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Restricted intake of dietary advanced glycation end products retards renal progression in the remnant kidney model

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Diet-derived advanced glycation end products (AGEs) contribute significantly to accumulation of AGEs in renal insufficiency. To test whether modulation of dietary AGEs would impact on progression of chronic renal disease, 5/6 nephrectomy rats were randomly placed on three diets that differed only in AGEs content (low AGEs diet (LAD), high AGEs diet (HAD), and standard rodent diet (SRD)) for 5–13 weeks. Compared with SRD- or HAD-fed rats, LAD-treated animals showed decreased proteinuria and retarded decline of creatinine clearance without alteration of blood pressure. Glomerular volume was reduced by 23% compared with HAD-fed rats at week 13 ($P<0.001$). Renal fibrosis progressed with time in the remnant kidneys from HAD-fed rats. However, LAD-fed animals presented a better-preserved structure of the kidneys. LAD-fed rats demonstrated significantly decreased serum and renal AGEs concentration ($P<0.01$ and $P<0.01$). This was associated with marked decrease of intrarenal advanced oxidation protein products and thiobarbituric acid reactive substances, as well as increase of glutathione peroxidase activity. LAD treatment also downregulated expression of monocyte chemoattractant protein-1 and transforming growth factor-β1 and ameliorated macrophage infiltration in the remnant kidney. These results demonstrated that restriction of dietary AGEs intake retards progression of renal fibrosis and dysfunction in the remnant kidney model.

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KEYWORDS: diet; AGEs; renal fibrosis; renal dysfunction

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Chronic kidney disease (CKD) is a significant interactive disease in patients with diabetes, hypertension, and cardiovascular disease with major morbidity consequences and high costs to the healthcare system.[1] Renal fibrosis, the final common pathway of CKD, predicts the degree of renal dysfunction and long-term prognosis for almost all forms of CKD.

Numerous mechanisms have been implicated in the initiation of this cascade including activation of renin-angiotensin system, overexpression of cytokines, redox imbalance, and metabolic toxins accumulation.[2] Advanced glycation end products (AGEs) might be one of these toxins. Endogenous AGEs are formed during nonenzymatic reaction between protein and glucose or glucose degradation products under hyperglycemia.[3,4]

Increased AGEs burden has also been demonstrated in euglycemic patients with renal insufficiency, in whom AGEs accumulation is attributed to either decreased renal clearance or increased formation of these compounds owing to enhanced oxidative stress.[5,6]

In addition to those endogenously formed, AGEs are abundant in exogenous sources such as foods, especially when prepared under elevated temperature.[7] Modulation of dietary AGEs in human subjects or animals with or without diabetes or renal disease modifies circulating AGEs levels, suggesting that dietary AGEs intake constitutes an important source of the body AGEs pool.[8,9]

It has been documented that AGEs, both endogenously formed and exogenously ingested, are bioreactive toxins[8,10,11] and contribute to the complications of diabetes and renal insufficiency, such as endothelial dysfunction, accelerated atherosclerosis, and β2-microglobulin amyloidosis.[10–12] Prolonged intravenous injection of pre-formed AGEs into normal animals results in albuminuria and glomerular sclerosis,[13] suggesting that AGEs might be a class of renal fibrogenic factors.

Kidney is the major organ for dealing with the daily load of dietary AGEs[7,14] and the predominant tissue of AGEs accumulation after oral administration.[15] Plasma AGEs levels are closely correlated with the progression of renal insufficiency.[16] Thus, it is important to clarify whether accumulating dietary AGEs contribute significantly

to the progression of CKD, especially in the presence of renal insufficiency. A short-term *in vivo* animal study has in part revealed this pathogenic link by showing that the administration of an AGEs-rich diet to the remnant kidney model for a short period (6 weeks) result in increase of proteinuria.[17] However, the impact of prolonged load of dietary AGEs on the structure and function of the kidney, particularly in the presence of renal dysfunction, has remained unclear.

This study was conducted to test the hypothesis that modulation of dietary AGEs intake may modify progression of CKD.

RESULTS

Effects of dietary AGEs on serum and renal AGEs levels

As shown in Table 1, the three kinds of diet were nutritionally equivalent and differed only in AGEs content. As the daily food intake was equal between high AGEs diet (HAD)-, low AGEs diet (LAD)- and standard rodent diet (SRD)-fed groups (Figure 1a), HAD-fed animals ingested threefold more AGEs and LAD-fed rats ingested onefold less AGEs as compared with SRD-fed animals.

Serum AGEs levels, assessed as N^{ε}-carboxymethyllysine (CML)-like immunoepitopes, were significant higher in 5/6 nephrectomy (5/6 Nx) rats than the sham-operated controls ($P<0.05$). Animals given HAD showed marked increase of serum AGEs as compared with SRD-fed 5/6 Nx rats. LAD-treated rats had significant lower serum AGEs levels than SRD-fed 5/6 Nx animals (Figure 1b). A similar pattern was observed in AGEs levels in the remnant kidney among animals received three kinds of diet (Figure 1c). There was no significant difference in lipid profile and albumin levels among 5/6 Nx rats given food containing different levels of AGEs (Table 2).

Effect of dietary AGEs on renal compensatory growth

As shown in Table 3, the weight of the remnant kidney increased with time in 5/6 Nx rats as compared with sham-operated controls. The increment, expressed as the left kidney weight/body weight, was significant higher in the HAD-fed

Table 1 | Characteristic of the diet[a]

	HAD	LAD	SRD
Nutrients			
Protein (g/100 g)	17.5	17.0	16.0
Fat (g/100 g)	6.3	6.1	5.0
Carbohydrate (g/100 g)	61.0	60.0	65.0
Fiber (g/100 g)	2.5	2.6	2.4
Calcium (g/100 g)	1.3	1.3	1.2
Phosphate (g/100 g)	0.4	0.5	0.4
Soluble chloride (g/100 g)	0.3	0.4	0.4
Lysine (g/100 g)	0.8	0.8	0.7
Methionine (g/100 g)	0.4	0.4	0.4
Vitamin A (IU/100 g)	253	240	238
Vitamin D_3 (IU/100 g)	130	120	110
Vitamin E (IU/100 g)	5.5	5.6	5.0
Total calories (kcal/g)	3.5	3.7	3.6
AGEs[b]			
CML(ng/mg)	785	112	217
Total fluorescent AGEs (AU/mg)	63.8	23.1	28.3

