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Beneficial effects of Phellodendri Cortex extract on hyperglycemia and diabetic nephropathy in streptozotocin-induced diabetic rats

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This study investigated the effect of Phellodendri Cortex extract on hyperglycemia and diabetic nephropathy in streptozotocin-induced diabetic rats. Male Sprague-Dawley rats were divided into normal control (NC), diabetic control (DC), and diabetic treatment with Phellodendri Cortex extract (DP). Over a 4-week experimental period, Phellodendri Cortex extract was administered orally at 379 mg/kg BW/day. The final fasting serum glucose level, urine total protein level, and relative left kidney weight in the DP group were significantly lower than the DC group. Renal XO and SOD activities in the DP group were significantly lower than the DC group and renal CAT activity in the DP group was significantly higher than the DC group. Tubular epithelial change was reduced in the DP group compared to the DC group. These results indicated that Phellodendri Cortex can reduce glucose level and prevent or retard the development of diabetic nephropathy in streptozotocin-induced diabetic rats. [BMB reports 2008; 41(10): 710-715]

INTRODUCTION

Diabetes is a chronic disease that cannot be completely cured and may patients develop complications if not properly treated. The most devastating complication of diabetes is nephropathy, which causes 14% of all deaths in diabetes patients (1) and accounts for 40% of end-stage renal cases (2). Recently, attention has been drawn to the theory that oxidative stress is involved in the development of complications associated with chronic diabetes (3). Oxidative stress has been considered as a common pathogenetic factor in diabetic nephropathy and other complications (4, 5).

Diabetic nephropathy is characterized by glomerular hypertrophy, thickening of glomerular and tubular basement membranes, increased amounts of extracellular matrix (ECM) in the mesangium, and increased glomerular permeability (6, 7). Excessive excretion of glycogen through the glomeruli is reabsorbed into the cytoplasm of tubules. These phenomena are especially prominent in the straight portion of proximal tubules. In histological preparations, these glycogen inclusions are washed out and result in a clearing effect within the tubular epithelial cells. This phenotype is referred to as Almanni-Ebstein cells and is a clear morphological characteristic of a diabetic kidney (8).

While management of diabetes mellitus includes diet, exercise, oral hypoglycemic agents and insulin, these treatments do not effectively prevent the complications of diabetes, such as nephropathy, neuropathy and hypertension (9). Therefore, there is an increasing demand for natural products and traditional herbal medicines with antidiabetic activities. Herbal plants have been recently popularized in modern medicine, since many therapeutically important compounds are derived from them (10).

Phellodendri Cortex, the dried trunk bark of *Phellodendron amurense* Ruprecht, contains a number of alkaloids, e.g. berberine, palmatine and jatrorrhizine. Phellodendri Cortex is a known anti-inflammatory agent (11, 12) and has been widely used as drug in East Asia. This study investigated the effects of Phellodendri Cortex extract on the progression of diabetes, antioxidant enzyme activities, renal function, and histological renal changes in streptozotocin (STZ)-induced diabetic rats.

RESULTS

Water intake, food intake, body weight gain and food efficiency ratio

Water intake and food intake in diabetic groups were significantly higher ($P < 0.001$) than the NC group and they did not differ between the two diabetic groups. Body weight gain and food efficiency ratio in diabetic groups were significantly lower ($P < 0.001$) than the NC group and they were higher in the DP group than the DC group by 268% and 279%, respectively (Table 1).

Blood glucose levels

At the end of the 4 week experimental period, blood glucose level in the DC group was significantly higher than the NC group (626.1 ± 64.5 vs. 101.8 ± 30.0 mg/dl, $P < 0.001$) and it was significantly lower in the DP group (536.5 ± 77.5

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Table 1. Water intake, food intake, body weight gain and food efficiency ratio

Items	Gripus[a]		
	NC	DC	DP
Water intake(ml/day)	49.40 ± 3.85	294.29 ± 20.64$^{###}$	248.67 ± 65.95$^{###}$
Food intake (g/day)	27.40 ± 2.30	51.00 ± 3.65$^{###}$	50.33 ± 7.55$^{###}$
Body weight gain (g/day)	3.15 ± 0.29	-0.34 ± 0.29$^{###}$	0.57 ± 0.93$^{###,*}$
Food efficiency ratio[b] (%)	11.56 ± 1.49	-0.67 ± 0.61$^{###}$	1.20 ± 2.47$^{###}$

[a] NC: Normal Control, DC: Diabetic Control, DP: Diabetic Phellodendri Cortex.
[b] Food efficiency ratio = (body weight gain/ food intake) × 100.
Values are means ± SD of 7 rats. The value with a sharp-note is significantly different from NC group by t-test ($^{###}$; $P < 0.001$). The value with an asterisk is significantly different from DC group by t-test (*; $P < 0.05$).

