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ORIGINAL ARTICLE

Effects of aminoguanidine and vitamin C on collagen type IV in diabetic nephropathy rats

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Abstract The aim of this article is to investigate the effects of Aminoguanidine and vitamin C (VitC) on type IV collagen in diabetic nephropathy rats. Diabetic nephropathy rats were induced by intraperitoneal injection of STZ. Rats were randomly divided into five groups: normal control group ($n = 10$), diabetes group ($n = 10$), aminoguanidine group ($n = 10$), VitC group ($n = 10$), aminoguanidine and VitC group ($n = 10$). After 16 weeks, the general conditions, blood gloucose, glycosylated hemoglobin, blood urea nitrogen, serum creatinine, serum type IV collagen, urinary albumin excretion rate, and creatinine clearance rate were detected, type IV collagen protein was determined by immunohistochemical analysis as well as the expression of collagen type IVα1 mRNA were determined by in situ hybridization analysis in the kidneys of each group. The results were (1) diabetes mellitus and renal lesions occurred in the diabetes group, aminoguanidine group, VitC group, VitC and aminoguanidine group; (2) aminoguanidine and VitC improved the general conditions of diabetic nephropathy rats, decreased blood urea nitrogen, serum creatinine, and urinary albumin excretion rate as well as increased creatinine clearance rate. The expressions of collagen type IV were significantly down-regulated in treatment groups in contrast to the diabetes group. Aminoguanidine and VitC protect renal lesions in diabetic nephropathy, respectively, by inhibiting expression of type IV collagen, while aminoguanidine and VitC have a synergistic effect on them.

Keywords Aminoguanidine · Vitamin C · Diabetic nephropathy · Type IV collagen

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Introduction

Diabetic nephropathy is characterized by glomerular extracellular matrix (ECM) accumulation, glomerulosclerosis, and progressive tubulointerstitial fibrosis, yet the pathogenesis is not completely clear. Early diagnosis and treatment are very important. Type IV collagen accounts for 50% of dry weight of glomerular basement membrane (GBM) in the normal mesangial matrix. It is one of the important components of glomerular capillary basement membrane and a marker of the mesangium, reflecting the dynamic state of the ECM and other related substances, and is an essential indication of early pathological changes of diabetic nephropathy [1]. Researchers have proved that oxidative stress and non-enzymatic glycosylation reaction play an important role in morbidity of diabetic nephropathy under conditions of high glucose [2, 3]. It is reported that

aminoguanidine can inhibit the production of advanced glycation end products (AGEs) in vivo or vitro. Retarding oxidated glycosylation can postpone or lessen diabetic nephropathy [4]. Vitamin C (VitC) is a traditional anti-oxidizing agent [5]. We purport that aminoguanidine and VitC can protect diabetic nephropathy by observing the effects of aminoguanidine and VitC on type IV collagen in streptozotocin-induced diabetic nephropathy rats. We will discuss the effects and mechanisms of aminoguanidine and VitC on type IV collagen in diabetic nephropathy.

Method

Experimental animals

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

Instruments and reagents

Type IV collagen protein radioimmunoassay (RIA) kit (Shanghai Navy Medical Science Research Institute). Yichen SENTEST hand-held blood glucose test instrument. RiLi 7170 auto bio-chemical FJ-2008 immunity cell recording instrument and SN-682 r radio-immunity counter (Produced by Shanxi 262 State-Operated Factory). STZ (American Sigma Company). VitC(C) (Jiao Zuo Company Limited of TianJin Medicine Industry). Aminoguanidine (D) (HeNan Shuai Ke Pharmacy Company limited, certificate number 20050602). S-P immunity staining kit, anti-collagen IV, ISH (in situ hybridization) testing reagent box, collagen IVα_1, CIV$\alpha_1$tide probe, (S′-marked by cardiox, Sequence5′ > GTG AGG GAG ACC TGC GGG TGC TGC GAC TGT GAG AAG CGC TGT GGC GCC CT < 3′) (WuHan Boshide Biological Science Company). Antibodies for CIV and CIVα_1 tide probes were obtained for immuno-histochemistry or ISH.