AGEs, advanced glycation end products; CML, N^{ε}-carboxymethyllysine; HAD, high-advanced glycation end products diet; LAD, low-advanced glycation end products diet; SRD, standard rodent diet.
[a]Nutrients were quantified by the Guangdong Province Institute of Animals Feed.
[b]Content of AGEs was determined based on enzyme-linked immunosorbent assay using an anti-CML polyclonal antibody[12,20] and on fluorospectrophotometry.[19]

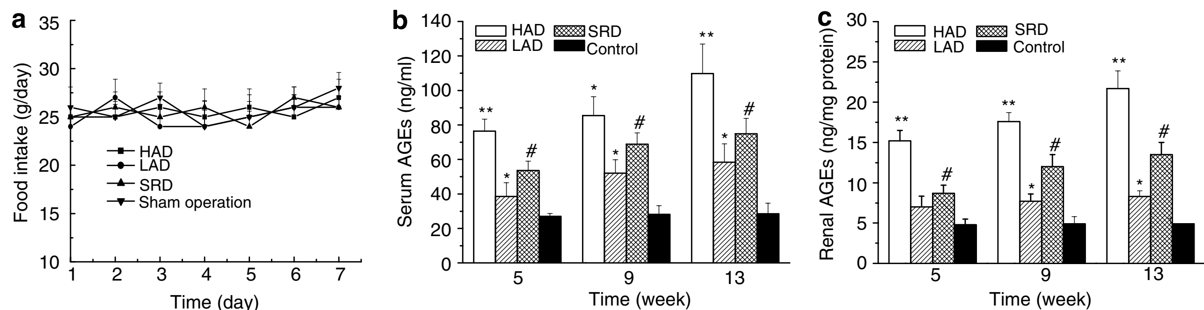

Figure 1 | Individual daily food intake (a), serum AGEs concentration (b), and renal AGEs levels (c) of 5/6 Nx rats on HAD, LAD, SRD, and control rats on SRD. Data are expressed as mean ± s.d., $n=10$ in each group at each time point. (**b**) ANOVA, $P<0.001$, (**c**) ANOVA, $P<0.001$ at week 5, 9, and 13. *$P<0.05$ vs SRD; **$P<0.001$ vs SRD; $^{\#}P<0.05$ vs control.

Table 2 | Serum lipid profile and albumin

	N	Total cholesterol (mmol/l)	Triglycerides (mmol/l)	Total protein (g/l)	Albumin (g/l)
HAD-fed 5/6 Nx rat	10	3.03 ± 0.30	1.42 ± 0.29	43.6 ± 2.6	14.1 ± 1.8
LAD-fed 5/6 Nx rat	10	2.51 ± 0.60	1.25 ± 0.23	48.2 ± 3.5	16.3 ± 2.7
SRD-fed 5/6 Nx rat	10	2.75 ± 0.15[a]	1.34 ± 0.20[a]	47.4 ± 2.6	15.5 ± 2.5[a]
Sham-operated control	10	1.36 ± 0.11	0.50 ± 0.02	57.6 ± 3.0	27.1 ± 1.4

HAD, high-advanced glycation end products diet; LAD, low-advanced glycation end products diet; SRD, standard rodent diet; Nx, nephrectomy; N, number of rats.
Data were mean ± s.d. Blood samples were collected at week 13 of the study after overnight fast.
[a]$P<0.05$, compared with sham-operated control.

Table 3 | Body weight, left kidney weight/body weight, and glomerular volume

	Week 5	Week 9	Week 13
BW (g)			
HAD-fed 5/6 Nx rat	319.1±16.3	374.1±18.4	422.2±20.5
LAD-fed 5/6 Nx rat	324.1±13.6	364.9±14.3	420.5±8.9
SRD-fed 5/6 Nx rat	320.6±17.4	376.7±26.7	416.7±15.3
Sham-operated control	326.1±23.9	368.8±11.7	418.3±12.4
KW/BW (g/kg)			
HAD-fed 5/6 Nx rat	4.1±0.1[a]	4.6±0.1[a]	4.8±0.1[a]
LAD-fed 5/6 Nx rat	3.6±0.2	3.6±0.1	3.7±0.1[a]
SRD-fed 5/6 Nx rat	3.7±0.4	3.8±0.1[b]	4.2±0.1[b]
Sham-operated control	3.6±0.2	3.1±0.1	3.2±0.1
Glomerular volume ($\times 10^6 \mu m^3$)			
HAD-fed 5/6 Nx rat	1.84±0.01	2.31±0.05[a]	2.54±0.07[a]
LAD-fed 5/6 Nx rat	1.62±0.05	1.70±0.14[a]	1.96±0.08[a]
SRD-fed 5/6 Nx rat	1.69±0.05	2.0±0.06[b]	2.17±0.04[b]
Sham-operated control	1.02±0.02	1.06±0.02	1.06±0.03

BW, body weight; HAD, high-advanced glycation end products diet; KW, kidney weight; LAD, low-advanced glycation end products diet; Nx, nephrectomy; SRD, standard rodent diet.
Data are expressed as mean±s.d.
[a]$P<0.05$, compared with SRD-fed 5/6 Nx rat.
[b]$P<0.05$, compared with Sham-operated control.

rats than in SRD-fed animals. Glomerular volume also increased with time in the remnant kidney and the increment was higher in HAD-treated animals than in SRD-treated rats. In contrast, LAD-fed rats showed significant lower weight of the remnant kidney at week 13 and decreased glomerular volume from week 9 as compared with SRD-fed animals.

Effect of dietary AGEs on renal tissue damage

Glomerulosclerosis and interstitial fibrosis progressed with time in 5/6 Nx rats (Figure 2). Compared with SRD-fed rats, HAD-fed animals showed marked increase of glomerulosclerosis index (Figure 2a) and interstitial fibrosis score (Figure 2b) from week 5. Animals given LAD showed significant improvement of glomerulosclerosis and interstitial fibrosis in the remnant kidney as compared with rats received SRD. There was a close correlation between serum AGEs levels and glomerulosclerosis index ($r=0.601$, $P<0.001$, $n=90$) or interstitial fibrosis score ($r=0.696$, $P<0.001$, $n=90$) in 5/6 Nx rats (Figure 3a and b).

