Original Article
Changes of urinary angiotensinogen concentration and its association with urinary proteins in diabetic rats

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Abstract: Objective: It had been reported that angiotensinogen might be a marker for activation of renin-angiotensin system, which was associated with the development of diabetic nephropathy. The purpose of this study was to investigate the functional roles of AGT in DN in vitro. Methods: Diabetic rat models were built by single intraperitoneal injection of streptozotocin. The diabetic rats were divided into three groups, two of the three groups were treated with different doses of losartan, the other diabetic group was as control and normal rats acted as healthy control. In a 12-week investigation, we detected the changes of AGT in all rats' blood and urine and the association between AGT concentration and RAS activation and urinary proteins were analyzed in this study. Results: The serum AGT of rats had no significant differences (P>0.05 for all). The urinary AGT of the diabetic rats was significantly different from the control group, moreover, the urinary AGT of the diabetic rats under different treatments was also obviously different (P<0.05 for all). Besides, the results of immunohistochemical assay indicated that AGT expression level was correlated with renal tissues damage. The level of AGT was positively associated with urinary protein (r=0.493, P<0.01) and negatively correlated with CCr (r=-0.474, P=0.007) and the dose of ARB (r=-0.575, P=0.001). Moreover, the dose of ARB was independently associated with urinary AGT (B=-2.963, P=0.024) in diabetic rats. Conclusion: Urinary AGT may be a marker for the activation of local RAS in kidney and independently associated with ARB.

Keywords: AGT, RAS, diabetic nephropathy, diabetic rat

Introduction

Diabetic nephropathy (DN), one of the chronic complications of diabetes, is the main reason of end-stage renal disease (ESRD). In the western countries, DN is the leading reason for ESRD, moreover, the prevalence is increasing year by year [1, 2]. It has been reported that approximately one third to half of patients with diabetes develops renal manifestations and the prevalence is increasing [3, 4]. Therefore, investigating the pathogenesis of DN might supply effective ways for prevention and treatments of diabetes.

Recently, it has been reported that glucose metabolism dysfunction and the changes of renal hemodynamics contribute to the pathogenesis of DN, as well as abnormal expressed cyctokineses, genetic predisposition and oxidative stress factors [5, 6]. However, renin-angiotensin system (RAS) involves in almost patho-

genesis, especially the renal RAS [7]. The main elements of RAS includes AGT, renin, angiotension I (Ang I), angiotension (Ang II), angiotensin converting enzyme (ACE) and Ang II type 1 receptor (AT1) [8]. It has been reported that renal RAS is the key factor for DN occurrence and development. Angiotensin II (Ang II) is the most active molecule of RAS, but it can freely through the glomerular filtration barrier due to small molecular weight. So urinary Ang II is not only from the local formation of kidney, but also from the circulation. Therefore, the Ang II in urine does not accurately reflect the renal RAS activation. Angiotensinogen (AGT) is the only substrate for renin and the root of RAS. The level of AGT in human and rats is close to the Km value of renin [9], so the changes of its concentration can directly affect the activity of RAS. AGT with big molecular weight dose not freely through the glomerular filtration barrier. Some reported had indicated that urinary AGT might be a useful biomarker for activation of

RAS in DN [10-12]. Therefore, AGT may help improve the treatments for patients with diabetes.

In this study, we investigated the changes and functional roles of AGT in diabetes via diabetic models, in order to improve the prevention and treatments of DN.

Materials and methods

Main agents and equipments

The metabolism cage of rats was bought from Suzhou animal experiment instrument factory, China. Automatic biochemical analyzer (7600-020) was from Hitachi, Japan. The optical microscope (CH20-BIM) and fully automatic digital microscope camera system were purchased from Olympus Corporation, Japan. Electronic analytical balance (BS210S) and the balance for weigh were separately from Sartorius Company, Germany and Shanghai Qihong instrument and apparatus company, China. High-speed centrifuge at low temperature (GRX-299) was bought from TOMY Company, USA. Ultrapure water device (Milli-Q Plus) was from Millipore Company, USA. Electric-heated thermostatic water bath was purchased from Beijing Changfeng instrument company, China. Ultraviolet spectrophotometer was from Shimadzu, Australian. Enzyme-linked immunometric meter was bought from Biotek, USA. The automation-tissue-dehydrating machine, embedding center and histotome were purchased from Leica, Germany. Pipettes were from Gilson, France. Computer automatic blood pressure meter (Bp-98A) was obtained from Softron Co. Ltd, China. AGT ELISA kit was bought from IBL, Japan. Losartan and Streptozotocin (STZ) were purchased from Hangzhou MSD pharmaceutical Co. Ltd, China and Sigma, USA. Sodium citrate and PBS were from Shanghai Jiahe biotechnology co. Ltd, China. Methyl aldehyde was from Tianjin Guangfu chemical industry research institute, China.

Animals and feeding

43 adult male rats with the SD stage were purchased from Department of Laboratory Animal Science in Beijing University at the age of 8 weeks. The average weight of the rats was 250±2 g. 3-4 rats were housed in a metabolic cage and the housing conditions were standardized (25±3°C, 55±15% relative humidity, 12 h: 12 h light-dark cycle). All the rats were free access to water and food, which treated with sterilization. The rats chow was supplied by Department of Laboratory Animal Science in Beijing University.