Table 2. Serum and urine parameters

Items	Groups		
	NC	DC	DP
Serum			
Glucose (mg/dl)	167.00 ± 7.04	668.40 ± 41.29$^{###}$	552.20 ± 66.91$^{###,*}$
Insulin (pmol/l)	156.41 ± 80.80	18.14 ± 1.24$^{###}$	22.65 ± 8.80$^{###}$
BUN (mg/dl)	17.98 ± 2.20	42.25 ± 8.14$^{###}$	38.60 ± 4.52$^{###}$
Creatinine (mg/dl)	0.58 ± 0.08	0.55 ± 0.08$^{##}$	0.55 ± 0.08$^{##}$
Urine			
Volume (ml/kg/day)	64.13 ± 12.76	815.52 ± 75.17$^{###}$	677.16 ± 172.91$^{##}$
Total protein (mg/day)	16.04 ± 3.88	40.35 ± 5.38$^{##}$	23.85 ± 5.42**
Creatinine clearance (ml/min)	3.00 ± 0.80	2.54 ± 0.75	3.19 ± 0.46

Values are means ± SD of 7 rats. The value with a sharp-note is significantly different from NC group by t-test ($^{##}$; $P < 0.01$, $^{###}$; $P < 0.001$). The value with an asterisk is significantly different from DC group by t-test (*; $P < 0.05$, **; $P < 0.01$).

mg/dl, $P < 0.05$) than the DC group by 14% (Fig. 1).

Biochemical measurement

A significant elevation in final fasting serum glucose level was observed in diabetic groups compared to the NC group ($P < 0.001$) and it was significantly lower in the DP group than the DC group by 17% ($P < 0.05$). Serum insulin level in the DC group was significantly lower than the NC group ($P < 0.001$) and it was higher in the DP group than the DC group by 25%. Serum BUN level in the DC group was significantly higher than the NC group ($P < 0.001$) and it was lower in the DP group than the DC group by 9%. Serum creatinine level in the DC group was significantly lower than the NC group ($P < 0.01$). However, they did not differ between the DC and DP groups.

Urine volume in the DC group was significantly higher than the NC group ($P < 0.001$) and it was lower in the DP group than the DC group by 17%. Urine total protein in the DC group was significantly higher than the NC group ($P < 0.01$) and it was significantly lower in the DP group than the DC group by 41% ($P < 0.01$). Urine creatinine clearance in the DC group was lower than the NC group by 15% and it was higher in the DP group than the DC group by 26% (Table 2).

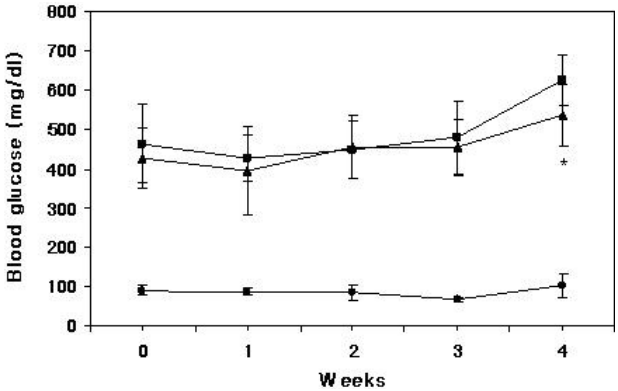

Fig. 1. Changes in blood glucose levels of normal and diabetic rats fed Phellodendri Cortex extract for 4 weeks. Values are mean ± SD of 7 rats. Values with an asterisk are significantly different from DC group by t-test (*; $P < 0.05$). ●, NC; ■, DC; ▲, DP.

Organ weight

Relative weight of liver ($P < 0.001$), kidney ($P < 0.001$) and heart ($P < 0.05$) in the DC group were significantly higher than

the NC group. Relative left kidney weight in the DP group was significantly lower than the DC group ($P < 0.05$) (Table 3).