To determine the glomerulotubular neck sclerosis, 72±44 glomeruli were studied per slide. About 6.9–29.5% of the

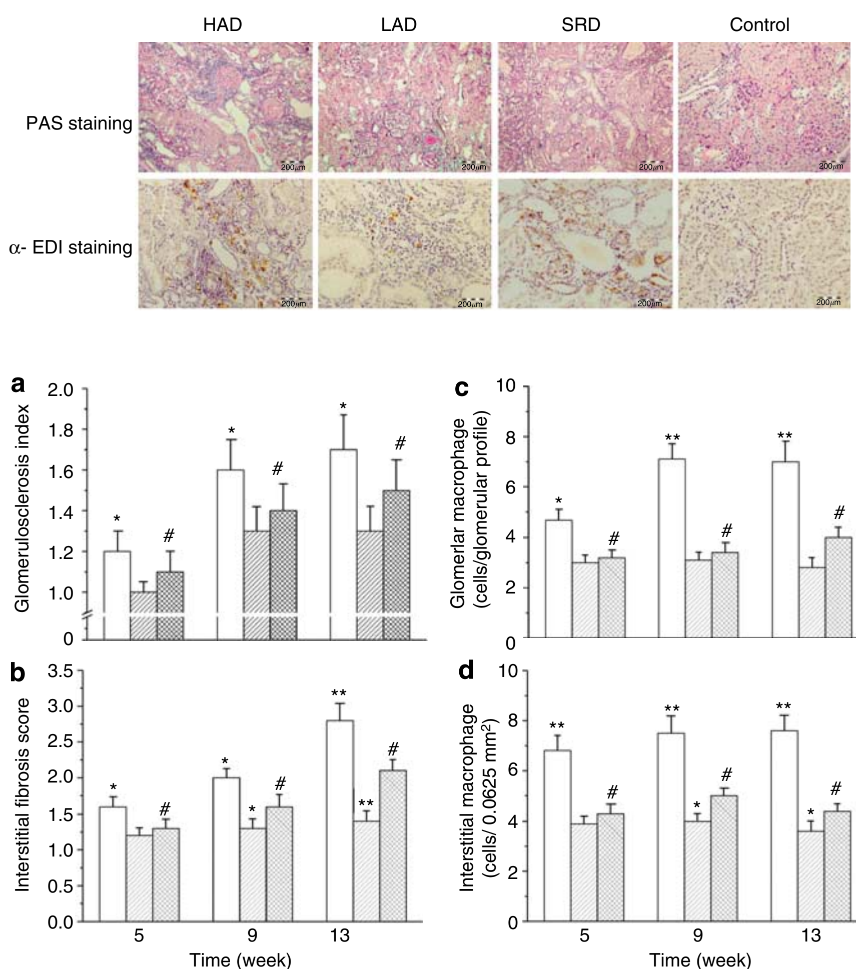

Figure 2 | Glomerulosclerosis index (a), interstitial fibrosis score (b), macrophage infiltration in glomeruli (c), and in interstitium (d) of 5/6 Nx rats on HAD (□), LAD (▨), SRD (▩), and control rats on SRD (■). Data are expressed as mean±s.d., $n=10$ in each group at each time point. (**a**) ANOVA, $P<0.01$, (**b**) ANOVA, $P<0.01$, (**c**) ANOVA, $P<0.05$, (**d**) ANOVA, $P<0.001$ at week 5, 9, and 13. *$P<0.05$ vs SRD; **$P<0.001$ vs SRD; [#]$P<0.05$ vs control.

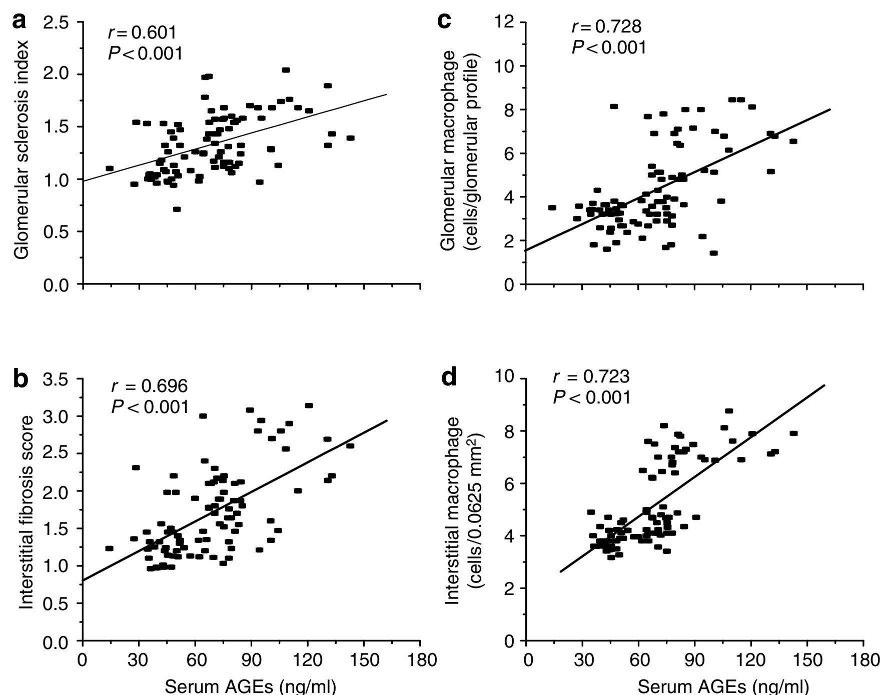

Figure 3 | Relationship between serum AGEs levels and glomerulosclerosis index (a), interstitial fibrosis index (b), and macrophage infiltration in glomeruli (c) and in interstitium (d).

glomeruli had identifiable glomerulotubular junctions. The average internal glomerulotubular junction lumen diameter was $24.0 \pm 7.5\,\mu m$ in sham-operated controls, $8.8 \pm 3.6\,\mu m$ in 5/6 Nx rats fed with HAD, $10.7 \pm 4.0\,\mu m$ in SRD, and $18.7 \pm 6.7\,\mu m$ in rats treated with LAD (analysis of variance (ANOVA), $P<0.001$; LAD vs HAD, $P<0.001$; LAD vs SRD, $P<0.001$).

There were few ED1-positive cells in normal renal tissue removed at the time of the surgery and in sham-operated animals. Macrophage infiltration was evident in both glomeruli and interstitium of the remnant kidney. From week 5, the number of infiltrated macrophages was markedly increased in HAD- vs SRD-fed 5/6 Nx rats. Restriction of dietary AGEs significantly decreased macrophage infiltration in the remnant kidney (Figure 2c and d). A close correlation was found between serum AGEs levels and the number of ED1-positive cells in both glomeruli ($r=0.728$, $P<0.001$, $n=90$) and in interstitium ($r=0.723$, $P<0.001$, $n=90$) in the remnant kidney (Figure 3c and d).