Diabetic rat's preparation and grouping

The blood glucose and urine glucose of the 43 rats were normal before the study. After fed adaptively for 3 days, 33 rats were treated with Streptozotocin (STZ) to induce experimental diabetes mellitus. STZ dissolved in newly prepared 0.1 mM citric acid buffer (pH4.5) with the end concentration of 1% (10 g/L). Without being fed for 12 hours, the rats in diabetic groups were injected with 60 mg/kg of STZ, while the control group was injected with the same dosage of citric acid buffer. The glucose of serum collected from the rats tails was detected by a glucometer and the concentration more than 16.7 mM was considered as diabetic models [13]. The results suggested that 96.97% (32/33) rats were successfully induced by STZ which enrolled in the diabetic group (the one which unable to be induced would not be used in the following study). All the rats in this study were divided into four groups: group A with 10 diabetic rats without treated, group B with 11 rats treated with low doses of losartan, group C with 11 rats treated with large doses of losartan and group D with 10 normal rats for control. The rats in group B and C were administered with 5 mg/kg.d and 20 mg/kg.d losartan by gastric perfusion once a day, separately [14], while the group A and D were treated with the same doses of sterile normal saline. None of the group had received insulin or other drugs treatments. After investigation for 12 weeks, 41 rats finished the experiments and one of the rats in group B was death. During the investigation, the blood pressure and weight of the rats were detected every 2 weeks. Blood glucose was detected every 6 weeks and 24 h urine was collected for analysis, using metabolism cage.

Rats were narcotized by intraperitoneal injection of chloral hydrate (0.3 mL/100 mg) and blood were drawn from abdominal aorta. The rats were sacrificed and the heart, liver and kidney were used for pathological examination.

Urinary specimen collection

24 hours urinary samples were collected on 0, 6th and 12th week from every group of rats in metabolism cage. After weighting, the urinary specimens were centrifuged at 2,200 rpm for 15 min and 4 mL supernatant was collected and stored in -20°C, in order to detect urinary proteins (UP), creatinine (Cr) and AGT.

Serum specimen collection

After investigating for 6 weeks, 2 mL blood sample was collected from the epicanthus of each rat. On 12th week, rats were narcotized by intraperitoneal injection of chloral hydrate (0.3 mL/100 mg) and 6 mL blood was collected from abdominal aorta. Serum was separated from the collected blood sample and kept in -20°C, used for biochemical index analysis. In addition, 2 mL blood was drawn and kept in a tube with 30 μL 10% EDTA and 40 μL apotinin, centrifuged at 2,200 rpm for 10 min. The plasma was separated and stored in -20°C to detect the activity of plasma renin. Beijing north biotechnology research institute gave assistance to detect plasma renin activity and Ang II via radio immunoassay.

Blood pressure detection

Standard tail-cuff method was used to measure the caudal artery aystolic blood pressure of rats on 0, 1st, 4th, 8th, 10th and 12th week via computer automatic blood pressure meter (Bp-98A Softron Co. Ltd). Every detection was repeated six times and the mean value was used for analysis.

Blood and urinary AGT detection

The human AGT ELISA kit (IBL, JP) was used to detect urinary AGT and the method was as followed: (1) The agents and standard substance were prepared according to manufacturer's introduction. The assay is performed at room temperature. (2) ELISA diluent was used to dilute blood and urine at 1:6400 and 1:16 separately. (3) The wells were added with 100 μL standard substance or dilute sample. The wells contained with 100 μL ELISA diluent acted as control. (4) The plate was incubated for 60 min at 37°C. (5) Wash 7 times with 300 μL washing buffer per well and add 100 μL biotinylated human Angiotensin II antibody to each well. (6)

The plate was incubated for 60 min at 37°C. (7) Wash 9 times with 300 washing buffer per well and add 100 μL TMB color agent. (8) After being incubated for 30 min without light at room temperature, each well was added 100 μL stop solution. (9) Read the absorbance on a microplate reader at a wavelength of 450 nm. The concentration-OD standard curve was drawn according to the concentration and absorbance of standard substance, then the concentration of the samples were read from the curve.

Kidney pathological examination

Kidney tissues: Abdominal incision was used to separate bilateral kidney, the right kidney was snap-frozen in liquid nitrogen immediately and stored in -80°C until used, while the left kidney was washed with ice-saline and dried to weight. The tissues specimen of left kidney was fixed in 10% buffered neutral formalin.

Paraffin section preparation: ① Immobilization: The kidney tissues specimens were fixed in over 20 times than the tissues 10% buffered neutral formalin. ② Dehydration: The tissues specimens were dehydrated with 85% alcohol and 95% alcohol for 30 min, separately. Then anhydrous alcohol was used for dehydrating for 20 min and repeated for twice. ③ Clearing: The specimens were treated with xylene for 10 min and repeated twice until the specimens was clear. ④ Wax immersion: The tissues were wrapped in wiping paper according to the number and putted in the embedding box for wax immersion. The specimens were paraffined in Paraffin cylinder I, II and III for 30 min separately. ⑤ Embedding: The paraffined tissues were putted in the embedding box with molten paraffin. The progression was performed in a 70°C embedding instrument. ⑥ Section: The paraffin-embedded tissues were cut into 4 μm slices. The slices were put into glass slides with glue in a exhibition pieces box at 42°C. After slightly dry in the air, the glass slides were put into a oven at 60°C overnight. The preparative sections were stored at dry environment without light for pathomorphological and immunohistochemical analysis. ⑦ Dewaxing: The specimens used for staining were needed dewaxed. The approaches were as followed: xylene I for 15 min; xylene II for 15 min (crystal clear); absolute alcohol for 5 min; 95% alcohol for 5 min; 80% alcohol for 5 min;

50% alcohol for 5 min; water supply washed for 2 min.