Antioxidant enzyme activities

XO, SOD ($P < 0.001$) and GST ($P < 0.05$) activities in the DC group were higher than the NC group. However, they were lowered in the DP group than the DC group by 28% ($P < 0.05$), 14% ($P < 0.05$) and 10%, respectively. CAT activity in the DC group was significantly lower than the NC group ($P < 0.05$) and it was significantly higher in the DP group than the DC group by 48% ($P < 0.05$) (Table 4).

Histological analysis

In an assay via light microscopy, the DC group showed no glomerular enlargement or sclerotic change. Proximal tubular epithelial cells showed obvious changes with PAS-positive grains at the periphery of cytoplasm (Armanni-Ebstein cells). This change was especially prominent in the straight portion of proximal tubules. In the DP group, this cytoplasmic change was rarely seen and glomeruli showed no significant change (Fig. 2). Fig. 3 shows quantitative analysis of Armanni- Ebstein cells in renal cortex. Armanni-Ebstein cells in the DC group was significantly higher than the NC group (0.287 ± 0.050 vs. 0.066 ± 0.020, $P < 0.001$) by quantitative scoring and it was significantly lower in the DP group (0.148 ± 0.062, $P < 0.001$) than the DC group.

DISCUSSION

In this study we investigated the effect of the Phellodendri Cortex extract (PCE) on blood glucose level, antioxidant en-

Table 3. Organ weight

Organs	Groups		
	NC	DC	DP
Liver	10.80 ± 1.12^{a}	$9.27 \pm 0.96^{\#}$	9.94 ± 1.03
	3.00 ± 0.23^{b}	$4.46 \pm 0.34^{\#\#\#}$	$4.34 \pm 0.45^{\#\#\#}$
Kidney (right)	1.21 ± 0.11	1.31 ± 0.13	$1.39 \pm 0.13^{\#}$
	0.34 ± 0.03	$0.63 \pm 0.05^{\#\#\#}$	$0.61 \pm 0.10^{\#\#\#}$
Kidney (left)	1.21 ± 0.11	$1.39 \pm 0.12^{\#}$	1.36 ± 0.12
	0.34 ± 0.03	$0.67 \pm 0.04^{\#\#\#}$	$0.59 \pm 0.08^{\#\#\#,*}$
Heart	1.09 ± 0.15	$0.73 \pm 0.13^{\#\#}$	$0.75 \pm 0.08^{\#\#}$
	0.30 ± 0.04	$0.35 \pm 0.03^{\#}$	0.33 ± 0.02

[a]Absolute organ weight, Unit: g. [b]Relative organ weight, Unit: g/ 100 g body weight.
Values are means ± SD of 7 rats. The value with a sharp-note is significantly different from NC group by t-test ($^{\#}$; $P < 0.05$, $^{\#\#}$; $P < 0.01$, $^{\#\#\#}$; $P < 0.001$). The value with an asterisk is significantly different from DC group by t-test (*; $P < 0.05$).

Table 4. Effect of Phellodendri Cortex on kidney SOD, CAT, XO and GST activities

Enzymes	Groups		
	NC	DC	DP
XO[a]	1.64 ± 0.35	2.08 ± 0.52	$1.50 \pm 0.21^{*}$
SOD[b]	2.18 ± 1.34	$6.59 \pm 0.49^{\#\#\#}$	$5.65 \pm 0.75^{\#\#,*}$
CAT[c]	4.21 ± 0.59	$3.08 \pm 0.90^{\#}$	$4.57 \pm 0.72^{*}$
GST[d]	21.48 ± 3.52	$27.55 \pm 3.72^{\#}$	24.77 ± 1.38

[a]Unit: nmole uric acid formed/mg protein/min. [b]Unit: U (50% inhibition of autoxidation of hematoxylin) mg protein/min. [c]Unit: H_2O_2 nmole reduced/mg protein/min. [d]Unit: nmole 2, 4-dinitrobenzene-glutathione conjugate/mg protein/min.
Values are means ± SD of 7 rats. The value with a sharp-note is significantly different from NC group by t-test ($^{\#}$; $P < 0.05$, $^{\#\#}$; $P < 0.01$, $^{\#\#\#}$; $P < 0.001$). The value with an asterisk is significantly different from DC group by t-test (*; $P < 0.05$).