Effect of dietary AGEs on renal function

Subtotal nephrectomy resulted in renal dysfunction, as evidenced by progressive increase in serum creatinine (Figure 4a) and decline of creatinine clearance (Ccr) (Figure 4c). Systolic blood pressure (BP) did not change during the first 5 weeks, but progressively rose at latter time points (Figure 4d). HAD in 5/6 Nx rats significantly worsen renal dysfunction and proteinuria without altering systolic BP. In contrast, LAD-treatment significantly retarded renal dysfunction and decreased proteinuria at week 13 (Figure 4a–d). There was a close correlation between serum AGEs levels and urinary protein excretion ($r=0.724$, $P<0.001$, $n=90$) (Figure 4e) or Ccr ($r=-0.342$, $P=0.001$, $n=90$) (Figure 4f) in the 5/6 Nx rats.

Effect of dietary AGEs on expression of monocyte chemoattactant protein-1 and transforming growth factor-β1

Immunohistological studies revealed that monocyte chemoattactant protein-1 (MCP-1) and transforming growth factor-β1 (TGF-β1) staining was limited to minimal positive reaction of tubular cells and negative in glomeruli. Among 5/6 Nx rats, positive staining of MCP-1 (Figure 5) and TGF-β1 (Figure 6) was observed in both tubulointerstitium and glomeruli. Compared with SRD-fed 5/6 Nx rats, higher staining scores were observed in HAD-fed rats and lower scores were found in LAD-fed animals (Figures 5a and b and 6a and b). Likewise, expression of MCP-1 (Figure 5c) and TGF-β1 mRNA (Figure 6c) were upregulated in HAD-fed rats and downregulated in LAD-fed 5/6 Nx rats as compared with SRD-fed animals.

Uninary MCP-1 (Figure 5d) and TGF-β1 (Figure 6d) were markedly increased in 5/6 Nx rats as compared with sham-operated controls. HAD-fed rats showed higher levels of urinary MCP-1 and TGF-β1 excretion than rats fed with SRD. Restriction of dietary AGEs significantly decreased urinary MCP-1 and TGF-β1 levels from week 9 (Figures 5d and 6d).

Effect of dietary AGEs on renal redox reaction

As shown in Figure 7, thiobarbituric acid reactive substance (TBARS) and advanced oxidation protein products (AOPPs) levels in renal cortical homogenates significantly increased in

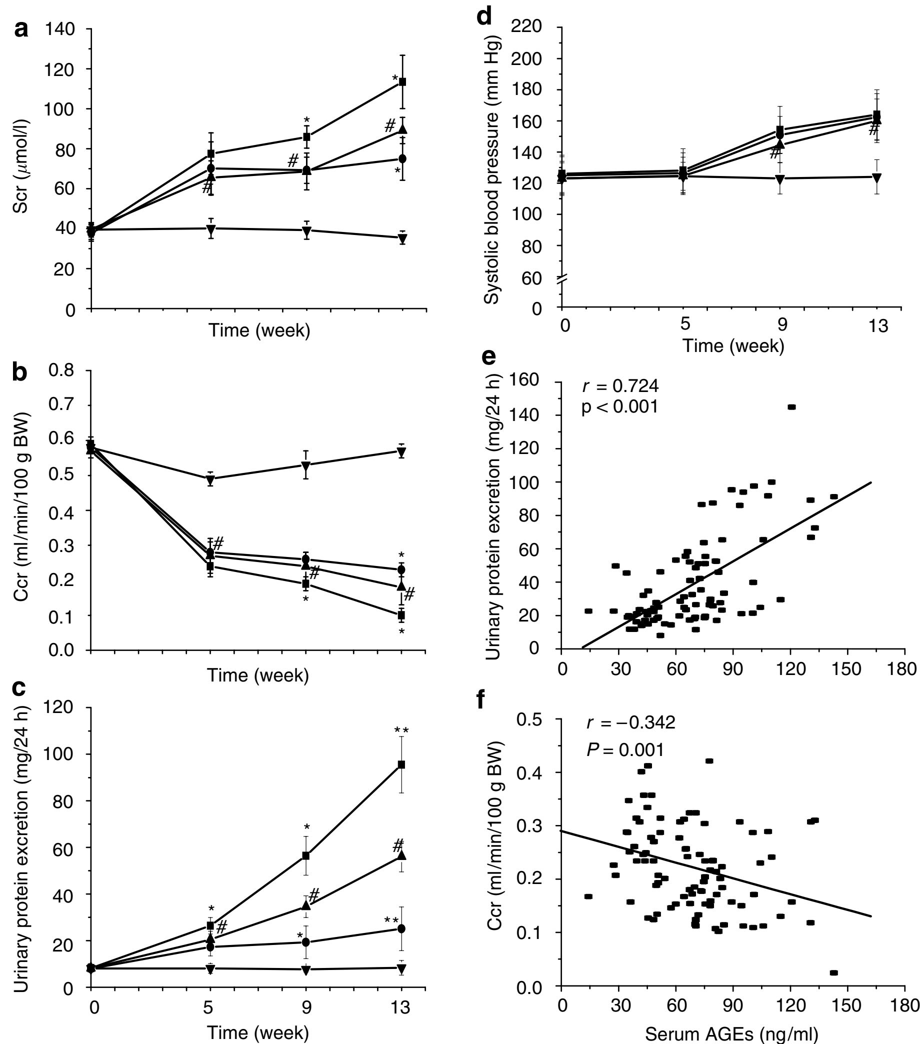

Figure 4 | Serum creatinine (a), Ccr (b), urinary protein excretion (c), and systolic BP (d) of 5/6 Nx rats on HAD (—■—), LAD (—●—), SRD (—▲—) or control rats on SRD (—▼—). The relationship between serum AGEs levels and (**e**) urinary protein excretion and (**f**) Ccr. Data are expressed as mean ± s.d., $n = 10$ in each group at each time point. (**a**) ANOVA, $P < 0.05$, (**b**) ANOVA, $P < 0.05$, (**c**) ANOVA, $P < 0.001$ at week 5, 9, and 13. *$P < 0.05$ vs SRD; **$P < 0.001$ vs SRD; $^{\#}P < 0.05$ vs control.

5/6 Nx rats. HAD treatment significantly increased TBARS levels (Figure 7a) and AOPPs levels (Figure 7b) from week 9. Glutathione peroxidase (GSHPx) activity decreased markedly in HAD-fed rats from week 9 as compared with SRD-fed animals (Figure 7c). At week 13, TBARS and GSHPx activity tended to restore in SRD-fed 5/6 Nx rats, but sustained in HAD-fed animals. LAD-treatment significantly decreased renal TBARS and AOPPs levels and increased GSHPx activity (Figure 7a–c). There was a close correlation between renal AGEs levels and renal TBARS ($r = 0.654$, $P < 0.001$, $n = 90$) (Figure 7d), renal AOPPs ($r = 0.815$, $P < 0.001$, $n = 90$) (Figure 7e), or renal GSHPx ($r = -0.677$, $P < 0.001$, $n = 90$) (Figure 7f).