Haematoxylin-eosin staining (HE): The HE staining was carried out as the following steps: The haematoxylin was used for dying nuclear for 3 min, washed with supply water for 1 min, 1% hydrochloric acid-alcohol differentiated for a few seconds, supply water was for washing for 1 min, then eosin dyed 10 min; supply water washed for 1 min, then dehydrated by gradient alcohol (85% alcohol for 20 seconds, 90% alcohol for 20 seconds, 95% alcohol for 20 seconds, absolute alcohol for 10 min), transparent with xylene for 2 min and cemented with neutral balsam.

Periodic Acid-Schiff stain (PAS): 1% periodic acid stained for 15 min, supply water washed for 2 min, Schiff was for 10 min, then the specimens were washed with supply water for 10 min, dyed with haematoxylin for 3 min, washed with supply water for 10 min, and 60°C distilled water for 15 min. The xylene was used for making the specimens clear for 2 min and neutral balsam was used for cementing. The basement membrane of glomerular basement, renal tubular and kidney blood capillary were dyed with red.

Histopathologic diagnosis: The histopathological diagnosis were made by two trained clinical physicians with reference to the WHO (1995) glomerular disease type organization credits revision plan, combining with the characteristics of clinical data, laboratory examination, immune pathological and the ultrastructural changes, discussing with clinician.

Immunohistochemistry analysis

(1) The sections were dewaxed with xylene following the conventional procedure. (2) 3% H_2O_2 was added and incubated for 10 min at 37°C to eliminate endogenous peroxidase activity. After being washed with supply water and distilled water, PBS buffer was used for washing for 3 times and 3 min for each time. (3) The sections were then reparation by microwave at 92-98°C for 10-15 min. (4) Sealing agent was added into the sections and incubated for 20 min at 37°C. (5) After removing sealing agent, suitable concentration of primary antibody was added and incubated for 45 min at room temperature. (6) PBS buffer was used for washing for 3 times, 3

min each time. (7) Appropriated concentration of second antibody was added and incubated for 30 min at 37°C. (8) Step 6 was repeated. (9) 0.04% DAB was used for coloration and the time for color developing was 5-10 min (kidney tissues were colored with clay bank) and the coloration was finished with supply water for 3 min. (10) The sections were stained by hematoxylin for 3 min and finished with supply water at room temperature. (11) The sections were dehydrated by alcohol, cleared with xylene and mounted with neutral gums.

Statistical analysis

SPSS 15.0 software was used for statistical analysis. The data were as mean ± SD. The significant differences between two groups were analyzed by student's t test and the association between two variables was analyzed by linear correlation analysis. Multiple factors regression analysis was using regression method of stepwise regression equation. The differences between multiple groups were detected by one-way -ANOVA test. $P<0.05$ was considered as statistical significance.

Results

General data

During the 12-week investigation, the rats in diabetic groups (group A, B, C) were angular, depressed, dull in response, lumbering and curled body with vertical hair without gloss, while the growth conditions of the control rats (group D) were well.

The results of serum glucose changes were shown in **Figure 1** and from the figure, we found that there were no significance differences between fasting blood-glucose in different groups ($P>0.05$). On the tine of 1, 6, 12 week, the fasting blood-glucose of the rats in group A, B and C was significantly increased, compared with the start time ($P<0.001$). The fasting blood-glucose of group A, B, C was higher than that in group D at the same time ($P<0.001$), while there were no significant difference between group A, B, C at the same time ($P>0.05$).