Experimental procedure

After being fed for 1 week, 10 of the 70 rats were randomly taken as the normal control group. The other 60 rats, after fasting for 12 h, were administered an intraperitoneal injection of STZ (60 mg/kg). After 72 h, the blood glucose of each rat was measured by a Yicheng blood glucose analyser. If the blood glucose was >16.7 mmol/l, the rat was considered to be diabetic. 40 of the 60 rats developed diabetes and were divided into four groups: the diabetes group (D group, $n = 10$), the aminoguanidine group (D + A group, $n = 10$), VitC treatment group (D + C group, $n = 10$) and VitC plus aminoguanidine treatment group (D + C + A group, $n = 10$). The rats in the aminoguanidine group were given intragastric administration of aminoguanidine (100 mg/kg; lg dissolved into 1 l) once a day [6]. The rats in the VitC treatment group were given intragastric administration of VitC (100 mg/kg; lg dissolved into 1 l of water) once a day [7]. The rats in the VitC plus aminoguanidine treatment group were given intragastric administration of aminoguanidine 100 mg/kg and VitC 100 mg/kg once a day (lg dissolved into 1 l of water). The treatment lasted for 16 weeks. To avoid ketosis and ensure survival, diabetic rats (except normal control group) were given 2–4 units of long-acting insulin to maintain the blood glucose level about 25 mmol/l. Rats in the normal control group received an equal volume of water.

General observation of the rats

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

Sample collection

Urine was collected for 24 h using a metabolic cage to measure the urinary albumin excretion in different groups after 16 weeks. The rats were weighed after being anaesthetized. 5 ml 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 each rat to remove the left kidney. Then the left kidney was weighed, washed with saline repeatedly until it appeared white. After removing the capsule of the left kidney, 2 blocks of renal tissue of about 0.5 cm in length were obtained and fixed in *para*-formaldehyde (quality score of para-formaldehyde in the phosphate buffer solution was 0.04) for 12 h. Sections of 4 μm thickness were prepared and used for haemotoxylin and eosin (HE), in situ hybridization and immunohistochemical staining.

Image analysis of immunohistochemistry and in situ hybridization

Ten fields for each slide were randomly chosen by the computerized microscopic image processing system under a magnification of 400 times. Grey scale scanning was applied to the IHC positive result, with the average relative grey value used as the value of expression levels (mean [SD]). The results were identified by the color 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 were expressed as mean (SD) and all analyses were done using SPSS 11.0. A P value <0.05 was considered statistically significant.

Results

General conditions of the rats

Normal control group: the rats visibly gained weight and were active with lustrous fur, spontaneous movements and keen reactions. Diabetes group: the rats in diabetic group looked lethargic, were obviously emaciated, polyuric, polydipsia, hyperhidrotic, slow to act, stooping like a snake with lusterless fur. Beddings needed to be changed once or twice a day. Two of them developed cataracts and one of them had repeated diarrhea. Treatment groups: the rats in each treatment group were in good spirits, and had no cataract with intact tail and feet. They could act freely. But their reaction sensitivities were slightly worse than in the normal control group. Two rats in D group died from obvious dehydration in the first and fourth week, respectively. At the 5th week, one rat in D + C group appeared unremitting diarrhea, and finally died from presumed hypoglycemia.

Effects of aminoguanidine and vitamin C on different parameters in the diabetic and treated rats

The body weights of the rats in D group were reduced significantly compared with those in the control group, while the urine volume and blood glucose increased significantly ($P < 0.05$). After treatment, compared with the D group, the body weight obviously increased, urinary volume, kidney weight/body weight ratio decreased in D + A, D + C, and D + A + C groups ($P < 0.05$). Comparing D + C group with D + A group, there were no differences in weight, urinary volume, and kidney weight/body weight ratio ($P > 0.05$). Comparing with D + A, D + C groups, the weight increased whilst urinary volume and kidney weight/body weight ratio decreased in D + A + C group. Compared with the normal group, the body weight in D + A + C group showed marked differences ($P < 0.05$, Table 1).

Compared with the control group, the urinary albumin excretion rate, serum creatinine and blood urea nitrogen of the diabetic rats increased significantly, while the creatinine clearance rate decreased ($P < 0.05$). Compared to the diabetic group, the treatment groups showed significantly lower urinary albumin excretion rate, serum creatinine and blood urea nitrogen, and higher creatinine clearance rate ($P < 0.05$). After treatment, compared to the D group, the urinary albumin excretion, blood urea nitrogen, and glycosylated hemoglobin of D + A, D + C, and D + A + C groups were obviously elevated ($P < 0.05$). Comparing D + C and D + A groups, there were no differences in urinary albumin excretion, blood urea nitrogen and glycosylated hemoglobin ($P > 0.05$). Comparing D + A and D + C groups, the urinary albumin excretion, blood urea nitrogen, and glycosylated hemoglobin in D + A + C group were elevated ($P < 0.05$), but were still lower than in the normal group ($P < 0.05$, Table 2).