DISCUSSION

This study demonstrated that prolonged intake of food rich in AGEs in the remnant kidney model resulted in accelerated progression of renal damage and that certain aspect of this pathology were largely prevented by the restriction of dietary AGEs intake. Such restriction improves renal function, as evidenced by reduction of proteinuria and decrease of tubular fibrosis and glomerulosclerosis. It also retarded decline of renal function without alteration of BP. Although there is extensive evidence from both animals and clinical studies showing significant changes in circulating AGEs levels as a result of dietary AGEs modulation,[8,9,17,20] to the best of our knowledge, this is the first study demonstrating that restriction of dietary AGEs intake in chronic renal insufficiency provides renal protection not only on proteinuria, but also on progression of renal fibrosis and dysfunction.

Consistent with the previous reports,[9,17] our results showed significant increase of serum CML immunoreactivity in 5/6 Nx rats fed with HAD. CML has been recognized as marker of glycoxidant burden *in vivo*[19,21] and is thought to

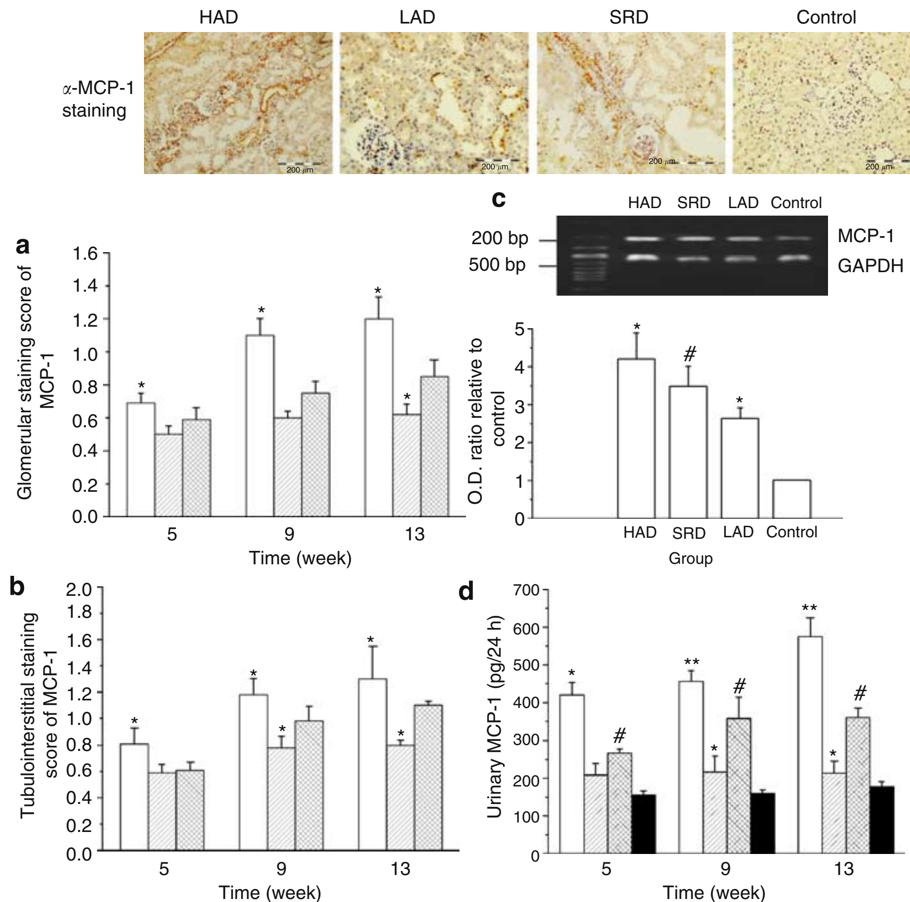

Figure 5 | Glomerular α-MCP-1 staining score (a), tubulointerstitial α-MCP-1 staining score (b), MCP-1 mRNA expression (c), and urinary MCP-1 levels (d) of 5/6 Nx rats on HAD (□), LAD (▨), SRD (▩), and control rats on SRD (■). Data are expressed as mean ± s.d., $n = 10$ in each group at each time point. (**a**) ANOVA, $P < 0.05$, (**b**) ANOVA, $P < 0.001$, (**c**) ANOVA, $P < 0.05$, (**d**) ANOVA, $P < 0.01$ at week 5, 9, and 13. *$P < 0.05$ vs SRD; **$P < 0.01$ vs SRD; $^{\#}P < 0.05$ vs control.

contribute to intracellular oxidative stress and tissue damage.[19] However, the mechanisms by which dietary glycoxidation products increase serum CML remain to be investigated, as some studies report that CML may not be actively absorbed.[22,23]

Furthermore, we found that increased circulating AGEs were associated with enhanced burden of AGEs in renal tissue. Previous studies have demonstrated that approximately 10% of ingested AGEs enter the circulation, but only one-third are excreted within 3 days of ingestion.[24] Chronic renal dysfunction or diabetes is associated with impairment in AGEs excretion.[25] Thus, dietary AGEs intake might be an important contributor to the body AGEs burden, particularly in renal insufficiency and diabetes. The increase in kidney-associated AGEs noted in HAD-fed rats can be attributed in large part to the covalent attachment of the exogenous AGEs onto to renal tissue through reactive moieties such as collagen or to the deposition of filtrated degradation products of AGEs proteins.[13]

Renal function, as determined by Ccr, significantly decreased after nephrectomy. However, Ccr decreased by 68% at week 13 in 5/6 Nx rats corresponding to 83% resection of renal mass, suggesting that compensatory glomerular hyperfiltration occurred in this model. Supporting this notion, glomerular volume in 5/6 Nx rats significantly increased after renal mass reduction. The glomerular volume in the remnant kidney was higher in HAD-fed than SRD-fed rats. In contrast to the other report,[17] it was not accompanied by any significant increase in body weight. Likewise, the protective effects of LAD were also marked against the glomerular hypertrophy, indicating that dietary intake of AGEs might be involved in the pathogenesis of renal mass hypertrophy and remodeling.

