

Original Article

Histopathology and urinary metabonomics reveal the role of dietary salt on the pathogenesis of fructose-induced kidney injury

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Abstract: Although increasing evidence demonstrates that dietary salt is involved in hypertension induced by high fructose levels (HF), it is not known how dietary salt plays a role in kidney injury caused by HF. Therefore, we investigated the effects of different diets on abnormal renal histology and urinary metabolites. SD rats were randomly divided into five groups: a normal diet (0.5% NaCl, w/w); a high-salt diet (HS; 8% NaCl); 10% fructose in water (HF; w/v); high-salt diet and 10% fructose water (HF-HS); and low-salt diet (LS; 0.07% NaCl) with 10% fructose water (HF-LS). Eight weeks of the HF diet induced marked glomerular hypertrophy (PAS-staining), podocyte injury (desmin), tubular damage and inflammatory cell infiltration. However, the HS diet produced glomerular sclerosis (α -SMA) and podocyte injury. Importantly, metabonomic analysis showed that compared with the normal diet, the HS diet elicited the urinary excretion of trimethylamine oxide (TMAO) and hippurate (urinary microbial metabolites), whereas high fructose primarily affected the activity of the tricarboxylic acid (TCA) cycle. Notably, switching to LS diet halted the progression of HF-induced renal injuries. Further, the excretion of TMAO and hippurate from HF-LS rats was significantly reduced compared with normal rats, whereas the HF+HS diet promoted excretion. Our findings indicate that dietary salt is involved in renal injury induced by HF, probably by affecting gut microbiota activity.

Keywords: Dietary salt, high fructose water, renal injury, urinary metabonomics, urinary metabolites

Introduction

Renal interstitial fibrosis involves various pathogenic processes, including interstitial hypercellularity, matrix accumulation, and atrophy of epithelial structures, which lead to a loss of normal function and renal failure [1]. Moreover, interstitial fibrosis is the strongest morphological predictor of clinical outcomes and is the most tightly linked variable to disease progression [2]. Although most renal diseases originate from glomerular injuries such as diabetic nephropathy, interstitial involvement indicates a more ominous outcome [3]. The relationship between excessive sodium intake in the modern diet and cardiovascular risk factors, particularly hypertension, volume overload and chronic kidney disease (CKD), has been extensively investigated [4-6]. Recent research has revealed that high salt intake may enhance the

production of transforming growth factor- β (TGF- β) and lead to renal fibrosis [7]. Meanwhile, compelling evidence implicates the increased consumption of both fructose and salt in our daily diet [8]. High fructose intake is believed to play a role in the increasing epidemic of metabolic disorders [9]. The administration of high fructose to rats led to afferent arteriolar thickening, glomerular hypertension, glomerular hypertrophy, renal inflammation, tubulointerstitial injury and cortical vasoconstriction [10]. Both salt [11, 12] and fructose [13-15] can be absorbed in the small intestine and kidney proximal tubule via different mechanisms. Previous studies have shown that a high fructose diet significantly decreases the daily excretion of chloride and sodium in the rat kidney [16]. Recent studies in rodents have also demonstrated that increased dietary fructose intake stimulates salt absorption in the small intestine