Effects of treatment on glycosylated hemoglobin and serum collagen IV

The results showed that: compared with the normal group, glycosylated hemoglobin and serum collagen IV increased in the diabetic model group ($P < 0.05$) After treatment, compared with the D group, glycosylated hemoglobin and serum collagen IV of D + A, D + C, and D + A + C groups were obviously elevated ($P < 0.05$). Comparing D + C group and D + A groups, there were no differences in glycosylated hemoglobin and serum collagen IV ($P > 0.05$). Compared to D + A and D + C groups, the glycosylated hemoglobin and serum collagen IV were

Table 1 Changes of urine volume, body weight, ratio of kidney to body weight, and blood glucose in different groups ($\bar{x} \pm s$)

Groups	n	Urine volume (ml)	Body weight (g)	Ratio of kidney to body weight ($\times 10^{-3}$)	Blood glucose (mmol/l)
CN	10	12.9 2 ± 4.22	303.20 ± 20.92	3.20 ± 0.46	3.98 ± 0.33
D	8	129.41 ± 17.60[a]	150.83 ± 17.71[a]	5.88 ± 0.38[a]	24.65 ± 2.28[d]
D + A	10	114.55 ± 16.32[a,b]	241.61 ± 26.77[a,b]	4.71 ± 0.38[a,b]	24.09 ± 3.88[d]
D + C	9	120.24 ± 18.50[a,b]	242.52 ± 18.98[a,b]	4.71 ± 0.44[a,b]	23.48 ± 2.87[d]
D + A + C	10	78.09 ± 20.36[a,b,c]	290.46 ± 20.90[a,b,c]	4.77 ± 0.92[a,b,c]	23.97 ± 2.80[d]

[a] $P < 0.05$ vs. CN group, [b] $P < 0.05$ vs. D group, [c] $P < 0.05$ vs. D + A and D + C group, [d] $P < 0.05$ vs. CN group

Table 2 Changes of urine albumin, serum creatinine, clearance rate of creatinine and blood urea nitrogen (BUN) in different groups($\bar{x} \pm s$)

Group	n	Urine albumin (mg/24 h × 10^{-3})	Serum creatinine (μmol/l)	Clearance rate of creatinine (ml/min)	BUN (mmol/l)
CN	10	2.82 ± 0.35	26.20 ± 7.92	0.92 ± 0.15	3.25 ± 0.33
D	8	43.41 ± 0.40[a]	97.83 ± 7.71[a]	0.19 ± 0.03[a]	10.25 ± 0.28[a]
D + A	10	18.40 ± 0.37[a,b]	72.96 ± 7.95[a,b]	0.60 ± 0.24[a,b]	8.70 ± 0.51[a,b]
D + C	9	17.24 ± 0.30[a,b]	75.52 ± 7.98[a,b]	0.61 ± 0.14[a,b]	7.98 ± 0.47[a,b]
D + A + C	10	9.89 ± 0.75[a,b,c]	59.65 ± 8.82[a,b,c]	0.68 ± 0.29[a,b,c]	5.80 ± 0.92[a,b,c]

[a] $P < 0.05$ vs. CN group, [b] $P < 0.05$ vs. D group, [c] $P < 0.05$ vs. D + A and D + C group

Table 3 Changes of serum glycosylated hemoglobin and serum collagen IV in different groups ($\bar{x} \pm s$)

Groups	n	HbA1c (%)	Serum collagen IV (pg/ml)
CN	10	4.03 ± 1.54	81.46 ± 4.88
D	8	13.73 ± 1.21[a]	160.28 ± 11.33[a]
D + A	10	8.11 ± 1.62[a,b]	113.65 ± 29.03[a,b]
D + C	9	9.05 ± 0.56[a,b]	120.78 ± 30.69[a,b]
D + A + C	10	7.02 ± 1.30[a,b,c]	95.46 ± 35.78[a,b,c]

[a] $P < 0.05$ vs. CN group, [b] $P < 0.05$ vs. D group, [c] $P < 0.05$ vs. D + A and D + C group

higher in the D + A + C group ($P < 0.05$), but were still lower than the normal group ($P < 0.05$, Table 3).