It is interesting to note that LAD rats had less extent of glomeruli hypertrophy and more preserved Ccr. This might be related to the significant improvement of interstitial fibrosis in LAD as compared with HAD or SRD. It has been well established that interstitial fibrosis is a better predictor for renal impairment than glomerular changes.[26] Sclerosis in the glomerulo-tubular neck, which is associated with interstitial fibrosis, provides an anatomic and functional link between tubulointerstitial fibrosis and loss of glomerular function.[27] The less sclerosis of glomerulo-tubular neck seen in LAD fed rats might decrease resistance to tubular flow, and contribute to more preserved glomerular filtration rate.

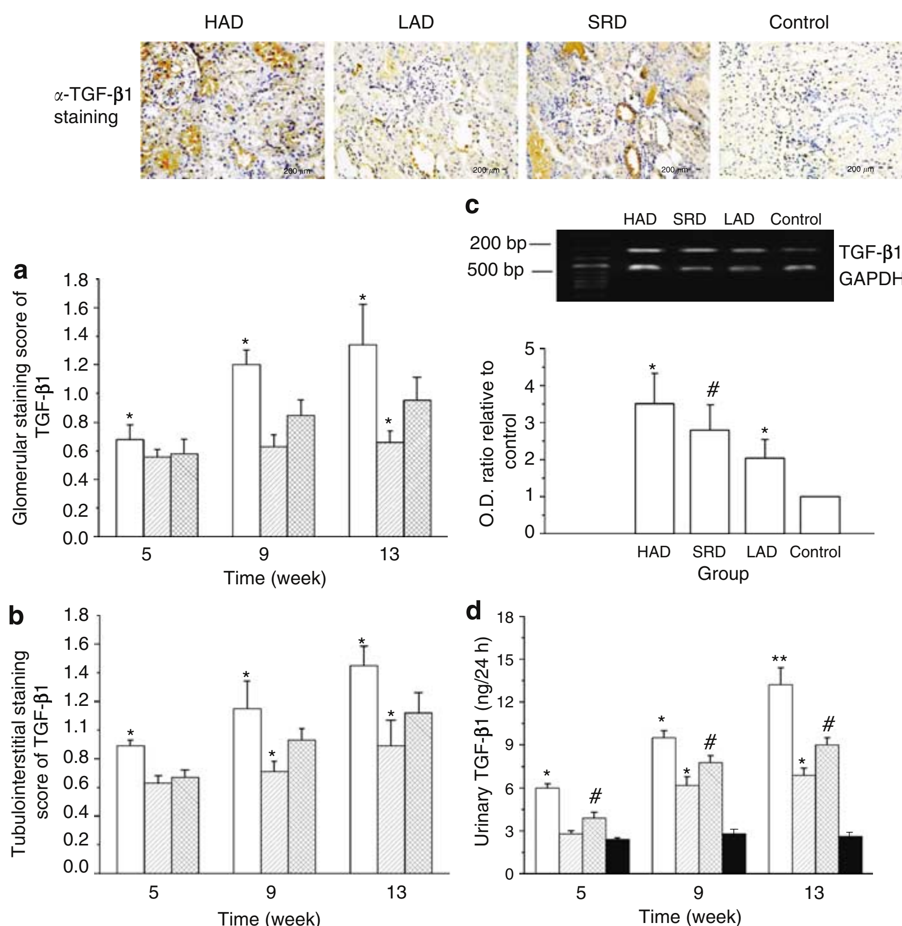

Figure 6 | Glomerular α-TGF-β1 staining score (a), tubulointerstitial α-TGF-β1 staining score (b), TGF-β1 mRNA expression (c), and urinary TGF-β1 levels (d) of 5/6 Nx rats on HAD (□), LAD (▨), SRD (▩), and control rats on SRD (■). Data are expressed as mean ± s.d., $n = 10$ in each group at each time point. (**a**) ANOVA, $P < 0.01$, (**b**) ANOVA, $P < 0.001$, (**c**) ANOVA, $P < 0.05$, (**d**) ANOVA, $P < 0.01$ at week 5, 9, and 13. *$P < 0.05$ vs SRD; **$P < 0.001$ vs SRD; $^{\#}P < 0.05$ vs control.

Our results showed that modulation of diet AGEs content affected renal mass remodeling associated with renal mass reduction. The basic nutrients of the three diets and the daily food intake among the three groups of 5/6 Nx rats were comparable, and, there was no significant difference in serum albumin and lipid profile among groups during the study period, suggesting that the observed effects were not attributable to differences in nutrition or lipid profiles. AGEs can inhibit the activity of nitric oxide in previous study.[28] However, there is no evidence to support such a mechanism of action in this study, based on the absence of changes in BP. A recent *in vivo* study demonstrated that activity of renin-angiotensin system is elevated by infusion of AGEs-modified protein and this is associated with glomerular and tubular hypertrophy.[29] Although not tested, changes in glomerular volume and kidney weight were found in response to the modulation of dietary AGEs, suggesting that food-based AGEs may have the similar effect on renal hypertrophy.

The accelerated renal fibrosis observed in HAD-fed rats may be mediated at least in part by the induction of renal oxidative stress and inflammation. Intake of dietary AGEs in the remnant kidney model significantly enhanced the oxidative stress in renal tissue, as evidenced by increase of renal AOPPs, markers of oxidant-induced protein damage, and increase of TBARS, which reflects the levels of lipid peroxidation. HAD administration also reduced the activity of anti-oxidative system *in vivo*, as demonstrated by decrease of intrarenal GSHPx activity. Furthermore, intake of dietary AGEs in rats with renal impairment upregulated proinflammatory chemokine, MCP-1. Overexpression of MCP-1 has been found to be associated with the cellular inflammation and the myofibroblastic activity in renal parenchyma.[30] HAD treatment also increased expression of TGF-β1, a well-documented fibrogenic growth factor that plays a major role in the pathogenesis of renal inflammation and fibrosis.[31] More important finding is that HAD associated intrarenal oxidative stress and proinflammatory reaction can be largely inhibited by restricted intake of dietary AGEs, suggesting that food-based glycoxidation products might be potential inducers of oxidative stress and inflammation in CKD. The close relationship between renal AGEs and the parameters of redox and inflammatory reaction provide further evidence