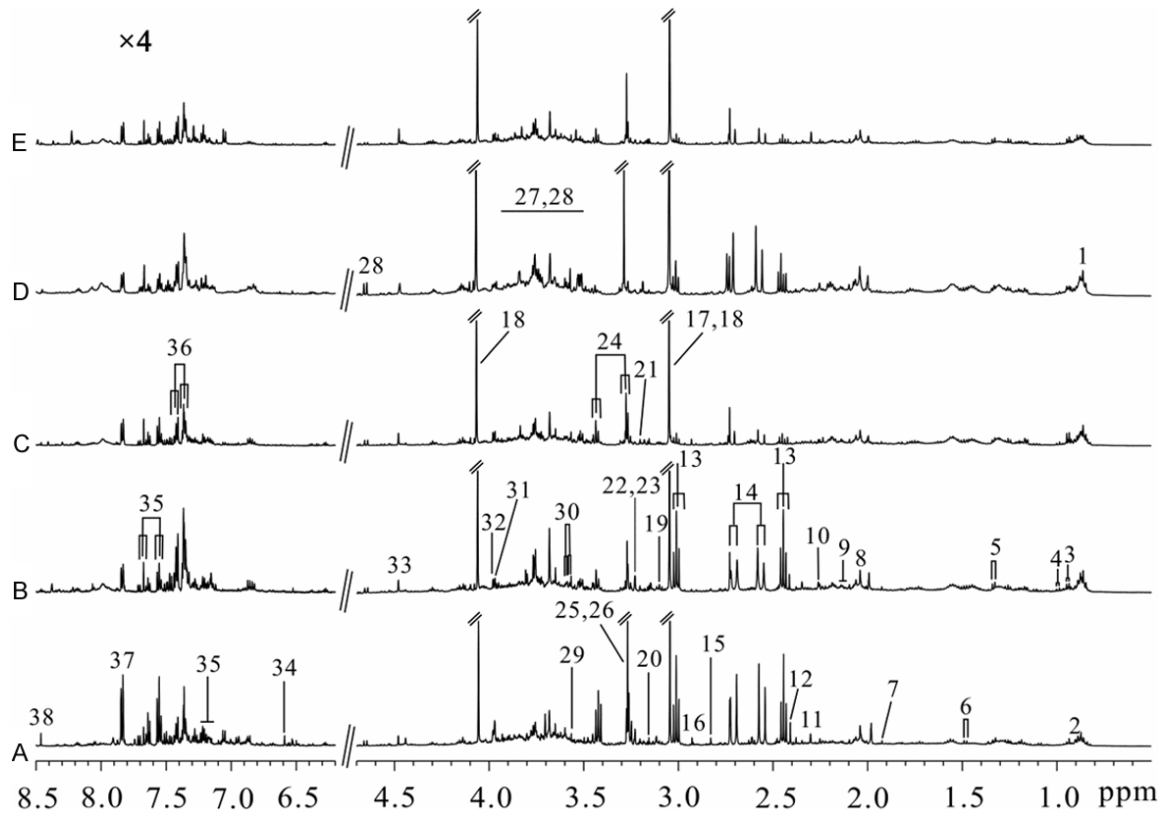


Figure 1. Representative urine (A: Normal, B: HF, C: HS, D: HF+HS, E: HF+LS) at the end of the 8th week. Keys: 1. 2-hydroxybutyrate; 2. isoleucine; 3. leucine; 4. valine; 5. lactate; 6. alanine; 7. acetate; 8. N-acetylglycoprotein; 9. glutamine; 10. acetone; 11. acetoacetate; 12. succinate; 13. α -KG; 14. citrate; 15. DMA; 16. DMG; 17. creatine; 18. creatinine; 19. tyrosine; 20. N-acetylcarnitine; 21. choline; 22. PC; 23. GPC; 24. taurine; 25. TMAO; 26. betaine; 27. α -glucose; 28. β -glucose; 29. glycine; 30. glycerol; 31. phosphoethanolamine; 32. hippurate; 33. N-methylnicotinamide; 34. fumarate; 35. tryptophan; 36. phenylalanine; 37. uridine; 38. formate.

and renal proximal tubules, resulting in a state of salt overload and hypertension [17]. However, the role of salt in the pathogenesis of renal injury associated with high fructose is still unclear. Therefore, in the present study, we investigate the link between histopathologic changes and urinary metabonomic profiles in rats treated with high-fructose water and a high- or low-salt diet to understand the underlying mechanism of renal injury.

Materials and methods

Animals and treatments

Eight-week-old male Sprague-Dawley (SD) rats (180-200 g) were obtained from Guangdong Medical Laboratory Animal Center (permit number: SCXK (YUE) 2008-0002). All animal procedures were approved by the animal care and use committees at the Guangdong Phar-

maceutical University, and the rats were exposed to a 12-h light/dark cycle at $22 \pm 2^\circ\text{C}$ for 2 weeks with free access to food and water in a pathogen-free facility. Rats were randomly divided into five groups: (i) group Normal ($n=10$) was fed a normal diet (0.5 NaCl, w/w); (ii) group HS ($n=10$) was fed a high-salt diet [18] (8% NaCl, w/w, online [Supplementary Material](#)); (iii) group HF ($n=10$) received a high-fructose water (10% fructose in water, w/v) and normal salt diet (0.5% NaCl, w/w); (iv) group HF+HS ($n=10$) was fed a high-fructose water and high-salt diet; and (v) group HF+LS ($n=10$) received a high-fructose water and low-salt diet (0.07% NaCl, w/w). After 8 weeks on the diets, the rats were placed in metabolic cages, and 24-h urine samples were collected. The collection container was disinfected with 1% sodium azide (50 μL) before urine collection. All samples were stored at 80°C for NMR determination. Organs

