# *Original Articles*

## Effect of puerarin on the expression of extracellular matrix in rats with streptozotocin-induced diabetic nephropathy

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**ABSTRACT**

*Background.* Inhibition of the formation of advanced glycation end-products delays the development of diabetic nephropathy. Puerarin decreases the formation of these products. We studied the effect of puerarin in a rat model of diabetic nephropathy.

*Methods.* Three groups of rats were studied: a control group, a diabetic group in whom diabetic nephropathy was induced by intraperitoneal injection of streptozotocin, and a puerarin group in which diabetic rats were treated with puerarin. During and after treatment, measurements were made on the rats' general status, blood glucose level, blood urea nitrogen, serum creatinine, creatinine clearance rate and urinary albumin excretion over 24 hours. The expression of collagen I and heparan sulphate proteoglycan in the extracellular matrix of the glomerulus was assessed by immunohistochemistry.

*Results.* Rats in the puerarin group had a better general condition than those with diabetes. They also had lower blood urea nitrogen, serum creatinine and urinary albumin excretion rate over 24 hours compared with those in the diabetic group. The creatinine clearance and expression of heparan sulphate proteoglycan in the kidney also increased significantly in the puerarin group compared with that in the diabetic group.

*Conclusion.* Puerarin seems to have certain protective effects on diabetic nephropathy induced by streptozotocin. This is caused possibly by regulating the expression of glomerular extracellular matrix.

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INTRODUCTION

The extracellular matrix is located in the renal glomerulus and is an important part of the glomerular basement membrane. Collagen I (CI) is closely associated with renal fibrosis of diabetes.[1] Heparan sulphate proteoglycan (HSPG) has been shown to play a key role in maintaining the glomerular charge barrier;[2] hence, together with CI it has a place in the study of diabetic nephropathy.[3,4] There is increasing awareness of the role of advanced glycation end-products (AGEs) and oxidase stress in the occurrence of renal lesions of diabetic nephropathy.[5,6] In animal experiments, aminoguandine hydrochloride has been shown to inhibit the formation of AGEs, which in turn delays the development of diabetic nephropathy.[7] However, this has not been used in clinical practice because of its severe adverse effects. Therefore, a safer product with a similar effect as aminoguandine will be useful for people with diabetic nephropathy.

Natural products used in Chinese herbal medicine such as puerarin may be useful. Puerarin can be extracted from the root of kudzu, *Pueraria lobota*.[8] It has been reported that puerarin can decrease the production of AGEs.[9] We studied the mechanism of action of puerarin in an animal model of diabetic nephropathy by studying its effect on the expression of CI and HSPG in the extracellular matrix of the glomerulus.

METHODS

*Experimental animals*

Forty, 2-month-old Sprague Dawley (SD) male rats, weighing 200–250 g were used. The animals were provided by the Animal Department, Hunan Agricultural University. During the experiments, the rats were allowed free access to water and mixed feed provided by the animal centre of Central South University.

*Instruments and reagents*

Puerarin (brown-yellow powder, 90% pure) was provided by Hunan Kinglong Bioresource Company; S-P immunohistochemical staining kit, rabbit anti-rat CI antibody and rabbit anti-rat HSPG polyose antibody were provided by Beijing Bio-Lab Materials Institute; Streptozotocin (STZ) was provided by Sigma Co., USA. A Hitachi 7170 fully automatic biochemical analyser, high-speed handle glucose analyser and FJ-2008 immune cell counter were used for analysis.

*Experimental procedure*

After being fed for 1 week, 30 rats, fasting for 12 hours, were injected with STZ 60 mg/kg. After 72 hours, the blood glucose of the rats was measured by a Yicheng blood glucose analyser. If the

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blood glucose was ≥16.7 mmol/L, the rat was considered to have diabetes.