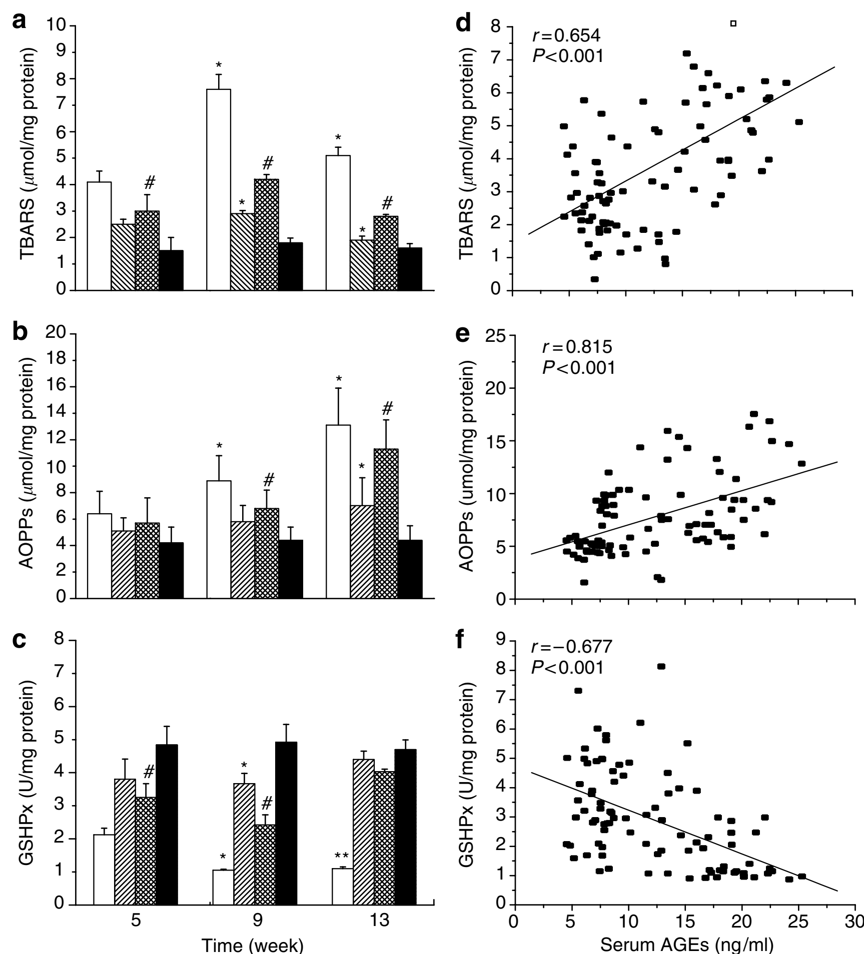

Figure 7 | Renal TBARS (a), AOPPs (b), GSHPx activity (c) of 5/6 Nx rats on HAD (□), LAD (▨), SRD (▩) and control rats on SRD (■). The relationship between renal AGEs and TBARS (**d**), AOPPs (**e**), and GSHPx activity (**f**). Data are expressed as mean ± s.d., $n = 10$ in each group at each time point. (**a**) ANOVA, $P<0.05$, (**b**) ANOVA, $P<0.05$, (**c**) ANOVA, $P<0.05$ at week 5, 9, and 13. *$P<0.01$ vs SRD; **$P<0.001$ vs SRD; #$P<0.05$ vs control.'

supporting the notion. Given the fact that AGEs accumulation occurs from early stage of CKD when Ccr remains at 60 ml/min,[16] it is plausible to propose that prolonged intake of food rich in AGEs may increase AGEs accumulation followed by renal mass reduction and enhanced the intrarenal oxidative stress and inflammation. Reactive oxygen species produced during oxidative stress and inflammation can further increase endogenous AGEs formation through promoting of glycoxidation.[5] This positive feedback loop could amplify or maintain the imbalance of redox reaction and inflammation status, and thus accelerate the renal fibrosis and the progression of CKD. If this hypothesis is true, restricted intake of food rich in AGEs might ameliorate the progression of CKD and should be considered as an intervention in CKD.

In summary, we demonstrated that intake of food rich in AGEs accelerates renal progression in the remnant kidney model. Restriction of dietary AGEs intake retards renal fibrosis and dysfunction, probably via improving renal oxidative stress and inflammation.

MATERIALS AND METHODS

Rat dietary formulas

The three diets used in this study were purchased from the Guangdong Province Institute of Animals Feed (Guangzhou, China). SRD is a rodent diet that is exposed to heat (50°C for 15 min) during pelleting and is fortified with supplement to offset heat-depleted micronutrients. LAD was prepared by SRD without exposure to heat, and HAD consisted of the same SRD exposed to the heat cycle (180°C for 30 min). The three dietary formulas were nutritionally equivalent (Table 1). However, as reported previously,[32] heat procedure increased AGEs content, based on assessment of enzyme-linked immunosorbent assay using an anti-CML polyclonal antibody[12,18] and on determination of total AGEs fluorescence by fluorospectrophotometry.[19] LAD contained sevenfold lower level of CML and 2.5-fold lower fluorescent AGEs as compared with HAD. The diets were kept at 4°C.[33]

Animals and treatment

Male 12-week-old Sprague–Dawley rats (initial weight, 220–240 g, $n = 120$) were purchased from the Southern Medical University Animal Experiment Center. Rats were given food and water *ad libitum* in a standardized environment at the center for Laboratory

Animal Science, Nanfang Hospital. The Animal Experiment Committee of Southern Medical University approved animal care and experimental procedures. After 1 week of adjustment, the rats were subjected either to 5/6 Nx ($n=90$, by performing a right nephrectomy with surgical resection of the two-thirds of the left kidney) or to sham operation (controls, $n=30$). One week after the operation, the 5/6 Nx rats were randomized by the percent remnant kidney weight removed ((right kidney weight−weight of two poles of left kidney)/right kidney weight $\times 100$), divided into three subgroups ($n=30$ in each subgroup), and were fed for indicated time with the three kinds of diet, respectively. The control rats were given SRD. To guarantee equal food intake, the food intake were recorded daily for 1 week.

At the end of 5, 9, and 13 weeks, the rats ($n=10$ in each time point) were anesthetized and exsanguinated by an abdominal aortic puncture. The left kidneys were collected after perfusion with 50 ml of ice-cold normal saline. The 24-h urine samples were collected in the metabolic cages before killed.

Assessment of AGEs content

The concentration of AGEs in all specimens was measured by a competitive enzyme-linked immunosorbent assay using a polyclonal antibody against CML, as described previously.[12,18] The antibody is highly reactive with soluble and structural proteins modified by CML, an established biomarker for AGEs.[18,19] We also quantitated fluorescent AGEs in the diet by using fluorospectrometry as described previously.[18] This method detects low molecular mass AGEs peptides or free adducts such as pentosidine crosslink and argpyrimidine.[34] The content of AGEs determined by the above methods has been compared with that measured by high-performance liquid chromatograph and by mass spectrometry.[12] Forty-three units/mg of AGEs as measured by fluorospectrometry were equal to 135 pmol/mg of pentosidine as measured by high-performance liquid chromatograph and 525 ng/mg of AGEs measured by enzyme-linked immunosorbent assay were equal to 43.8 nmol/mg of CML as determined by mass spectrometry.[12]

BP and kidney function

Systemic BP was measure before the rats were killed. A PE-50 catheter was inserted into the femoral artery and BP was determined by a pressure transducer (Gould, MA, USA) connected to a physiological recorder (Gilson Medical Electronics, Middleton, OH, USA).