The weight detection results were shown in **Figure 2**. The weight of rats in control group was significantly higher than that in the diabetic
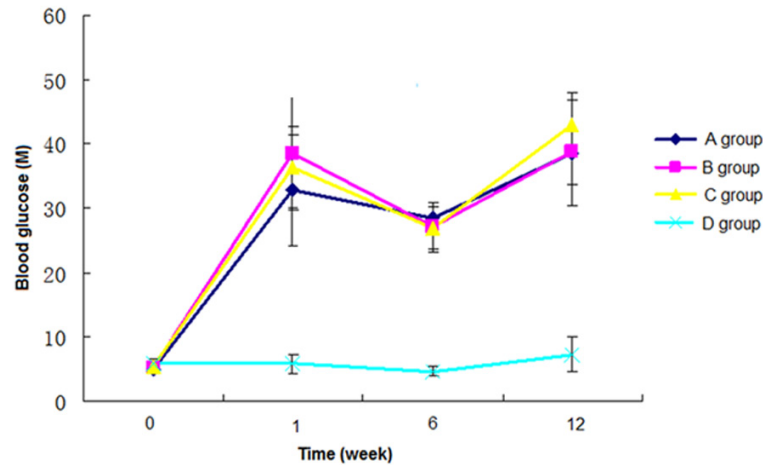
Figure 1. The changes of serum glucose. **$P<0.001$, Diabetic groups (A, B, C) vs. healthy control (D).
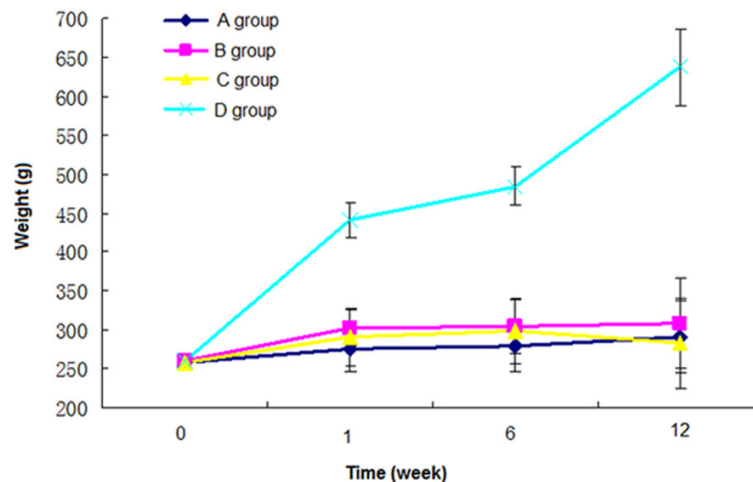
Figure 2. Changes of weight. **$P<0.001$, Diabetic groups (A, B, C) vs. healthy control (D) at the same time and comparison within (D) group at different time.

ously increased, the basement membrane of glomerulus incrassated, glomerular mesangial broadened, a few of glomeruluses appeared mild sclerosis, the renal tubular cells enlarged, vacuoles degenerated, the tube was narrowed, the hyaline degeneration occurred in renal interstitial small blood vessels and fibrosis was unclear. The pathological changes in group B were subdued, compared with group A and the pathological changes in the group C were weaker than that in group B. The results were shown in **Figure 3**.

Blood pressure of rats

The results of blood pressure analysis were shown in **Figure 4**. From the figure, we found that blood pressure of all the rats was similar at the beginning of the study ($P>0.05$). At 1 week, the blood pressure of all the rats was significantly increased, compared with the beginning ($P<0.05$). At 4, 8, 10, 12 week, the blood pressure was decreased and there were no significant difference from the beginning. However, the blood pressure of rats in group C was significant decreased at 4 week and the status lasted to the end of the investigation, which resulted in the significant deceased compared with group A, B or C ($P<0.001$).

group (group A, B and C) at the same time ($P<0.001$). Moreover, the weight of rats in control group was increased during the investigation, compared with that in the diabetic group ($P<0.001$). There were no significant differences between the diabetic groups at the same time ($P>0.05$) or in the group at different times ($P>0.05$).

Kidney tissues pathological changes

There were no obviously abnormal changes were seen in glomerular, renal tubule and interstitial of the control rats. The kidney tissues changed a lot compared with control group and the changes were as followed: glomerullar obvi-