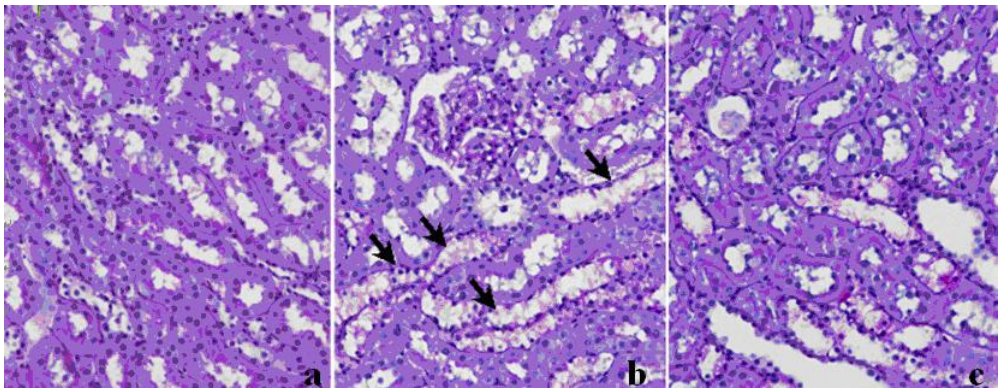

Fig. 2. Histological analysis of pathological renal changes in normal and diabetic rats fed Phellodendri Cortex extract for 4 weeks. Representative photomicrographs of periodic acid Schiff (PAS)-stained renal sections from a non-diabetic control (NC) rat (a), an untreated diabetic (DC) rat (b) and Phellodendri Cortex treated diabetic (DP) rat (c). Clear epithelial cells (Armanni-Ebstein cells) in proximal tubules (arrows) were frequently seen in DC rat and reduced in DP rat. However, these cells were not apparent in NC rat. Glomerulus demonstrated no increase in mesangial matrix even in DC rat. × 100.

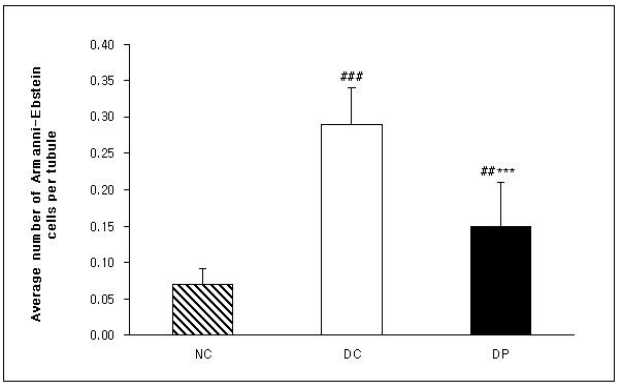

Fig. 3. Quantitative analysis of Armanni-Ebstein cells in renal cortex. Values are mean ± SD. Values with a sharp-note are significantly different from NC group by t-test ($^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$). Values with an asterisk are significantly different from DC group by t-test (*** $P < 0.001$).

zyme activities, histological changes and renal function in STZ-induced diabetic rats (70 mg/kg body weight i.p.). Male Sprague-Dawley rats were divided into three groups of seven animals each. The oral administration of PCE (379 mg extracted powder/kg body weight/day) was performed over a 4-week experimental period.

Diabetic rats showed increased food and water intake and less body-weight gain than non-diabetic rats, which were attributed to a polyphagic condition and weight loss due to excessive break down of tissue protein (13). The treatment with PCE improved food efficiency ratio and increased body weight gain compared to diabetic control rats, indicating control over polyphagia and muscle wasting caused by the hyperglycemic condition to some extent.

The treatment with PCE also significantly reduced serum glucose level and increased serum insulin level to some extent. This may be beneficial in the amelioration of the diabetic state and could explain the significantly lowered blood glucose levels in rats fed PCE.

Hyperglycemia also generates reactive oxygen species (ROS) that in turn cause lipid peroxidation and membrane damage (14). Previous studies have reported that lipid peroxidation in the liver, kidney, and brain of diabetic rats was increased (15, 16). Antioxidant enzymes are capable of eliminating ROS and lipid peroxidation products, thereby protecting cells and tissues from oxidative damage. Diabetic nephropathy is one of the most important microvascular complications of diabetes mellitus. Recent studies have indicated that ROS plays a key, intermediate role in the development of diabetic nephropathy. High glucose level directly increases hydrogen peroxide production of mesangial cells and lipid peroxidation of glomerular mesangial cells (17). XO has been proposed to be a major source of ROS in diabetes mellitus (18). SOD accelerates dismutation of superoxide radicals to hydrogen peroxide that in turn is removed by CAT and GPx (19). Endogenous antioxidant enzymes (SOD, CAT and GST) are responsible for the detoxification of deleterious oxygen radicals (20).