HE staining in kidney tissue and Immunohistochemistry

Hematoxylin and eosin staining (HE staining) results showed that there were no significant changes in renal glomerulus and tubulointerstitial pathology as detected by the microscope in the normal control group. Renal glomeruli in the diabetes group (D group) had a greater diameter, renal glomerular cells proliferating, scattered renal tubular epithelial cells swelling, degenerating and desquamating. Diffuse thickness of glomerular capillary basement membranes was noted. Expansion of the mesangial area, mesangial cell hyperplasia, and moderate or severe matrix proliferation with focal inflammation and lymphocyte infiltration were also apparent. The above pathological changes in the treatment groups, especially in the D + A + C group, were lesser in the extent compared to the D group.

Immunohistochemical results showed, that compared with the normal control group, obvious brown yellow granules were seen on mesangial cells, vascular endothelial cells and renal tubular epithelial cells, suggesting positive results. Collagen IV protein expressed in the glomerular ECM in the D group were strongly positive compared with the normal control group ($P < 0.05$). Compared with the D group, collagen IV protein expression was lower in the D + A group and D + C groups, showing positive expression ($P < 0.05$), while weakly positive expression ($P < 0.05$) in the D + A + group. There were no differences in collagen IV protein expressions between D + C group and D + A groups ($P > 0.05$, Table 4; Fig. 1a–e).

Situ hybridization analysis on collagen IVα_1 mRNA

Situ hybridization analysis results showed that, compared with the normal control group, obvious brown yellow granules were seen on mesangial cells, vascular endothelial cells and epithelial cells of renal tubules, suggesting positive results. Collagen α_1 mRNA expressed in glomerular ECM in D group were strongly positive compared with the normal control group ($P < 0.05$). Compared with D group, collagen IVα_1 mRNA expressions were reduced in D + A group and D + C group showing positive expression($P < 0.05$), while weakly positive expression ($P < 0.05$) in the D + A + C group. There were no differences in collagen IVα_1 mRNA

Table 4 Changes of in situ hybridization optical density CollageIV protein and CollageIVα_1 mRNA in different groups ($\bar{x} \pm s$)

Groups	n	Collagen IV Determine the nature	Protein Ration	Collagen IVα_1 Determine the nature	mRNA Ration
CN	10	−∼±	61.46 ± 7.88	−∼±	34.03 ± 7.54
D	8	+++	120.28 ± 11.33[a]	+++	118.83 ± 17.51[a]
D + A	10	++	124.08 ± 19.41[a,b]	++	87.05 ± 17.98[a,b]
D + C	9	++	110.78 ± 9.69[a,b]	++	90.25 ± 12.23[a,b]
D + A + C	10	+	91.74 ± 12.66[a,b,c]	+	59.55 ± 8.91[a,b,c]

[a] $P < 0.05$ vs. CN group, [b] $P < 0.05$ vs. D group, [c] $P < 0.05$ vs. D + A and D + C group