CCr analysis

The results of CCr detection were shown in **Figure 5**. The CCr of rats in group D was significantly increased over time ($P<0.001$). The CCr status of group A was significantly lower than that of group D ($P<0.001$). The levels of group B and C were between group D and A. There were no significant differences between group B, C and D or A at 6 week, while at 12 week, group B was significantly lower than group D and group C, higher than group A ($P<0.01$ for all).
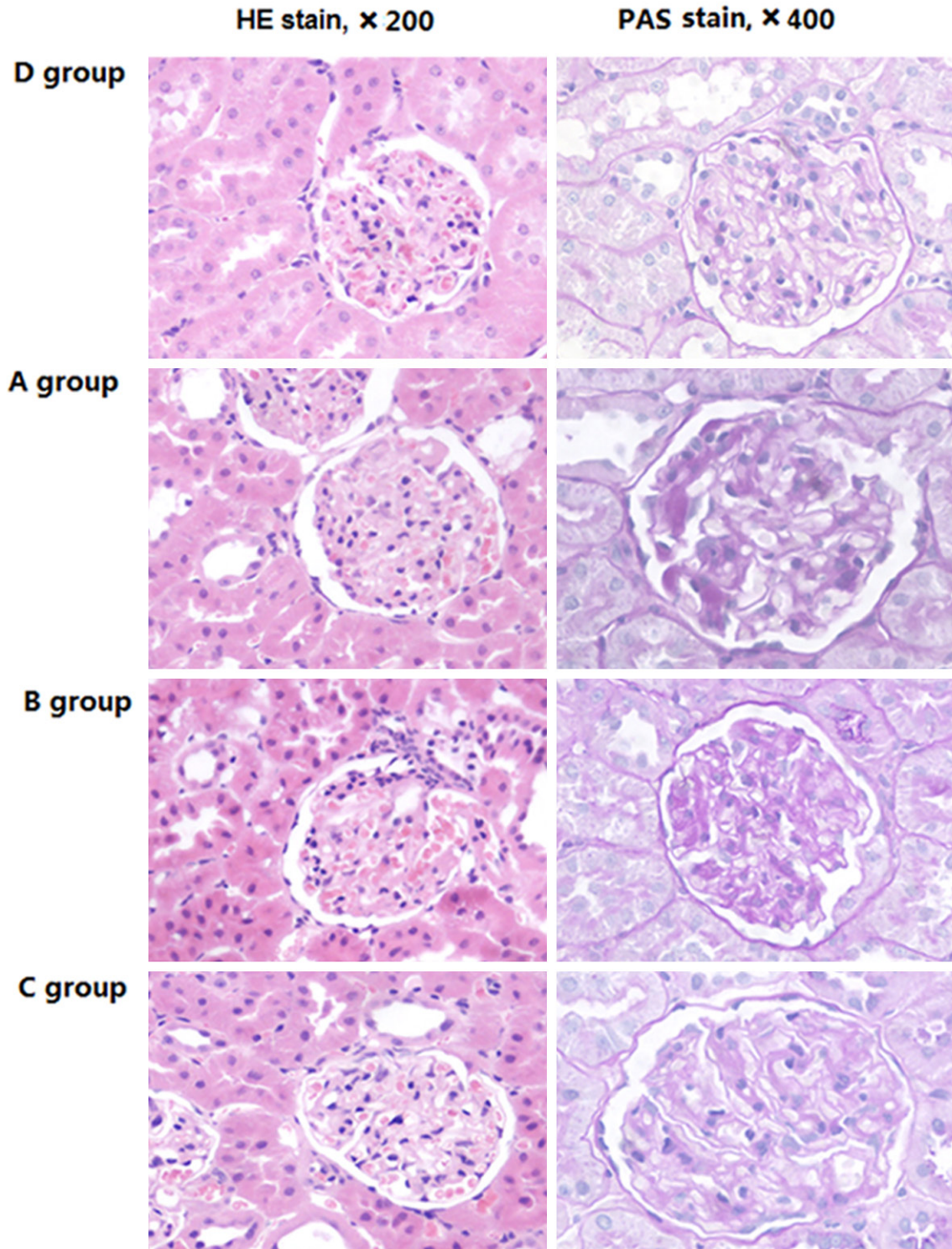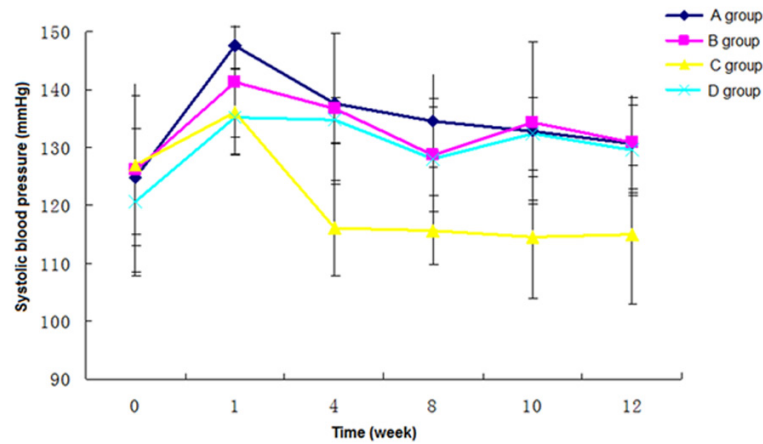
**Figure 3.** The pathological changes of kidney.

UP/Cr assay

The value of UP/Cr in group D was stabilized during the investigation ($P>0.05$, **Figure 6**). The

level in group A was increased, compared with the beginning ($P=0.078$ for 6 week; $P=0.025$ for 12 week). There were significant differences between group B, C and A at 6 week ($P=0.029$
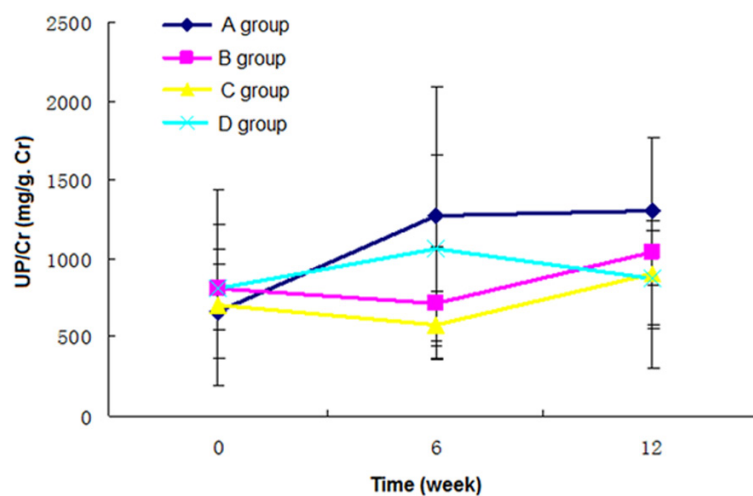
Figure 4. The changes of blood pressure. *$P<0.01$, Diabetic groups (A, B, C) vs. healthy control (D) at the same time.
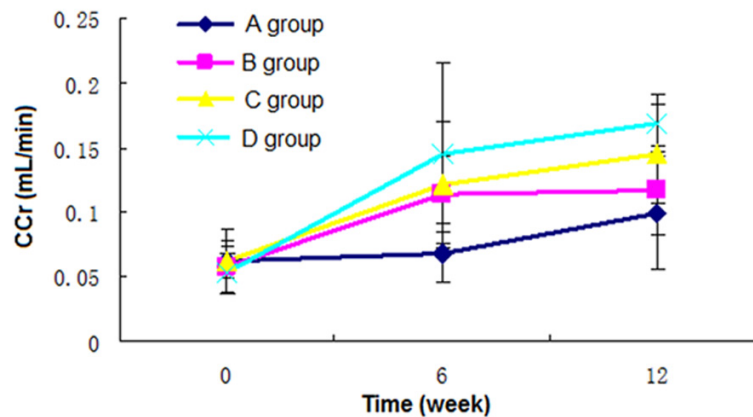
Figure 5. The changes of CCr *$P<0.01$, A group vs. C, B, D group on 12 week; **$P<0.001$, Comparison in D group at different time or A group vs. D group at the same time.