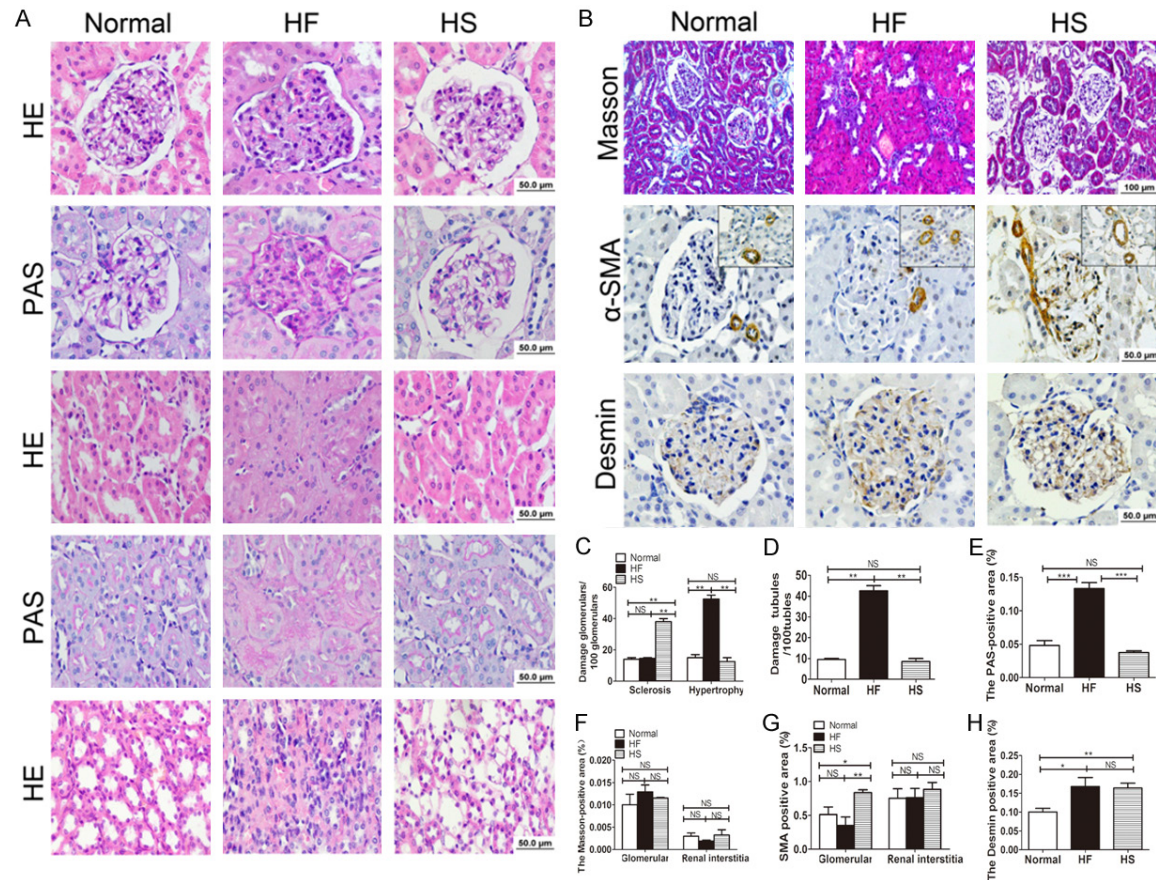


Figure 2. Effect of high fructose or high salt on the histology of renal glomerular and interstitial injuries. Representative images from the normal diet group (n=10), high-fructose water group (n=10) and high-salt diet group (n=10) after 8 weeks. A: Hematoxylin-Eosin and Periodic acid-Schiff staining. B: Masson-staining and immunohistological staining. C: Relative numbers of damaged glomeruli in 100 randomly-selected glomeruli. D: Relative numbers of damaged tubules in 100 randomly-selected tubules. E: Quantification of the PAS-positive area. F: Quantification of the Masson-positive area. G: Relative expression of α-SMA in the kidney. H: Relative expression of desmin in the kidney. HF: High-fructose; HS: high-salt. Data are the mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001. NS: no significance.

were dissected under sodium pentobarbital anesthesia (60 mg/kg, i.p.).

Histological assessment

Kidney tissues fixed with 10% paraformaldehyde were embedded in paraffin. Kidney sections (3 μm) were stained with periodic acid-Schiff (PAS) to measure glomerulosclerotic injury and mesangial expansion and with Masson's trichrome to assess the glomerular and tubulointerstitial ECM accumulation. Mesangial area and ECM accumulation (percentage of glomerular area) were evaluated from digital pictures of glomeruli (20 glomeruli per animal) using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) [19]. To evaluate glomerular hypertrophy

(glomerular diameter), an area containing approximately 25-30 glomeruli per kidney section was measured in the Image-Pro Plus software [20]. Glomerulosclerotic injury was graded based on the severity of glomerular damage.

Immunohistochemistry

Rehydrated paraffin-embedded sections were blocked in 5% of BSA, 5% of donkey serum and 0.01% of sodium azide in PBS buffer for 4 h in a humidity chamber. Following a brief rinse, sections were incubated with 1:100 of rabbit anti-α-SMA (Abcam) overnight in humidity chambers at 4°C. Then, the sections were washed with PBS buffer and incubated with 1:200 of the appropriate secondary antibody. All primary and secondary antibodies were di-

luted in a 10-fold diluted blocking agent. HRP-conjugated IgG was used as the secondary antibody (Boster ABC kit; Boster biotechnology company, Wuhan, China). As a negative normal, primary antibody diluent was used. The sections were examined using light microscopy.

Sample preparation for NMR analysis

The urine samples were thawed at room temperature prior to NMR analysis. First, 300 μ L of sample was mixed with 200 μ L of phosphate buffer (0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.4) to minimize the chemical shift variations, and the mixture was then centrifuged (14000 g, 10 min, 4°C) to remove any precipitates. The supernatant was then pipetted into a 5 mm NMR tube, and 80 μ L of D_2O containing 0.05% sodium 3-trimethylsilyl-(2,2,3,3- D_4)-1-propionate (TSP) was added.

^1H NMR spectroscopy

^1H NMR spectra of all samples were collected at 298 K on a Bruker Avance III 500 MHz spectrometer. The ^1H NMR spectra of the urine and fecal extract samples were recorded using the water-presaturated standard one-dimensional NOESYPR1D pulse sequence (recycle delay-901-t1-901-tm-901-acquisition) to obtain a representation of the total metabolite composition. Overall, 128 transients were collected into 32k data points using a spectral width of 10 kHz with a relaxation delay of 3 s and a mixing time (tm) of 100 ms.

Statistical analysis

All ^1H NMR spectra were manually phase- and baseline-corrected and then bucketed and automatically integrated with an automation routine in AMIX. Each ^1H NMR spectrum was segmented into regions of 0.02 ppm. The region δ 4.7-5.2 was discarded to eliminate the effects of residual water signals. For the urine spectra, the region containing urea (δ 5.2-6.2) was also discarded to eliminate the urea signals. The integrals of these buckets covered the region δ 0.5-8.5 and were normalized to the total sum of the spectral integrals. The resulting normalized integral data obtained from urine samples were saved in Microsoft Excel and submitted to PCA using the Simca-P+12.0 software (Umetrics, Sweden). The scores plot, which highlights inherent clustering trends in

the samples, and the loadings plot, which provides potential biomarkers, were both visualized. Statistical analyses were performed using student *t* tests, and $p < 0.05$ was identified as significant. Other results are presented as the means $\pm$ S.E.M. Statistical comparisons of the differences among groups were performed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Distinctive renal injuries between high-fructose- and high-salt-treated rats

Representative photomicrographs of the hematoxylin and eosin (H&E), PAS staining and Masson's trichrome staining of the kidney tissues from normal, HF and HS diet groups following 8 weeks on their different diets are shown in **Figure 2A, 2B**, and the differential glomerular and interstitial injury scores are shown in **Figure 2C-H**. With H&E staining, kidneys from HF diet rats exhibited significant glomerular hypertrophy, inflammatory cell infiltration and marked tubular epithelial hyperplasia, whereas HS rats exhibited marked glomerular sclerosis and, thus, more Bowman's space than did rats that ate a normal diet (**Figure 2C**). Compared with normal rats, the amount of damaged tubules was markedly increased in HF rats but was not enhanced in HS rats (**Figure 2D**). HF rats had an increased accumulation of PAS-positive ECM proteins in glomeruli and the mesangium compared with normal rats, but there was no difference in the expression of PAS-positive ECM proteins between HS and normal rats (**Figure 2E**). Masson's trichrome staining showed that there was no difference among the groups (**Figure 2F**). Furthermore, consistent with the findings for glomerulosclerosis in HS rats, the expression of α -SMA, a marker of myofibroblasts, was higher in the glomeruli of HS rats than in normal and HF rats (**Figure 2G**). We also examined glomerular podocyte injury by desmin expression [21, 22], as evaluated by immunohistochemistry. Both HS and HF rats showed a markedly increased glomerular desmin-positive area compared with normal rats, and there was no difference between HF and HS rats (**Figure 2H**). Therefore, these observations indicated that HS alone mainly caused glomerulosclerosis, whereas HF