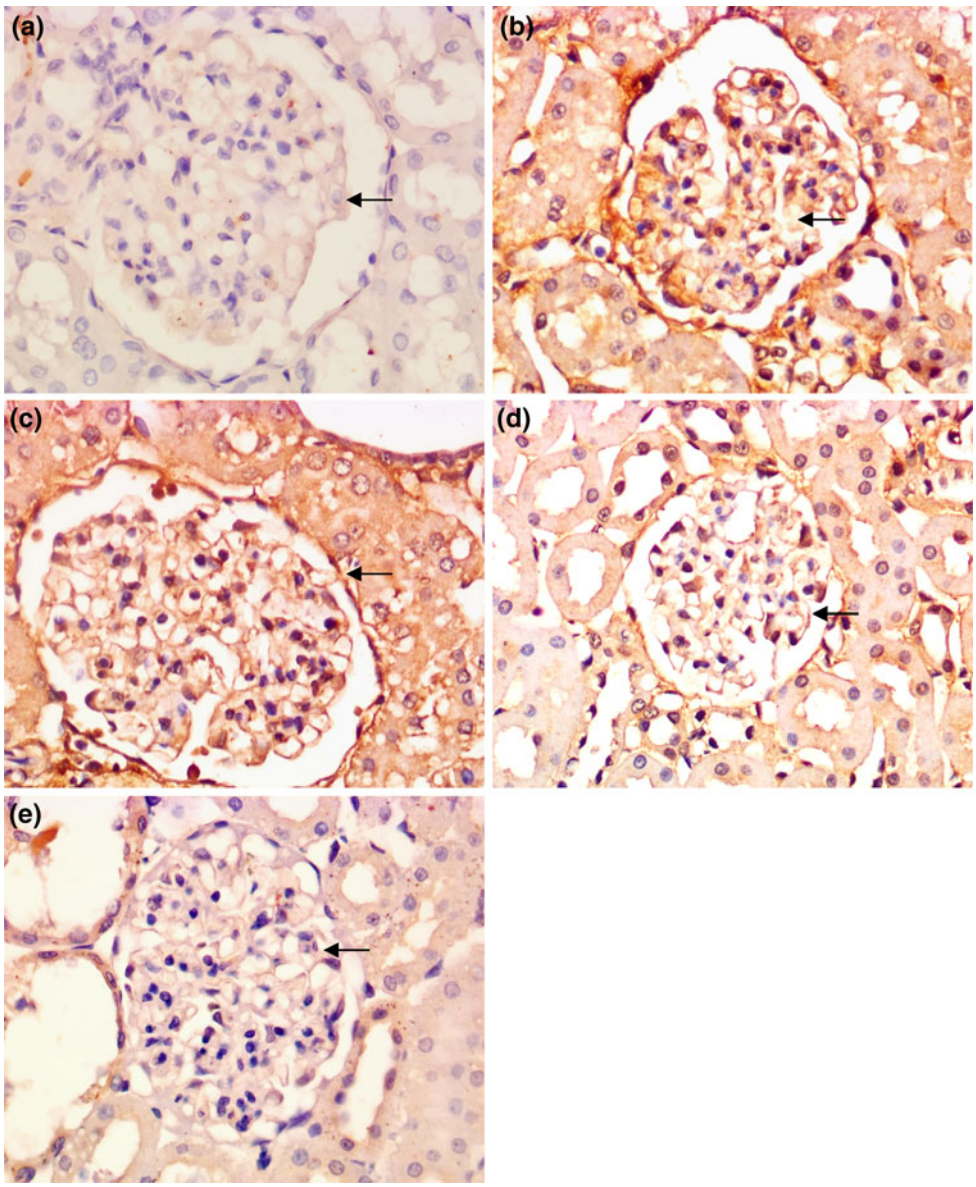

Fig. 1 IHC analysis for CIV expression in glomeruli of rat (immunohisto-chemistry, original ×400). Collagen IV protein of **a** control rat was low positive, **b** diabetic rat was strongly positive, **c** control rat was moderately positive after aminoguanidine for 16 weeks, **d** diabetic rat was moderately positive after VitC for 16 weeks, **e** diabetic rat was positive after aminoguanidine and VitC for 16 weeks in the glomerulus of kidney tissues. *Arrow* pointing to positive results

expressions between D + C and D + A groups ($P > 0.05$, Table 4; Fig. 2a–e).

Correlation analysis for serum collagen IV, collagen IV protein, and collagen IVα_1 mRNA

There were positive correlations between serum collagen IV and glomerular collagen IV protein ($P < 0.05$) and between collagen IV protein and collagen IVα_1 mRNA ($P < 0.05$) (Table 5).

Discussion

The incidence of diabetes in China has risen rapidly in recent years and the researches on the pathogenesis and treatment strategies of diabetes have made remarkable progresses. The effects of oxidative stress and glycosylation on diabetic complications have become increasingly important. Sufficient evidences show that high glucose and protein in the body can promote sugar and non-enzymatic reaction (glycosylation) and formation of AGEs, which play an important role in the incidence of diabetic nephropathy [8], while glycosylation and oxidation are two closely related biochemical processes which may have a synergistic role in the pathogenesis of diabetic nephropathy [2, 3]. The pathological features of diabetic nephropathy are renal hypertrophy, GBM thickening, and progressive accumulation of ECM in the glomerular mesangial regions.

