). Glucose itself and the glycosylated proteins known as Amadori rearrangement products are susceptible to autooxidation and may be a source of reactive oxygen species (Mullarkey et al 1990; Hunt et al 1993). Furthermore, enhanced oxidative stress due to diabetes may also result from a dysfunction in the defence system against free radicals, such as reduction of glutathione (Yabe-Nishimura 1998; Tachi et al 2001) or inactivation of superoxide dismutase (Arai et al 1987). 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, reflecting enhancement of oxidative stress. Oral administration of Keishi-bukuryo-gan for 15 weeks reduced these levels dose dependently. This finding agreed with our previous results (Nakagawa et al 2001), in which Keishi-bukuryo-gan strongly reduced lipid peroxidation after 5 weeks of treatment, which suggested that it exerts antioxidative effects even at later stages of diabetic nephropathy.

The enhancement of the polyol pathway in diabetes leads to the accumulation of sorbitol and fructose in the tissues. This would cause the occurrence of non-enzymatic fructosylation of collagen in diabetic rats (Suarez et al 1988) and the alteration of the cytosolic ratio of NADPH:NADH^{+} (Tilton et al 1992), leading to glomerular dysfunction. Sorbitol accumulation plays an important role in diabetic nephropathy, as well as in cataract, neuropathy and retinopathy. Aldose reductase is a key enzyme for sorbitol synthesis in the polyol pathway and inhibition of this step by aldose reductase inhibitors has been reported to improve some diabetic complications in animal experiments and clinical trials (Chang et al 1991; Pedersen et al 1991). In this study, the renal sorbitol level was higher in rats with diabetic nephropathy than in normal rats. Treatment with 100 mg kg^{-1} Keishi-bukuryo-gan daily significantly lowered it, but the 200 mg kg^{-1} daily dose did not reduce it further. Thus, it is likely that reduction of sorbitol accumulation is not the main effect of Keishi-bukuryo-gan.

Hyperlipidaemia is a causative factor in the progression of glomerular sclerosis (Moorhead 1991) and reduction of elevated serum triglyceride and total cholesterol levels has been reported to lead to the improvement of diabetic nephropathy (Mulec et al 1990). Our findings, that Keishi-bukuryo-gan effectively lowered serum triglyceride and total cholesterol levels in a dose-dependent manner, suggest strongly that some of its therapeutic properties are due to its hypolipidaemic effect.

Case reports indicating that traditional herbal medicines, including Keishi-bukuryo-gan, improved the quality of life of patients with diabetic nephropathy as well as prolonging the pre-dialysis stage of diabetic nephropathy have been published (Goto et al 2000). However, it is not clear how the bioactivity of these medicines contribute towards these effects. The aetiology of diabetic nephropathy is multifactorial and in the diabetic body, these aetiologic factors interdependently contribute to the progression of diabetic nephropathy. It is believed that traditional herbal medicines have the potential to become useful treatments for this complex disorder, because they are composed of several crude drugs and therefore exhibit various bioactivities.

In conclusion, we demonstrated in an experimental animal model that Keishi-bukuryo-gan effectively preserved renal function and ameliorated pathological changes in the kidney, resulting in retardation of the progression of diabetic nephropathy. In addition, our results from a previous study and this study provide insight into the possible therapeutic mechanisms of Keishi-bukuryo-gan, which exerts a renoprotective effect, at least in part, through its ability to reduce AGEs accumulation and oxidative stress and by virtue of its hypolipidaemic effect.

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