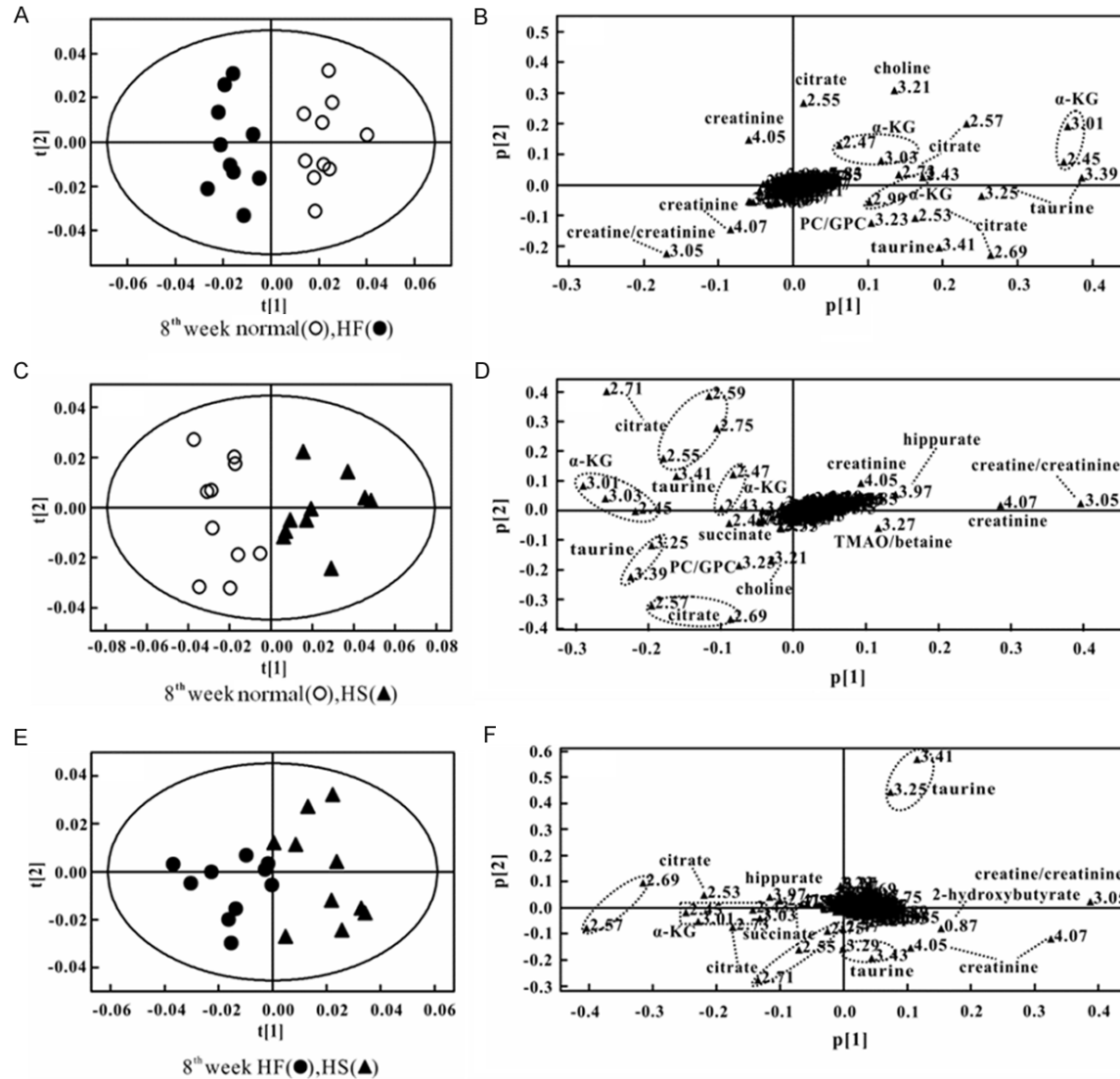


Figure 3. Effect of high fructose or high salt on urinary metabonomics profiles in rats. Multivariate analyses of urinary ^1H NMR spectra data at the end of the 8th week. A, B: Scores plot and loadings plot of PCA between the normal and HF groups ($R^2X=76.5\%$, $Q^2=56.5\%$). C, D: Scores plot and loadings plot of PCA for the normal and HS groups ($R^2X=77.2\%$, $Q^2=46.9\%$); E, F: Scores plot and loadings plot of PCA between the HF and HS groups ($R^2X=79.4\%$, $Q^2=41.9\%$).

induced glomerular hypertrophy and renal tubule damage.

Metabolic variation and biomarker identification between high fructose and high salt groups

Representative ^1H -NMR spectra of urine obtained from normal, HF and HS groups are shown in **Figures 1** and **3**. Principal component analysis (PCA) was performed on the urinary metabolite concentrations between the normal and HF or HS groups at week 8 (**Figure 3A, 3B**) and between the HF and HS groups (**Figure 3C**).