In our study, XO activity was increased in the kidneys of diabetic rats, which indicates increase in the generation of ROS. SOD and GST activities were increased in the kidneys of diabetic rats, which could be due to the compensatory reaction against increase in ROS. Data presented in our investigation indicate that the progression of diabetes results in augmentation of oxidative stress accompanied by impaired enzymatic antioxidative defense system in the kidneys of diabetic rats. The treatment with PCE reduced oxidative stress as evidenced by the restoration of the enzymatic antioxidative defense system. CAT activity was significantly decreased in diabetic control rats compared to the normal control rats. This could be due to the increased utilization for scavenging of free radicals. CAT activity in diabetic group treated with PCE was significantly increased compared to the diabetic control group. These results indicate that treatment with PCE restored the SOD, CAT, GST, and XO activities and reduced oxidative stress in diabetic rats.

Diabetic nephropathy is characterized by increased urinary protein, loss of renal function, excessive deposition of extracellular matrix proteins in the mesangium, and clear cytoplasm of the proximal tubular epithelial cells due to excessive reabsorbed glycogen (Armanni-Ebstein cells). In this study, the most significant change in the diabetic rats was a clear cytoplasm in the proximal tubular epithelial cells, a phenotype called Armanni-Ebstein cells. This clearing effect is a result of removal of the accumulated cytoplasmic glycogen particles resulted by the diabetic condition, during the staining procedure (21). The accumulation of glycogen occurred mainly in the proximal tubular epithelium. Tubular epithelial changes and the appearance of Armanni-Ebstein cells were reduced in the diabetic group treated with PCE. Glomerulosclerosis or increase in mesangial matrix was not detected in our study because of the limited 4-week duration of the experiment.

It has been reported that an increase in kidney weight in STZ-induced diabetic rats. It also has been hypothesized that this may be due to an increase in urinary output and renal blood flow (6). A similar finding was observed in this study, treatment with PCE leading to significant decreased relative kidney weight of the STZ-induced diabetic rats. Microalbuminuria or evident proteinuria is the result of histopathological changes within the kidneys. In this study, urinary total protein in the diabetic group treated with PCE was significantly lower than the diabetic control group. Furthermore, tubular epithelial changes, such as Armanni-Ebstein cells, was reduced in the diabetic group treated with PCE. These findings suggest that treatment with PCE can prevent the development of diabetic nephropathy by a reduction in renal damage through the restoration of enzymatic antioxidative defense system.

In conclusion, these results indicated that Phellodendri Cortex can prevent or retard the development of diabetic nephropathy via its beneficial effects for correcting the hyperglycemia, antioxidant enzyme system, renal dysfunction and protecting

against histopathological changes in the kidneys of diabetic rats.

MATERIALS AND METHODS

Phellodendri Cortex aqueous extraction

Phellodendri Cortex (the peel of *Phellodendron amurense* Ruprecht) was collected from the Yeongju region, Gyeongbuk, Korea and authenticated by a doctor of Oriental medicine Y.G. Choi, Department of Oriental Medicine, College of Oriental Medicine, Sangji University (Gangwon-Do, Korea). Three-hundred gram of Phellodendri Cortex with 5000 ml distilled water was boiled at 100°C for 2 hours in a heating extractor (COSMOS-660, Kyungseo Machine Co., Korea) and concentrated to 1500 ml. Thereafter, the aqueous extract was distributed into pouches containing daily volume each and stored at cold temperature. The calculated yield rate of the aqueous extract was 19% (w/w) using a lyophilization method.