Figure 6. The changes of UP/Cr. ▼$P<0.05$, *$P<0.01$.

for group B and A; $P=0.006$ for group C and A).

Serum RAS components analysis

The serum AGT concentration had no significant difference between different groups at the same time or in the same group at different time (**Figure 7**).

The serum Ang II of rats in group A and D was stabilized ($P>0.05$). With the developing of the investigation, the level of group B and C increased significantly, compared with group A and D or the beginning of the study ($P<0.05$ for all), but there were no obvious difference between group B and C (**Figure 8**).

Urine RAS components analysis

With the progression of the experiment, the value of UAGT/Cr in group D did not changed and the data in group A was increasing ($P<0.05$). Besides, the data of group B and C were higher than that in group D ($P>0.05$), lower than group A ($P<0.05$ for all). There were no significant difference between group B and C ($P>0.05$), but at 12 week the data in group C was significantly lower than that in group B ($P<0.05$, **Figure 9**).

The results of Ang II/Cr analysis were shown in **Figure 10**. From the figure, we found that the level of control group was stabilized at different time ($P>0.05$), while the level in group A increased significantly ($P<0.05$ for all). The value of group B was higher than that in the control group ($P<0.05$) and not obviously different
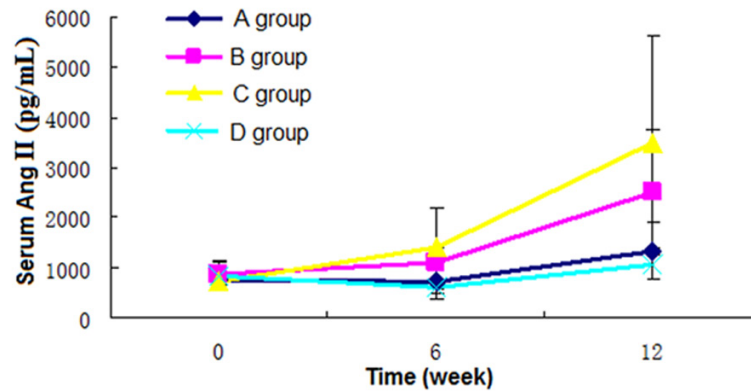
Figure 7. The changes of serum AGT concentration.
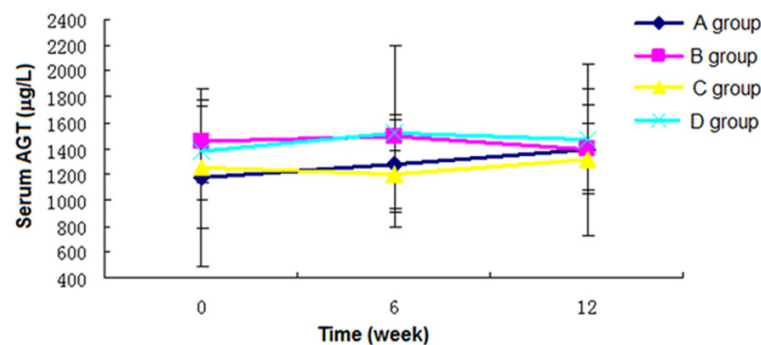
Figure 8. The changes of serum Ang II in all the rats during 12-week investigation. ▼$P<0.05$, *$P<0.05$, **$P<0.001$.
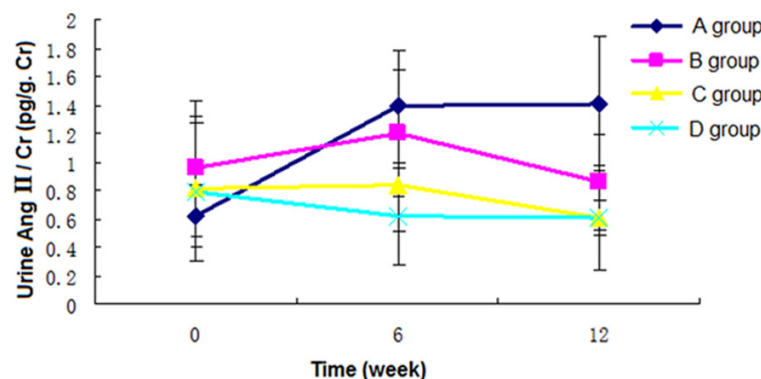

Figure 9. The results of UP/Cr in the 12-week investigation. *$P<0.05$.

from group A ($P>0.05$). Meanwhile, the level of group C was significantly different from group A and B ($P<0.05$ for all).