A clear separation in metabolic states was observed between the normal and HF/HS groups at week 8, which indicated a marked alteration of the urinary metabolites in the HF or HS groups. Interestingly, HF or HS affected the metabolite variations in the same directions (**Table 1**). However, the changes in 2-hydroxybutyrate, succinate, TMAO and hippurate were not significantly different between HF and normal rats, whereas the TMAO and hippurate concentrations were significantly increased in HS rats compared with normal rats. Previous studies have demonstrated the impor-

Table 1. Statistical analysis of the metabolites in urine at the end of the 8th week

Metabolites	Chemical shift	Variations		
		HF vs. normal	HS vs. normal	HF vs. HS
2-hydroxybutyrate	0.88(t)	-	-	↓*
Succinate	2.41(s)	-	↓*	↑*
α-KG	2.45(t), 3.01(t)	↓**	↓*	↑*
Citrate	2.56(d), 2.72(d)	↓**	↓**	↑*
Creatine + creatinine	3.04(s)	↑**	↑**	↓**
Creatinine	4.05(s)	↑**	↑**	↓*
Choline	3.21(s)	↓**	↓**	-
PC+GPC	3.23(s)	↓**	↓**	-
Taurine	3.25(t), 3.43(t)	↓**	↓**	↓*
TMAO + betaine	3.27(s)	-	↑**	-
Hippurate	3.97(s)	-	↑**	↑*

For the direction of change in ANCOVA analysis, (↑) indicates a relatively higher metabolite urinary excretion, and (↓) indicates a relatively lower excretion. A dash indicates no statistical significance in ANCOVA analysis ($P>0.05$). *Statistical significance from the baseline (t-test, $P<0.05$) indicates significant changes * $P<0.05$; ** $P<0.01$.

tance of the gut microbiota in contributing to the excretion of metabolites such as TMAO and hippurate, which are often referred to as urinary microbial co-metabolites [23-25]. Therefore, our results suggest an increase in microbiota activity in HS rats but not in HF rats (**Table 1**). Because the intermediates in the TCA cycle are located mainly in liver mitochondria, the amount of intermediates such as citrate, succinate and α-KG of in the urine of HF or HS are reduced compared with normal (**Table 1**), whereas the increased amount of these intermediates in the urine of HF rats compared with HS rats, suggesting that although both HF and HS diets affect TCA cycle, the activity of mitochondrial enzymes involved in the TCA cycle are mainly regulated by HF diet. Further, the creatine and creatinine concentrations were significantly higher in both HF and HS rats than in normal rats. Creatinine is excreted in the urine, and its serum concentration is the most commonly used indicator of renal function [26]. Our results thus indicate that renal function in was reduced in both HF and HS diet.

Effect of salt on renal injury induced by HF diet

Building on previous findings, we further investigated the degree of renal injury after a combined high fructose and high- or low-salt diet. In **Figure 4**, it can be seen that a high salt diet did not exacerbate glomerular hypertrophy in HF

rats and the glomerular hypertrophy scores in HF-HS rats were not higher than those in HF rats (**Figure 4C**). In contrast, the HS diet significantly promoted tubule damage (**Figure 4A HE**) in HF rats, and the PAS-positive ECM staining area scores were higher in HS-HF rat than in HF rats, especially in the renal medullary area (**Figure 4D**). Furthermore, our results showed that a HF+HS diet markedly increased the expressions of α-SMA and desmin compared with HF rats (**Figure 4F and 4G**). Notably, a low-salt diet almost reversed all of the pathological changes induced by high fructose water, which suggests that salt is involved in the progression of renal injury triggered by HF water and HS diet worsen glomerulosclerosis as well as tubule damage.

Impact of salt on urinary metabolites in rats given high-fructose water

Representative H-NMR spectra of urine obtained from HF+HS and HF+LS groups are shown in **Figures 1 and 5**. We further analyzed the urinary metabolites in the HF+HS and HF+LS groups (**Table 2**). Both diets affected metabolite variations in different directions, and PCA analysis showed a clear separation between the groups. **Table 2** displays the decreased levels of succinate, α-KG, citrate, creatine, taurine and hippurate in HF+HS 8-wk rats. Similarly, the HF+LS diet significantly reduced succinate, α-KG, citrate, taurine and hippurate in urine, which suggests that either a high- or low-salt diet might affect TCA activity and amino acid metabolism. Additionally, rats fed the HF+HS diet had increased levels of TMAO + betaine and decreased levels of creatine + creatinine compared with normal rats, which suggests that high salt exacerbates renal function and gut microflora in HF rats (**Table 2**). However, rats given the HF+LS diet exhibited increased 2-hydroxybutyrate, creatinine and creatine levels and reduced TMAO + betaine in urine compared with normal rats (**Table 2**), indicating that the low-salt diet partially improved renal function and the activity of microbiota.

Discussion

In the present study, we found that after 8 weeks of treatment, a high-salt diet alone main-