Of the 30 rats, 20 developed diabetes and were divided into 2 groups: the diabetes group and the puerarin group. Rats included in the puerarin group were injected with the drug in a dose of 80 mg/kg/day and were followed up for 16 weeks. To avoid ketosis and prevent mortality in rats with diabetes, rats in the diabetes and puerarin groups were given 2–4 units of long-acting insulin to maintain the blood glucose level around 25 mmol/L. Rats in the normal control group received an equal volume of sodium citrate buffer.

*General observation of the rats*

The following were measured and recorded daily for 16 weeks: Drinking water, diet, activity, urine volume and body weight.

*Sample collection*

Urine was collected for 24 hours by metabolic cage to measure the urinary albumin excretion in different groups after 10 weeks. The rats were weighed after being anaesthetized. A total of 5 ml of whole blood was obtained from the femoral artery to measure the blood glucose, blood urea nitrogen and serum creatinine and to calculate the creatinine clearance. A vertical incision was made on the back of the rat to remove the left kidney which was weighed and then washed with saline repeatedly until it appeared white. After removing the capsule of the left kidney, 2 blocks of renal tissue about 0.5 cmm were obtained and fixed in para-formaldehyde (quality score of para-formaldehyde in the phosphate buffer solution was 0.04) for 12 hours. Sections of 4 μ thickness were prepared and used for haemotoxylin and eosin (HE) and immunohistochemical staining.

*Urinary albumin excretion.* The 24-hour urine was collected. After mixing, 2 ml of urine was taken for detection of albumin by immune cell counter. Glomerular filtration rate was represented by clearance rate of creatinine.

Antibodies for CI and HSPG were obtained for immunohistochemistry which was performed as per the manufacturer's instructions.

*Image analysis of immunohistochemistry*

The microscope image processing system of the computer was used to choose 10-view areas at random in each image slice under a magnification of 400 times. Grey scanning was applied to the result of immunohistochemistry signals, with the average relative grey value as the value of expression vectors (mean [SD]). The result was identified by the colour seen under the microscope:

| | |
|---|---|
| Negative: | No yellow |
| Weak positive: | Light yellow |
| Positive: | Yellow |
| Strong positive: | Deep yellow or tawny |

*Statistical analysis*

Differences between groups were evaluated by ANOVA followed by independent *t*-test. All data are expressed as mean (SD) and all analyses were done using SPSS 10.0. A p value <0.05 was considered statistically significant.

## RESULTS

*General observation of rats during the experiment*

*Control group:* These rats showed increased body weight, good spirit, glossy fur, flexible and sensitive reaction.

*Diabetic group:* These rats showed gradual sagging of spirits, emaciation, were drinking more water and had a higher urine output requiring the mat to be changed 1–2 times every day. There was more movement in the early period, and laggard and slow reaction later. The eyesight of 1 rat was diminished, 2 rats had a pearl eye and 1 rat had repeated episodes of diarrhoea.

*Puerarin group:* These rats were in good spirits, did not have a pearl eye or necrotic tail and foot. However, their reaction was poorer than that of the control group of rats.

*Effect of puerarin on various parameters*

The body weight of the diabetic rats was reduced significantly compared with the control group, while the urine volume and blood glucose increased significantly ($p<0.05$). After treatment with puerarin, the body weight, kidney weight/body weight rose significantly compared with the diabetic group ($p<0.05$, Table I). The urine volume was also reduced though the blood glucose levels were not altered.

Compared with the control group, the urinary albumin excretion rate, serum creatinine and blood urea nitrogen of the diabetic rats increased significantly, and the creatinine clearance rate decreased ($p<0.05$). When the diabetic and the puerarin groups were compared, the urinary albumin excretion rate, serum creatinine and blood urea nitrogen decreased significantly, and the creatinine clearance rate increased ($p<0.05$, Table II).

*Effect of puerarin on CI, HSPG expression and serum glycosylated haemoglobin*

The diabetic group showed changes in renal histology typical of diabetic nephropathy including glomerular cell proliferation, swelling of the renal tubules and renal basement membrane thickening. These were relieved to some extent in the puerarin-treated group.