Twenty-four-hour urinary excretion was measured using the Coomassie Blue method.[35] Creatinine levels in serum and urine were measured by using a commercial kit (sarcosine oxidase-peroxidase-antiperoxidase, Zixing, Shanghai, China). Ccr was calculated and factored for body weight as described previously.[36]

Renal quantitative morphometry

Renal tissue was embedded in paraffin and sliced in 2 μm-thick sections. These were stained by periodic acid-Schiff and Masson's Trichrome. Morphological analyses were performed by an experienced pathologist blinded to the source of the tissue. The extent of glomerular sclerosis was assessed as described previously.[37] At least 50 glomeruli from each kidney were graded for individual glomeruli on the periodic acid-Schiff-stained sections according to the following criteria: 0, no sclerosis; 1, less than 25% cross-sectional sclerosis; 2, 25–50% exhibiting sclerosis; 3, 50–75% exhibiting sclerosis; and 4, over 75% cross-sectional sclerosis. The sclerosis index for each rat was calculated as follow: $(N1\times 1+N2\times 2+N3\times 3+N4\times 4)/n$, where N1, N2, N3, and N4 represent the numbers of glomeruli exhibiting grades 1, 2, 3, and 4, respectively, and n represents the number of glomeruli assessed.[38] Similarly, trichrome-stained sections from each kidney cortex were graded for the presence of interstitial fibrosis according to the following scale: 0, no evidence of interstitial fibrosis; 1, less than 25% involvement; 2, 25–50% involvement; and 3, more than 50% involvement.[39] The scale for each rat was reported as the mean of 20 random high-power ($\times 400$) fields per section. The average glomerular tuft volume was estimated at each time of killing as described previously.[40] The mean glomerular cross-sectional area (Ag) was determined by averaging approximately 50 glomerular sections. Individual glomerular volume (Vg) was calculated as $Vg=1.25\times(Ag)\ (3/2)$.[40] Glomerulo-tubular neck diameters were measured at $\times 400$ magnification with a microscope micrometer (HMIAS-2000, Champion Image, Wuhan, China) as described previously.[27] Five 5-μm serial sections stained by periodic acid-Schiff were mounted per slide. A total of 20 slides (five slides from each group) were studied in a masked manner.

Renal immunohistochemical analyses

The immunoperoxidase staining was performed as described previously,[41] with the following antibodies: macrophage infiltration were detected with monoclonal anti-ED1 (Serotec, Oxford, UK); TGF-β1 expression was detected with polyclonal rabbit anti-rat TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); MCP-1 was analyzed with polyclonal rabbit anti-rat MCP-1 (Boster Biological, Wuhan, China). Control experiments included omission of the primary antibodies and substitution of the primary antibodies with nonimmune rabbit or mouse immunoglobulin G.

Macrophage infiltration was quantitated by counting the number of ED1-positive cells in 30 glomerular profiles and in 20 randomly chosen 0.25×0.25 mm areas of tubulointerstitium for each kidney.[42]

The intensity of glomerular staining of TGF-β1 and MCP-1 was evaluated under $\times 400$ magnification according to the following scale: 0, no staining; 1, weak and spotty intraglomerular staining; 2, moderate and segmental intraglomerular staining; and 3, strong and diffuse (involving more than 50%) intraglomerular staining. Likewise, the intensity of tubulointerstitial staining in cortical areas was assessed under $\times 250$ magnification as the following scale: 0, no staining; 1, less than 25% involvement; 2, 25–50% involvement; and 3, more than 50% involvement. All, but at least 20 glomeruli and 20 randomly selected cortical tubulointerstitial areas from each sample were evaluated.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from renal cortex tissue using RNase mini kit (Qiagen, Valencia, CA, USA). Primers for MCP-1, TGF-β1, and glyceraldehydes-phosphate dehydrogenase were designed and synthesized based on published sequence of these genes.[42] The upstream and downstream of these primers are: (1) MCP-1: 5′-GTC ACC AAG CTC AAG AGA GAG A-3′, 5′-GAG TGG ATG CAT TAG CTT CAG A-3′; (2) TGF-β1: 5′-CTT CAG CTC CAC AGA GAA GAA CTG C-3′, 5′-CAC GAT CAT GTT GGA CAA CTG CTC C-3′; (3) glyceraldehydes-phosphate dehydrogenase: 5′-AAT GCA TCC TGC ACC ACC AA-3′, 5′-GTA GCC ATA TTC ATT GTC ATA-3′. Reverse transcriptase-polymerase chain reaction was performed using Qiagen One Step reverse transcriptase-polymerase chain

reaction kit (Qiagen) according to the manufacturer's instruction. Polymerase chain reaction products resolved in 2% agarose gels were photographed under ultraviolet light. Densities of bands were measured by scanning densitometry with UVIBAND V.99 Software (UVI, SJ, England). The results were expressed as optical density ratios relative to control (MCP-1 or TGF-β1: glyceraldehydes-phosphate dehydrogenase).

Parameters of biochemistry, oxidative stress, and inflammation

Serum levels of albumin and lipid were analyzed by an automatic biochemistry analyzer (Olympus AU540, Japan).

Lipid peroxides in the renal cortical homogenate were measured as TBARS by fluorometric assay.[43] GSHPx activity and AOPPs in the renal tissue were measured as described previously.[44,45] Urinary MCP-1 or TGF-β1 were quantitated by enzyme-linked immunosorbent assay using commercial kits (BioSource, Camarillo, CA, USA) according to the manufacturer's instruction.

Statistical analyses

All data are presented as mean$\pm$s.d. Main and interactive effect of AGEs and 5/6 Nx were analyzed by factorial analysis. Serum creatinine, Ccr, and urinary protein excretion were adjusted by covariates before intervention. Differences in the variables between groups were determined by one-way ANOVA followed by LSD method. Differences in the variables at each time point between 5/6 Nx and sham-operated rats were compared by independent samples *t*-test. The relationship between variables was assessed by Pearson correlation analysis. Statistical analysis was conducted with SPSS 12.0 for Windows. Significance was defined as $P \leqslant 0.05$.

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