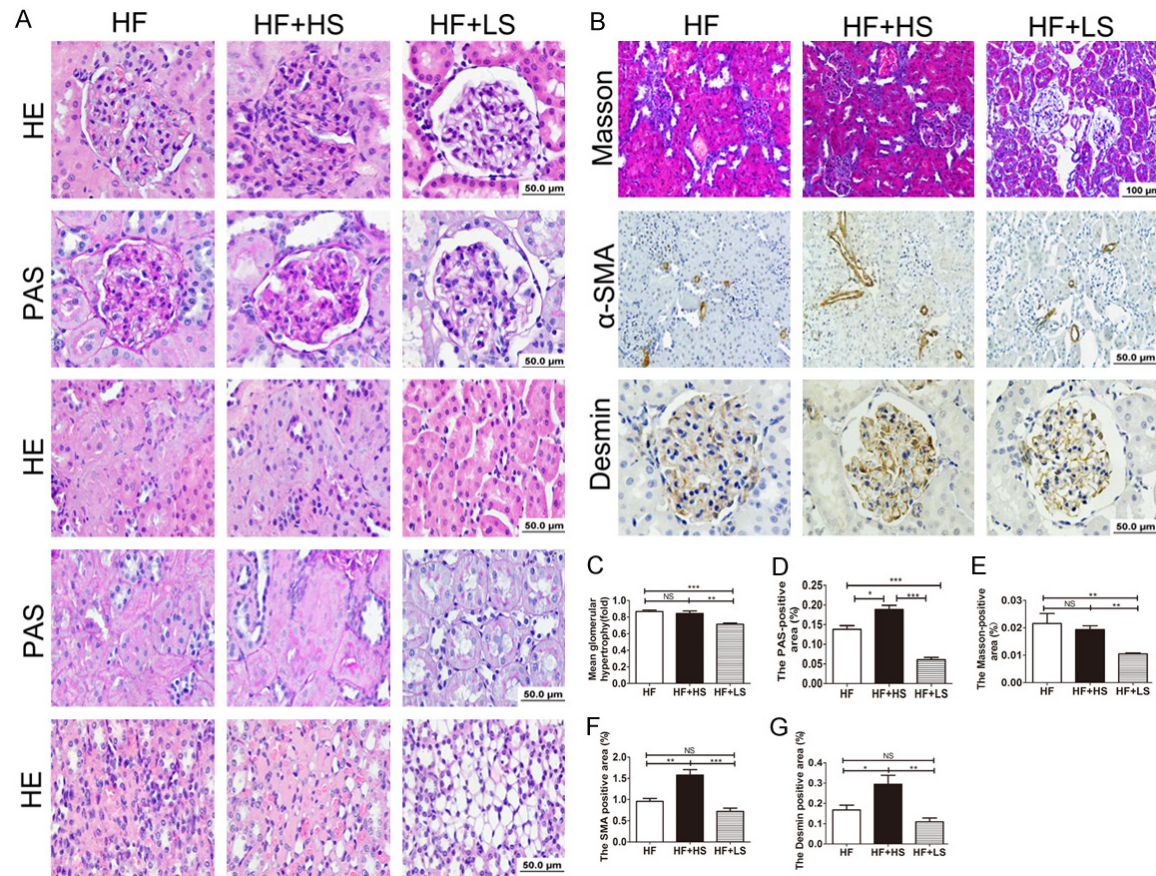


Figure 4. Influence of high/low salt on renal interstitial fibrosis of high fructose-induced rats for 8 weeks. Representative images from the high-fructose water group (n=10), the high-fructose water with high-salt diet group (n=10) and the high-fructose water with low-salt diet group (n=10) after 8 weeks. A: Hematoxylin-Eosin and Periodic acid-Schiff staining. B: Masson staining and immunohistological staining. C: Quantification of the glomerular hypertrophy. D: Quantification of the PAS-positive area. E: Quantification of the Masson-positive area. F: Relative expression of the α -SMA in kidney. G: Relative expression of the desmin in kidney. HF: High-fructose; HS: high-salt. Data are the mean $\pm$ SEM. * P <0.05; ** P <0.01; *** P <0.001. NS: no significance.

ly caused glomerulosclerosis, whereas a high fructose diet induced glomerular hypertrophy and renal tubule damage, reflecting the differential effect of these diets on renal injury. Fructose is a monosaccharide [27] that is predominantly metabolized in the liver and is involved in vital metabolic functions, including glycolysis and gluconeogenesis [28]. Furthermore, the urine metabolite analysis showed that compared with HS, HF may affect the activity of mitochondrial enzymes involved in the TCA cycle, resulting in the disruption of energy metabolism in the liver [29], which may be responsible for glomerular hypertrophy and tubule injury. Our evidence also consistently showed that fructose induced the proliferation of rat proximal tubular cells in vitro and was associated with focal tubular injury by up-regulating fructose transporters and the key enzyme in fructose metabolism [30].

Conversely, the HS diet mainly increased the activity of gut microbiota via the excretion of TMAO and hippurate compared with a normal diet. TMAO, a gut microbial mammalian metabolite of benzoic acid that can be generated by a range of gut microbes from low-molecular-weight aromatic compounds and polyphenols in the gut [31], has been associated with cardiovascular risk [32]. TMA is a byproduct of decomposition of choline by gut microbial flora, and its oxidation produces the toxic metabolite TMAO. Several studies have shown that TMAO correlates with the degree of renal injury inflicted by various mechanisms [33, 34]. Further, urinary hippurate has often been related to the activity of the gut microflora [35], suggesting that the gut microflora may vary with diet. Urinary levels of hippurate have also been shown to correlate with the obese phenotype in different animal models [36]. In addition, the