Expression of AGT in diabetic rats

Immunohistochemistry analysis was used to analyze the expression of AGT in diabetic rats. The results suggested that AGT expressed in

the proximal tubule epithelial cells in the cytoplasm in diabetic rats and there was a strong-positive area (**Figure 11**). The results also indicated that strong AGT-positive area was in group A and the positive area was in group B, while the group C and D was weakly positive (**Figure 12**).

Urinary RAS correlation analysis

The results of correlation analysis suggested that urine AGT/Cr in rats was positively correlative with UP/Cr (r= 0.493, $P<0.01$), negatively correlated with CCr (r=-0.474, $P=0.007$) and ARB (r=-0.575, $P=0.001$). However, there were no significant associated with blood pressure, serum glucose or AGT level ($P>0.05$ for all, **Table 1**).

The results of multiple regression equation analysis suggested that only ARB was independently associated with urine AGT in diabetic rats (B=-2.963, $P=0.024$, **Table 2**).

Discussion

In this study, we investigated the functional roles of AGT in DN via a diabetic rat model. STZ was used to induce diabetic rats in this study through single intraperitoneal injection. The results of growth conditions, fasting blood-glucose and weight indicated that we successfully constructed diabetic rat model. It was worth mentioning that the diabetic rats in this study did not show weight loss, polyuria (increased urination), polydipsia (increased thirst) and polyphagia (increased hunger) which were typical characteristics of diabetic rats in the previous studies [15]. On the contrary, the weight of diabetic rats in this study was increased; however, they were still hypogenetic compared with the healthy
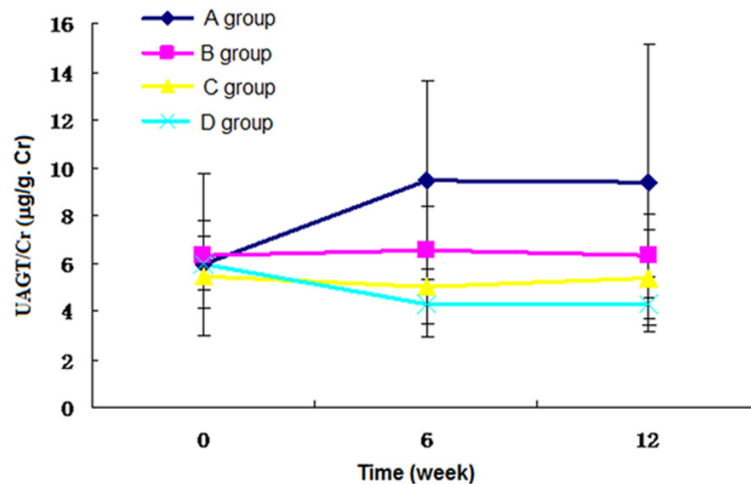
Figure 10. The changes of urinary Ang II. ▼$P<0.05$, *$P<0.05$, **$P<0.001$.
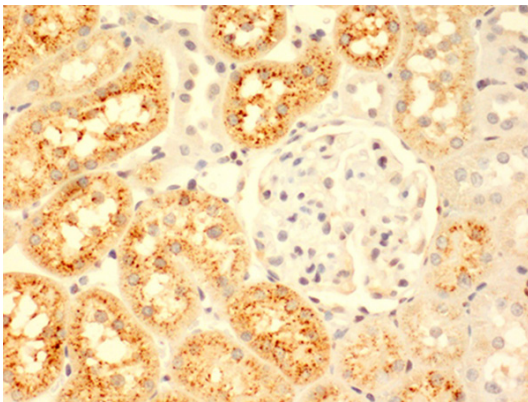
Figure 11. The positive area of AGT in diabetic rats. × 200.

controls. The increased of weight might due to small week age of the rats, free diet and short duration of diabetes. In a word, the diabetic rat model used in this study was successful and could be provide credible outcomes for this research.

In the previous studies, Wang et al. had reported that STZ induced diabetic rats showed early damage of kidney at 12 week [16]. However, the abnormal metabolism of urine protein occurred in 6 week in this study. Without obviously decreased in renal function, the increased of urinary protein indicated early damage of renal tissues. Some researchers had indicated that completely or partly unilateral kidney nephrectomy might contribute to shorten the experiment cycle and reducing the dose of

chemical drugs [17]. On 12 week, the diabetic rats appear typical symptoms of DN, such as glomerular swelling, congestion, and thickening of glomerular basement membrane. The difference might due to the short course and the diabetic rats at the end of the investigation were at Mogensen stage 3-4 [18].

RAS in the cells of kidney took part in all pathological progression of the renal disease caused by diabetes [19]. Ang II, the key factor of RAS, involved in almost pathophysiological processes during the development of DN, such as the changes of hemodynamic, growth factor induction, ROS formation and proteinuria [20]. Evidence had indicated that Ang II could inhibit the activity of Nephrin, a signal protein for inhibiting podocyte apoptosis, which leaded to cell apoptosis and proteinuria [21]. Ang II could interact with other ways which could promote the development of DN, for example AGEs and oxidative stress pathway. In vitro, AGEs were reported to activate RAS elements, such as ACE, AT1 receptor [21]. In the 12-week investigation, we detected the changes of RAS in serum, urine and kidney tissues of diabetic rats and its association with renal function and histological changes was analyzed. The results suggested that RAS played an important role in DN occurrence and development. Moreover, blocking local RAS in kidney by ARB could significantly improve the pathology of DN.