The results of immunohistochemistry showed that in the diabetic group as compared with controls the renal glomerulus staining of CI was increased. Brown yellow granules were seen on the mesangial cells, vascular endothelial cells and epithelial cells of renal tubules, the colour deepened and the quantity increased. HSPG expression was decreased in the diabetic group. Quantification showed that the differences were statistically significant. These changes were reversed significantly in the puerarin-treated group ($p<0.05$, Table III and Figs 1 and 2).

TABLE I. Comparison of body weight, urine volume and blood glucose in the three groups of rats

| Group | *n* | Urine volume (ml) | Body weight (g) | Kidney:body weight ratio ($\times 10^{-3}$) | Blood glucose (mmol/L) |
|---|---|---|---|---|---|
| Control | 10 | 17.86 (5.94) | 314.75 (19.63) | 3.54 (0.93) | 5.25 (0.33) |
| Diabetic | 10 | 159.41 (17.61)* | 161.55 (15.78)* | 5.74 (1.42)* | 25.63 (2.58)* |
| Puerarin | 10 | 100.24 (18.88)*† | 250.41 (16.90)*† | 4.65 (1.23)*† | 26.98 (2.81)* |

All values are mean (SD) * $p<0.05$ *v.* control group † $p<0.05$ *v.* diabetic group

TABLE II. Changes in renal function in the three groups of rats

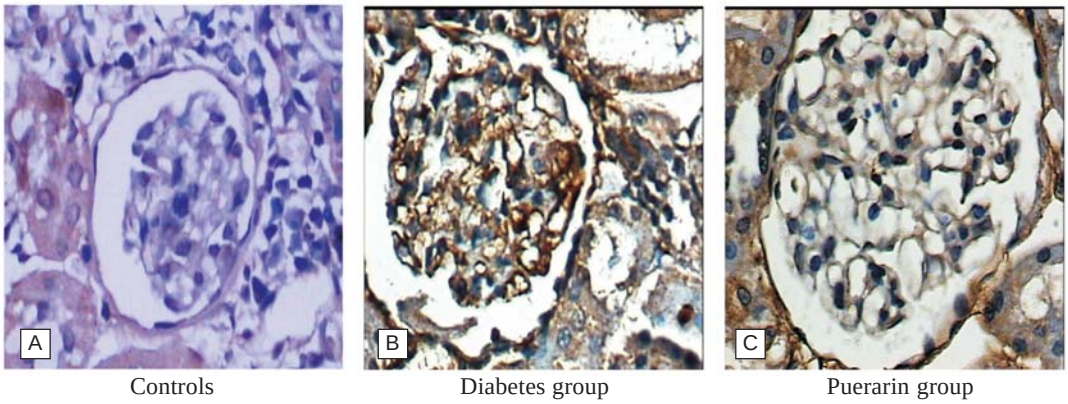
| Group | *n* | Urine albumin (mg/24-hour×$10^{-3}$) | Serum creatinine (mmol/L) | Creatinine clearance (ml/minute) | Blood urea nitrogen (mmol/L) |
|---|---|---|---|---|---|
| Control | 10 | 3.86 (0.79) | 27.11 (5.81) | 0.92 (0.35) | 3.25 (0.33) |
| Diabetic | 10 | 40.94 (5.40)* | 97.83 (11.62)* | 0.19 (0.03)* | 10.25 (0.28)* |
| Puerarin | 10 | 17.24 (1.32)*† | 70.81 (9.46)*† | 0.61 (0.24)*† | 7.18 (0.67)*† |

All values are mean (SD) * p<0.05 *v.* control group † p<0.05 *v.* diabetic group

TABLE III. Changes in immunohistochemistry optical density of collagen I, heparan sulphate proteoglycan (HSPG) and serum glycosylated haemoglobin (%) in the three groups of rats