Collagen IV is a major ECM in renal tissue. It has been confirmed that IVα-collagen polypeptide chain is classified into α1, α2, and α3 types according to the primary

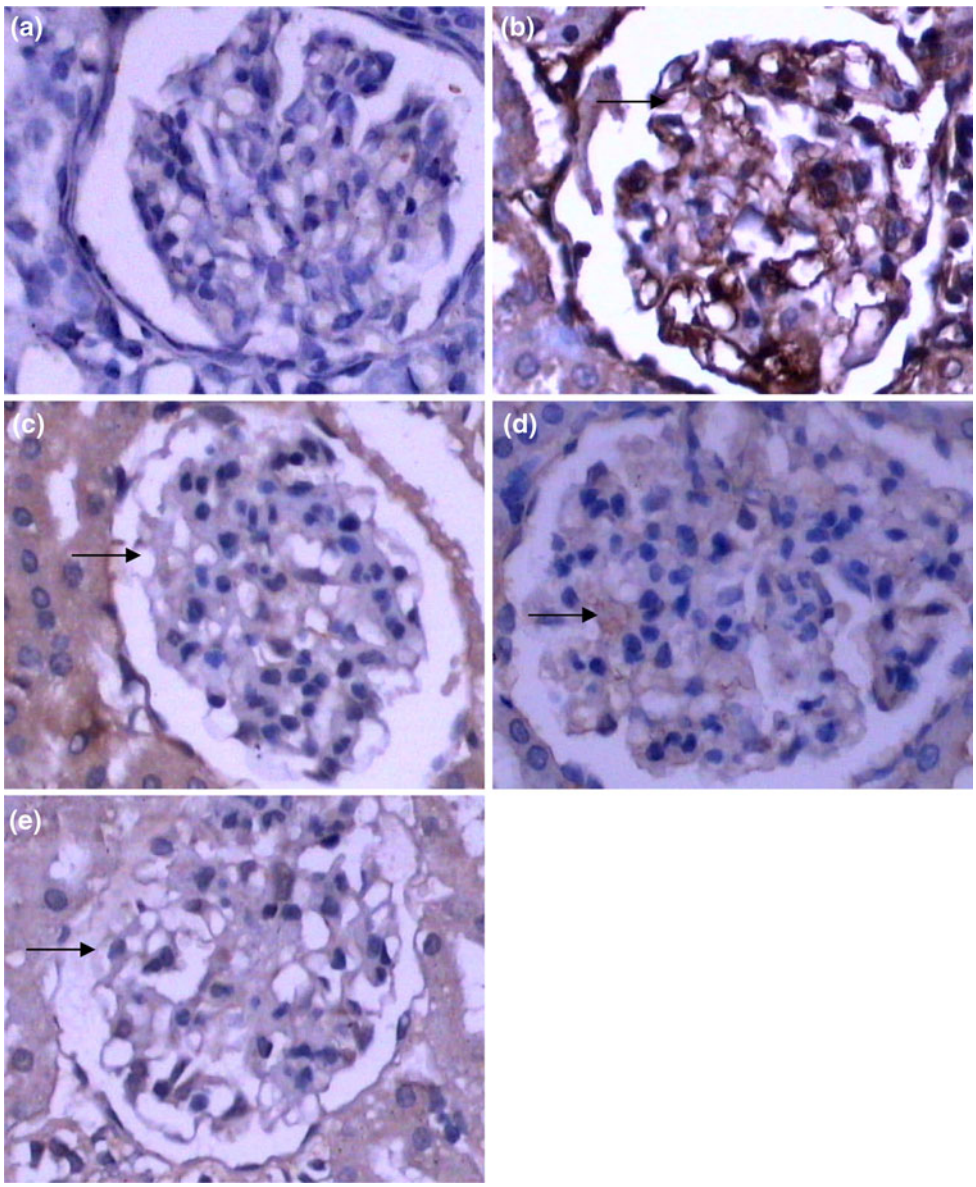

Fig. 2 In situ hybridization analysis for collagen IVα_1 mRNA expression in glomeruli of rat (in situ hybridization, DAB staining ×400). Collagen IVα_1 mRNA of **a** control rat of was low positive, **b** diabetic rat was strongly positive, **c** control rat was moderately positive after aminoguanidin for 16 weeks, **d** diabetic rat was moderately positive after VitC for 16 weeks, **e** diabetic rat was positive after aminoguanidine and VitC for 16 weeks in the glomerulus of kidney tissues