Recently, some studies had reported that the activity of plasma renin and ACE in patients with diabetes and DN did not increase, compared with the healthy, indicating RAS might not be activated [21]. Similar results were obtained in this study. The concentration of serum AGT and Ang II in diabetic rats was much the same as that in the control group. In the previous studies, excited RAS was reported to exit in locally diabetic kidney. The mRNA levels of some RAS components increased in the cultured cells, while the level in the culture supernatant was normal [22]. In the study of Wolf et al., RAS blocking drugs such as ACEI and ARB
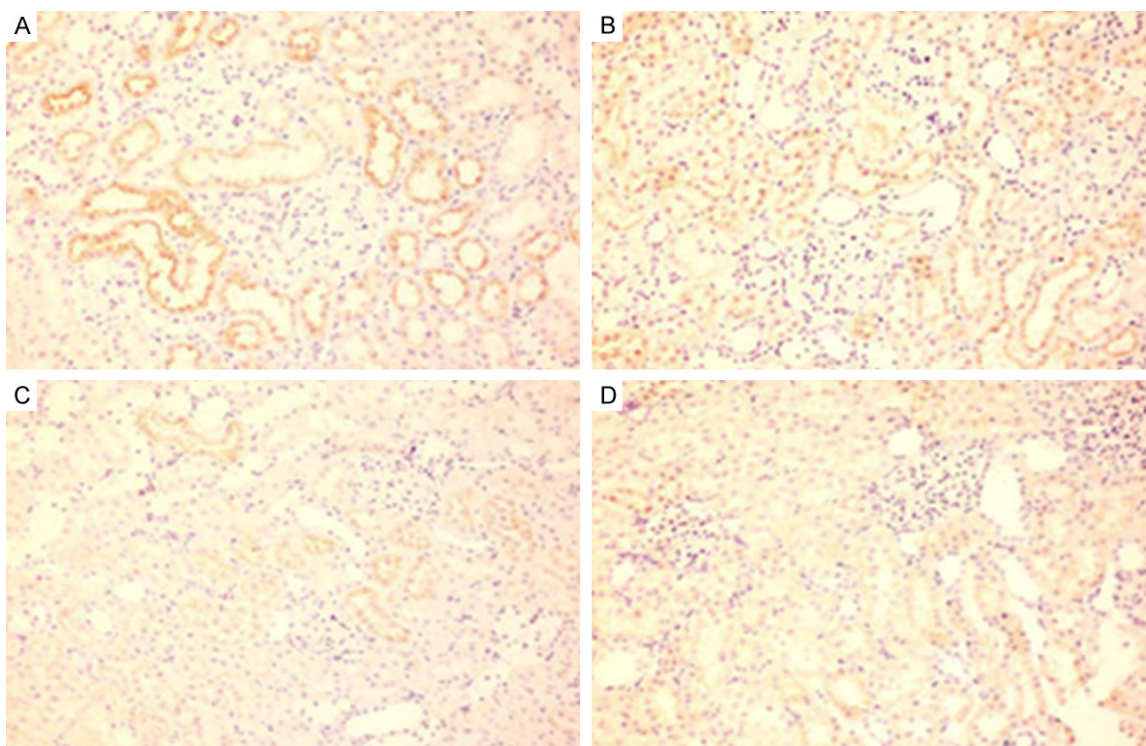
Figure 12. The expression level of AGT in all groups. × 100.

Table 1. The correlation analysis of AGT in diabetic rats

Factors	r	P
ARB dose	-0.575	0.001**
Arterial pressure (mmHg)	0.299	0.102
Fasting blood-glucose (mM)	0.010	0.956
CCr (mL/min)	-0.474	0.007**
Serum AGT (μg/L)	0.023	0.904
UP/Cr (mg/g. cr)	0.493	0.005**

*$P<0.05$, **$P<0.01$.

Table 2. Multi-factor regression analysis of AGT in diabetic rats

Factors	B	P
ARB dose	-2.194	0.001**
UP/Cr (mg/g. cr)	0.02	0.108
CCr (mL/min)	-15.024	0.242

*$P<0.05$, **$P<0.01$.

were reported to be more effective for treatment of diabetes than other drugs [23]. Therefore, the urinary AGT might be promising biomarkers for activity of RAS synthesized by kidney. In this study, we proved that urinary AGT/Cr was positively correlated with UP/Cr and negatively with CCr and ARB. Besides, the concentration of AGT was independently correlated with the dose of ARB. In clinical practice, UP/Cr and CCr were used for evaluating the severity of renal damage in DN. Therefore, urinary AGT was associated with renal damage and the results of immunohistochemical staining were also proved the conclusion.

In conclusion, the changes of kidney pathological and increase of urine protein may be directly related with local RAS activation in kidney tissues. The ARB drug can increase the level of serum Ang II and inhibit RAS activity, thus reduce urinary protein and protect the function of kidney.

Disclosure of conflict of interest

None.

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