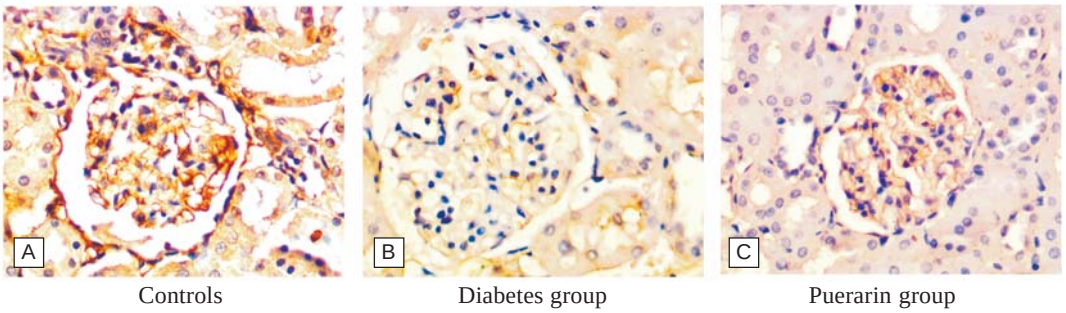
| Group | *n* | Collagen I | HSPG | Glycosylated haemoglobin |
|---|---|---|---|---|
| Control | 10 | 64.05 (9.56) | 159.84 (22.08)* | 3.97 (1.37) |
| Diabetic | 10 | 195.60 (12.66)* | 54.89 (7.07)* | 14.66 (4.31) |
| Puerarin | 10 | 98.61 (11.44)*† | 141.75 (24.94)*† | 8.14 (2.03)*† |

All values are mean (SD) * p<0.05 *v* control group † p<0.05 *v* diabetic group




Controls Diabetes group Puerarin group

FIG. 1 Immunohistochemistry analysis for collagen I expression in glomeruli of rats (×400)




Controls Diabetes group Puerarin group

FIG. 2 Immunohistochemistry analysis for HSPG expression in glomeruli of rats (×400)

## DISCUSSION

The role of AGEs in the induction of diabetic nephropathy is well known. Urinary albumin excretion is a key diagnostic feature of diabetic nephropathy.[10] It has been shown that puerarin can significantly reduce the levels of urinary albumin excretion in rats with diabetes. In puerarin-treated rats, there was concomitant reduction of serum creatinine and blood urea nitrogen as well as glycosylated haemoglobin, though there was no reduction in blood glucose. The glycosylated haemoglobin level was taken as a key indicator of AGEs, which in turn were critical for endothelial damage in diabetic nephropathy.[11,12] The important histopathological features of renal damage in diabetic nephropathy[13] were also reversed to some extent in the puerarin group.

The heparan sulphate side chain of HSPG is charged and is a major component of the charge selective barrier of the renal glomerulus. We suggest that the loss of heparan sulphate protein from the renal glomerular basement membrane in patients with diabetic nephropathy is an important cause of urinary albumin excretion. CI is an important vascular extracellular matrix protein and a key role in maintaining the integration, viscosity and permeability of vascular walls. It could promote the aggregation of thrombocytes after endothelial cell damage.[14] Since the extracellular matrix is affected in diabetic nephropathy,[15] it is

influenced by high blood glucose levels and consequent non-enzymatic glycosylation of proteins and nucleic acids, forming AGEs[16,17] leading to oxidative cell-induced cellular changes and other effects. Oxidative stress might in turn promote the formation of AGEs, and both[18,19] might cooperate with each other to participate in the pathogenic process of diabetic nephropathy.

Reversing changes in the extracellular matrix might play a key role in delaying the occurrence and progression of diabetic nephropathy.[20] Animal experiments[21] have shown that aminoguanidine could downregulate the expression of CI and CII genes and fibronectin in renal tissue. A study further showed that puerarin had a similar role as aminoguanidine in inhibiting the formation of AGEs in rats with diabetic nephropathy.[22] A pharmacological study showed that puerarin could reduce the viscosity of blood, relieve blood vessel spasm, reduce blood lipid, improve blood flow in the ischaemic area of the renal glomerulus, promote the function of endothelial cells, reduce the oxidative stress and block AGEs.[23] Our study demonstrates its effects on key glomerular proteins.

## ACKNOWLEDGEMENT

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