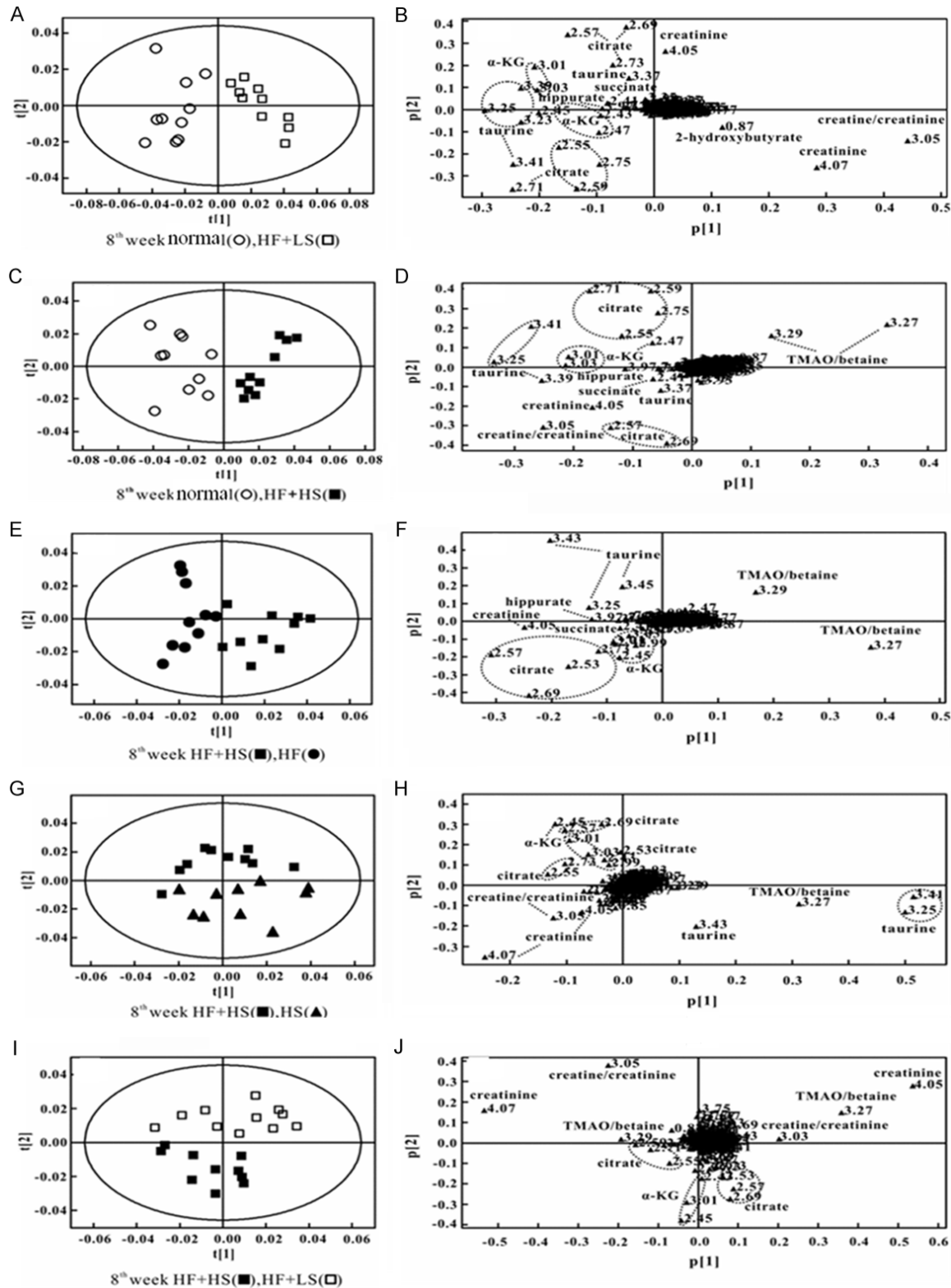
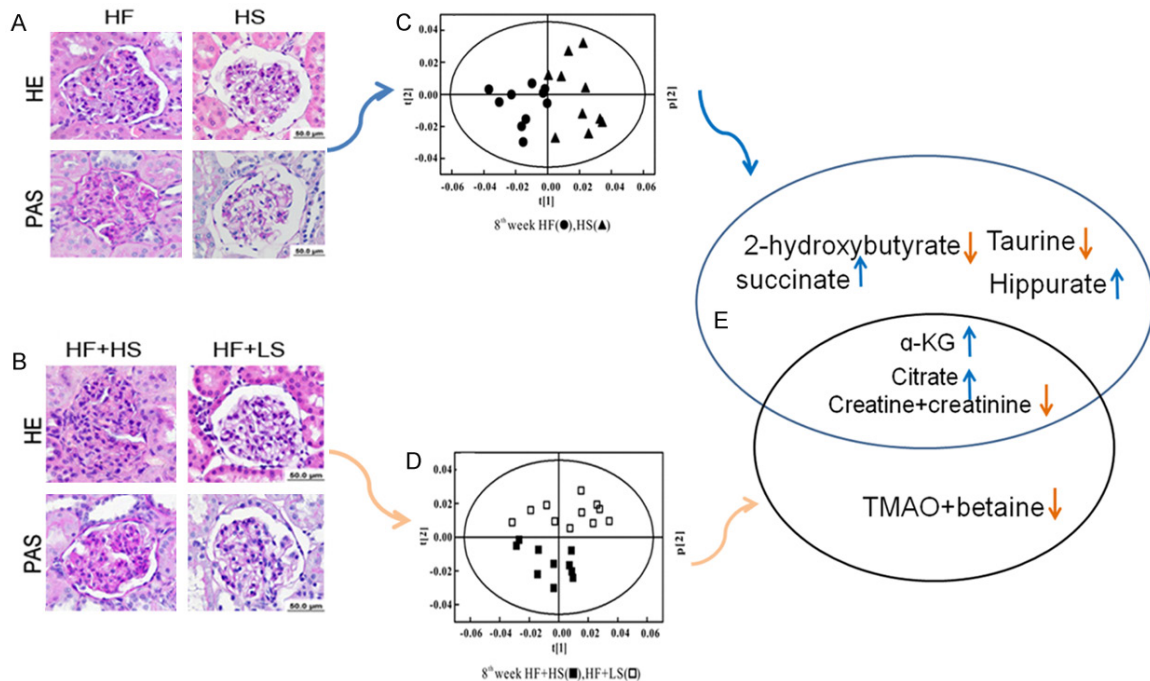


Figure 5. Multivariate analyses of urinary ^1H NMR spectra data at the end of the 8th week. A, B: Scores plot and loadings plot of PCA for the normal and HF+LS groups ($R^2X=75.8\%$, $Q^2=54.3\%$). C, D: Scores plot and loadings plot of PCA for the normal and HF+HS groups ($R^2X=75.8\%$, $Q^2=54.3\%$). E, F: Scores plot and loadings plot of PCA between the HF+HS and HF groups ($R^2X=84.7\%$, $Q^2=67.6\%$). G, H: Scores plot and loadings plot of PCA for the HF+HS and HS groups ($R^2X=72.2\%$, $Q^2=46.9\%$). I, J: Scores plot and loadings plot of PCA for the HF+HS and HF+LS groups ($R^2X=64.2\%$, $Q^2=31.9\%$).