Table 5 Linear correlation analysis between serum collagen IV, collagen IV protein expression and collagen IVα_1 mRNA

Groups	Collagen IVα_1 mRNA and serum collagen IV		Collage IVα_1 mRNA and collagen IV protein	
	r	*P*	*r*	*P*
CN	0.632	0.031	0.661	0.031
D	0.686	0.033	0.703	0.024
D + A	0.754	0.013	0.705	0.031
D + C	0.696	0.037	0.711	0.022
D + A + C	0.559	0.029	0.705	0.018

$P < 0.05$, relative levels of collagen IVα_1 mRNA, collagen IV protein expression were taken from glomerular

structure. The most common molecule of Collagen IV in GBM is $[\alpha1(IV)]_2\alpha2(IV)$, a tripolymer of triple helix. Because the collagen IV molecule in the GBM contains two α1(IV) chain, collagen IV expression in GBM can be detected efficiently by α1(IV) cDNA probe in our study [9]. UAE is a golden standard for DN diagnosis [10]. Combined analysis of collagen IV and UAE may constitute a monitoring index for DN [11].

In our experiment, the protein and mRNA expressions of collagen IV significantly increased in the regions of glomerular mesangium, GBM, and a few tubular basement membranes, which is similar with the results in Thallas-Bonke V's researches [8]. UAE and glycated hemoglobin increased in model DN rats, which HbA1c levels reflect the level of AGEs to a certain extent [12]. These results show that collagen IV may play an important role in the development of AGEs and diabetic nephropathy. The possible causes of upregulation of collagen IV in the kidney of DN include: first, high blood sugar can increase the stability and promote synthesis of collagen IV, which has been confirmed in our study that the levels of collagen IV in serum and kidney of DN increased. Second, non-enzymatic protein glycosylation reaction suppresses normal collagen IV crosslinking, enhances the stability of collagen IV and reduces its degradation [13]. Third, AGEs induce cytokines to promote ECM accumulation [9].

Aminoguanidine, a compound of hydrazines, can reduce blood viscosity, inhibit platelet aggregation, reduce the level of thromboxane A2, regulate blood lipids, and significantly increase plasma endothelin, renin activity and angiotensin to improve glomerular ischemia, hypoxia and hypercoagulable states and inhabit the formation of AGEs in vitro or in vivo [14, 15], which is known as one of the strongest blocking agents of glycosylation. VitC is an important antioxidant and can remove free radicals generated by the glycosylation reaction. It can inhibit the two-carbonyl intermediate products oxidised by ketoimine to suppress the activity and formation of AGEs and reduce oxidative stress [16, 17]. It has been confirmed that plasma levels of VitC in diabetes mellitus (DM) patients are lower than those of normal individuals by 40–50% [18]. VitC, a water soluble substance, cannot be stored in the body and needs to be added frequently whence intravenous or oral administration can increase the level of plasma VitC to achieve effective antioxidant effects.

Our experiments showed that aminoguanidine and VitC could improve the general states of rats and weight, decrease urinary volume and kidney weight/body weight ratio and reduce UAE, HbA1c, BUN, Scr, serum collagen type IV, and renal collagen IV protein expression, while the two drugs have a synergistic effect. This study also confirmed that serum collagen IV and renal collagen IV protein expression glomerular collagen α1 IV genes and collagen α1 IV mRNA were positively correlated. According to molecular biology results, changes of glomerular collagen IV synthesis may occur at the transcriptional level; the two drugs may inhibit glycosylation and oxidative stress in vivo, inhibiting collagen IV mRNA at the transcriptional level, thereby reducing collagen IV protein expression and secretion of collagen IV in the blood and inhibiting ECM accumulation to improve renal function. Though different protective effects in DN, aminoguanidine can inhibit formation of AGEs and oxidative stress [3] as well as can prevent impairment of blood antioxidant system in diabetic rats [19], while VitC may have an antioxidation effect and inhibit the activity and formation of AGEs [16], which two drugs have a better synergistic effect.

In short, inhibition of glycosylation and oxidative stress by aminoguanidine and VitC can be considered as a starting point for exploring strategies aiming at the treatment of diabetic nephropathy. They may yet have an important role in retarding the progress of diabetic nephropathy and preventing the occurrence of end-stage renal failure.

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