Animals and experimental design

Male Sprague-Dawley rats with body weight ranging from 200 to 220 g were obtained from a supply company, Daehan Biolink (Chungbook, Korea). All animal experiments were performed in accordance with the Guide Lines for Institutional Animal Care and Use Protocol in Kyemyung University (Approval No. KMU IACUC 2006-012). Animals were housed in plastic cages at 22 ± 1°C, with a relative humidity of 55 ± 5%, an alternating 12-hour light-dark cycle, and were allowed to have access to their respective diets ad libitum. The animals were allowed to acclimatize to the laboratory environment for 7 days and then were randomly assigned to one of three groups (seven animals each), for the 4-week experiment. The experimental groups were as follows: Group I, non-diabetic control rats (NC); Group II, diabetic control rats (DC); Group III, diabetic rats fed Phellodendri Cortex extract (DP). For repeated oral administration, rats were treated once daily for 4 weeks. Group I and II received distilled water, Group III received the aqueous extract at 10 ml/kg BW/day (379 mg extracted powder of Phellodendri Cortex /kg BW/day). Body weight change and blood glucose level were monitored weekly between nine and ten o'clock in the morning. Daily food and water consumption were determined by subtracting left-over amount from the total amount provided. Organs were weighed using an electronic balance to calculate weight. Each one of the removed kidneys was stored at -80°C and another each of the ones was fixed in 10% formalin solution.

Experimental induction of diabetes

Diabetes was induced by a single intraperitoneal injection of STZ (70 mg/kg BW; Sigma, USA) in freshly prepared citrate buffer (0.4 M, pH 4.5) after an overnight fast. NC group animals were injected intraperitoneally with an equivalent amount of buffer (0.4 M citrate buffer, pH 4.5). Diabetic rats were confirmed by measuring the 4-h fasting blood glucose level from the tail vein at 72 h after injection with STZ. Animals with a blood glucose level above 300 mg/dl were considered to be diabetic and included in the experiment. Blood glucose levels were determined using the glucose oxidase method with a Gluco card II™ (ARKRAY, Japan).

Blood analytical measurement

At 28 days after commencing the experiment, 4-h fasting blood samples were collected from the aorta of the abdomen under ether anesthesia. The serum glucose, blood urea nitrogen (BUN) and creatinine levels were measured using a commercial available assay kit (EIKEN, Japan) with a Konelab 20XT Analysis System (Thermo, Finland) by the method of Brandstrup *et al*. (22), Patton and Crouch (23), Fossati *et al*. (24), respectively. Serum insulin was measured using a rat insulin ELISA kit (Mercodia, USA) with a microplate reader (Molecular Devices, USA).

Urine analytical measurement

At 25 days after commencing the experiment, the animals were transferred individually to metabolic cages and urine was collected for 24 h. Urinary creatinine was estimated using a commercially available assay kit (EIKEN, Japan) with a Konelab 20XT Analysis System (Thermo, Finland). The urinary total protein was estimated by the pryogallol red method using a commercial available assay kit (Auto kit Micro TP, Wako Pure Chemical, Japan) with a ADVIA 1650 Analysis System (BAYER, USA). The urinary total protein content was calibrated to the urinary volume (urinary protein/urinary volume). The creatinine clearance (CCr) was calculated according to the following formula: (urine creatinine/serum creatinine) × (total volume/24 h × 60 min).

Antioxidant enzyme activities

Kidney tissues were homogenized in 0.25 M sucrose solution using a tissue homogenizer with a Teflon pestle at 4°C to give 20% homogenate (w/v). Homogenates were centrifuged at 600 × g for 10 minutes to remove any cell debris and supernatants were further centrifuged at 10000 × g for 20 minutes to remove the mitochondria pellets. Finally, the supernatants were ultracentrifuged at 105000 × g for 60 minutes to obtain the cytosol supernatants. Protein amounts in the mitochondrial and cytosolic fractions were measured by the method of Lowry *et al*. (25) with bovine serum albumin as the standard. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and xanthine oxidase (XO) were measured by the methods of Martin *et al*. (26), Aebi (27), Habig *et al*. (28) and Stripe and Della (29), respectively.

Histological analysis

Kidney tissues were routinely fixed in 10% formalin solution and the paraffin-embedded 2 μm sections were stained with periodic acid-Schiff (PAS). Samples were observed under Olympus BX50 light microscope equipped with a Polaroid DMCIe digital camera system (Polaroid, USA). The Armanni-Ebstein cells in the cortex and cortico-medullar junction area were quantified by the following method. Four images were taken by 80i microscope (Nikon) with a ProGres C14 Digital Camera (Germany)

from each animal. Armanni-Ebstein cells per tubule were counted at × 400 magnification.

Statistical analysis

Values are presented as means ± SD of 7 rats in each group. Data were analyzed by Student's t-test using SPSS-12.0. The limit of statistical significance was set at $P < 0.05$.

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