Table 2. Statistical analysis of the metabolites in urine at the end of the 8th week

Metabolites	Chemical shift	Variations				
		HF+HS vs. normal	HF+LS vs. normal	HF+HS vs. HS	HF+HS vs. HF	HF+HS vs. HF+LS
2-hydroxybutyrate	0.88(t)		↑*			
Succinate	2.41(s)	↓*	↓**	-	↓*	-
α-KG	2.45(t), 3.01(t)	↓*	↓**	↑**	↓*	↑*
Citrate	2.56(d), 2.72(d)	↓**	↓**	↑*	↓*	↑*
Creatine + creatinine	3.04(s)	↓**	↑**	↓**	-	↓**
Creatinine	4.05(s)	↓**	↑**	↓**	↓**	↓**
Choline	3.21(s)	-	-	-	-	-
PC+GPC	3.23(s)	-	-	-	-	-
Taurine	3.25(t), 3.43(t)	↓**	↓**	↓*	↓*	-
TMAO + betaine	3.27(s)	↑**	↓**	↓*	↑**	↓*
Hippurate	3.97(s)	↓**	↓**	-	↓**	-

+: indicates significant changes *P<0.05; **P<0.01.



TOC Graphic. As shown in TOC Graphic, although either HF or HS diets induced differential renal injury and urinary metabolites (A, C), the changes of α-KG, citrate and creatine + creatinine in urine from both diet treatments are similar, indicating both dietary fructose and salt impact TCA cycle activity (E). Unexpectedly, the LS diet apparently reversed renal injury induced by HF (B), which suggests that dietary salt indeed plays a key role in the mechanism of kidney damage caused by HF. Furthermore, urinary metabolites showed that LS diet mainly reduced changes in gut microbial flora like TMAO and betaine (D, E), which indicated the gut microbial flora interplayed with TCA cycles activity.

urinary taurine in both HF and HS rats was significantly reduced, which is consistent with reported findings in patients with advanced CKD [37]. Higher levels of creatine and creatinine were observed in both the HF and HS groups. The high excretion of creatine reflects

increased turnover in the creatine/creatinine pathway [38]. Therefore, glomerulosclerosis induced by the HS diet was mainly associated with the activation of gut microflora, whereas glomerular hypertrophy and tubule damage might be related to TCA cycle activity, both of

which exacerbate renal injury. Indeed, HS+HF rats showed marked tubule damage, glomerular podocyte injury and pathological fibrosis changes compared with HF rats. Urinary metabolite variation analysis showed that the HF+HS diet affected the intermetabolites of the TCA cycles and a toxic metabolite of gut microflora. The creatine and creatinine concentrations in this group were lower than those in the normal group, suggesting decreased kidney function. However, the LS diet significantly reversed most of the pathological changes induced by the HF treatment. Rats fed the HF+LS diet had increased 2-hydroxybutyrate, creatinine and creatine levels and reduced TMAO + betaine levels in urine compared with normal rats, indicating that the low-salt diet improved renal function and the activity of microbiota. Furthermore, with the HF+HS diet, metabonomic analysis indicated that gut microbiota might be interact with the activity of enzymes in the TCA cycle and corporately regulated the pathogenesis of kidney injury.

In summary, as shown in TOC Graphic, although either HF or HS diet induced differential renal injury and urinary metabolites (**Toc Graphic A, C**), the changes of α -KG, citrate and creatine + creatinine in urine from both diet treatments showed similar variations compared with the normal diet, indicating both dietary fructose and salt impacted on TCA cycle activity (**Toc Graphic E**). Unexpectedly, the LS diet apparently reversed renal injury induced by HF (**Toc Graphic B**), which suggests that dietary salt indeed plays a key role in the mechanism of kidney damage caused by HF. Furthermore, urinary metabolites showed that LS diet mainly reduced metabolite changes in gut microbial flora like TMAO and betaine (**Toc Graphic D-E**), which indicated the gut microbial flora interplayed with TCA cycles activity. However, we need to further investigate the underlying mechanism of how gut microbial flora involved in regulating the enzymes activity in TCA cycle.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Composition of normal and experimental diets (based on NIH-07)

Ingredient	Percentage by Weight (Total 100)		
	Normal	High-salt diet (8%)	Low-salt diet (0.07%)
Dried skim milk	5.00	5.00	5.00
Fish meal (60% protein)	10.00	10.00	10.00
Soybean meal (49% protein)	12.00	12.00	12.00
Alfalfa meal (dehydrated 17% protein)	4.00	4.00	4.00
Corn gluten meal (60% protein)	3.00	3.00	3.00
Ground#2 yellow shelled corn	24.25	24.25	24.25
Ground hard winter wheat	23.00	23.00	23.00
Wheat middlings	10.00	10.00	10.00
Brewer dried yeast	2.00	2.00	2.00
Dry molasses	1.50	1.50	1.50
Soy oil	2.50	2.50	2.50
Salt*	0.50*	8*	0.07*
Dicalcium phosphate	1.25	1.25	1.25
Ground limestone	0.50	0.50	0.50
Choline Cl-70	0.10	0.10	0.10
Mineral premix	0.15	0.15	0.15
Vitamin premix	0.25	0.25	0.25

*Different percentage of salt in diet.

Supplementary Table 2. Quantitative analysis of body weight, the ratio of kidney and body weight, plasma creatinine and blood pressure at 8th week

Group	8 th week			
	Body weight (g)	Kidney/body weight (%)	Plasma creatinine (mg/dl)	Blood pressure (mmHg)
Normal	388.73±11.44	0.32±0.02	0.26±0.01	111.03±9.1
High-fructose	424.45±18.61*	0.33±0.02	0.38±0.02*	128.08±7.51*
High-salt	411.7±17.55	0.39±0.03*, ^Δ	0.48±0.04*, ^Δ	133.94±5.86*
High-fructose and high-salt	359.87±19.07*, ^Δ	0.39±0.02*, ^Δ	0.65±0.03**, ^{ΔΔ}	140.97±9.75*, ^Δ
High-fructose and low-salt	355.04±15.84*, ^Δ	0.32±0.01	0.31±0.02 ^Δ	106.04±9.59 ^{ΔΔ}

Values are presented as means ± SD. *: to compare with Normal group *P<0.05; **P<0.01; Δ: to compare with high-fructose group ^ΔP<0.05; ^{ΔΔ}P<0.01.