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This study was to clarify the pathogenesis of CRF and action mechanism of TAES.
A Urine Metabonomics Study of Chronic Renal Failure and Intervention Effects of Total Aglycone Extracts of *Scutellaria Baicalensis* in 5/6 Nephrectomy Rats

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Abstract

Chronic renal failure (CRF) is a severe disease that can lead to decline of life quality. *Radix Scutellariae* is a well-known traditional Chinese medicine (TCM). Our previous study has demonstrated that the Total Aglycone Extracts of *Scutellaria Baicalensis* (TAES), can improve renal fibrosis induced by mercuric chloride in rats. However, no research has investigated the efficacy and mechanism of TAES in treating CRF. In the present study, we investigated the effects of TAES on some closely related parameters in 5/6 nephrectomy CRF rats, and studied the pathogenesis of CRF and the mechanism of TAES treatment using a metabonomics method based on gas chromatography coupled with mass spectrometry (GC/MS). Rats with CRF were divided into six groups with rats subjected to sham operation as normal control. After eight weeks of treatment by TAES, the levels of serum creatinine (Scr) and blood urea nitrogen (BUN) were...
decreased, and the metabolic perturbations induced by 5/6 nephrectomy were reversed according to pattern recognition analysis. Meanwhile, 18 potential biomarkers associated with CRF were identified, and the affected metabolic pathways in 5/6 nephrectomy rats were extracted based on the differential metabolites. Our findings suggest that TAES have positive effects on 5/6 nephrectomy-induced CRF in rats and show therapeutic potentials in CRF treatment. Our findings also indicate that metabonomics analysis based on GC/MS is a useful tool for studying the effect of drugs on the whole body, exploring biomarkers involved in CRF and elucidating the potential therapeutic mechanisms of TCM.

Keywords: Metabonomics, GC/MS, Total aglycone extracts of *scutellaria baicalensis*, Chronic renal failure, 5/6 nephrectomy

1 Introduction

Chronic renal failure (CRF) refers to the progressive renal injury resulted from primary or secondary chronic kidney diseases (CKD). It is often accompanied by a series of clinical syndromes and metabolic disorders, and could eventually develop into end stage renal disease (ESRD). Besides the increase in chronic diseases commonly observed in an expanding elderly population, such as hypertension, diabetes and abuse of nephrotoxic drugs, the prevalence and incidence of common disorders like CKD has also risen in past years, all of which impose a rising demand on the healthcare systems [1]. Currently, there is no effective treatment for CRF due to its unclear pathogenesis [2]. Therefore, it is of great significance to study CRF pathogenesis and to develop effective drugs for the treatment of CRF. The rat model 5/6 nephrectomy is characterized by glomerulosclerosis, tubular injury and interstitial fibrosis. Due to its similar pathological process with human CRF, 5/6 nephrectomy is commonly used to study CRF pathogenesis [3] and potential mechanisms of drug effect [2].

Metabonomics is a branch of system biology that is based on the analysis of an entire spectrum of metabolites rather than focusing on individual ones. Unbiased measurement and holistic analysis of biological samples are the critical steps of metabonomics studies. Due to its powerful separation efficiency and detection sensitivity, gas chromatography coupled with mass spectrometry (GC/MS)
is considered as one of the most useful approaches in the field of metabonomics research [4,5]. For example, metabonomics studies using GC/MS have been widely adopted in the evaluation of therapeutic efficacy of traditional Chinese medicine (TCM) [6].

*Radix Scutellariae* is a well-known TCM with the efficacy for heat-clearing, dampness-drying, fire-purging, detoxicating, maintaining hemostasis and preventing abortion [6]. It is listed in the Pharmacopoeia of the People’s Republic of China and mainly contains flavonoids such as baicalin, wogonoside, baicalein, wogonin and oroxylin A [7,8,9]. *Radix Scutellariae* also has a higher content of glycosides (also known as baicalin, wogonoside, et al.) than that of aglycones (also known as baicalein, wogonin, et al.). Several studies showed that the glycosides in *Radix Scutellariae* could be absorbed only after it has been hydrolysed to flavonoid aglycones by intestinal flora [10,11]. We have previously hydrolysed flavonoid glycoside to aglycones using enzymes found within *Radix Scutellariae*, and then extracted total flavonoid aglycone with ethyl acetate. We optimized the methodology of the extraction process and obtained the corresponding Chinese patent (CN 1583775A).

The key to treating CRF is to protect the function of residual kidney tissues. Many clinical and experimental studies declared that TCM processes unique protecting effects on renal function in patients with CRF [12]. It has been reported that Total Aglycone Extracts of *Scutellaria Baicalensis* (TAES) can improve renal fibrosis induced by mercuric chloride in rats [13]. However, the potential efficacy and mechanism of TAES as a treatment for CRF induced by 5/6 nephrectomy remain unclear. In order to fill this knowledge gap, we studied the pharmacology of TAES intervention in 5/6 nephrectomy rats, analyzed the pathogenesis of CRF in a holistic environment using metabonomics and explored the efficacy and mechanism of TAES on CRF.
2 Experimental

2.1 Chemicals

Losartan Potassium Tablets were purchased from MSD of Hangzhou in China. Methoxyamine hydrochloride, N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA + TMCS 99 : 1), urease and Myristic acid were purchased from Sigma Corporation of America. Chloral hydrate, heptanes, methanol, anhydrous ethanol, ethyl chloroformate, pyridine, and chloroform were of analytical grade and were supplied by China National Pharmaceutical Group Corporation in Shanghai, China. L-2-Chlorophenylalanine and heptadecanoic acid, used as internal quality standards, were provided by Sigma Corporation of America. The ultrapure water was obtained from Milli-Q system (Millipore, USA).

2.2 Preparation of TAES

Dried Scutellaria was purchased from Inner Mongolia. The effective fraction (EF) used in the present study was extracted by ethyl acetate after a three-hour enzymolysis at 37°C using enzyme present in Scutellaria. The total content of baicalein, wogonin and oroxylin-A in the EF was more than 60%. The extraction process of the EF is protected by the Chinese patent (CN 1583775A).

2.3 Animals and 5/6 nephrectomy

105 male Wistar rats weighing 180 ± 10 g were purchased from Shanghai Sippr BK Laboratory Animals Ltd. (Shanghai, China). All rats were housed in an air-conditioned room at 20 - 25°C with a 12 h light / 12 h dark cycle. The animals were allowed free access to food pellets and water. All experimental procedures were approved by the Ethics Committee of the Institute of Shanghai University of TCM. After one week, 5/6 nephrectomy was performed as described previously [14]. Briefly, rats were put under anesthesia with chloral hydrate (300 mg/kg body weight, i.p.). Then approximately 2/3 of the left kidney was ablated and then the right renal pedicle was ligated seven days later.

2.4 Groups and Treatment

Animals were randomly divided into 7 groups, namely, one control group, one positive group, one model group and four treatment groups, with 15 animals in each group. The rats that underwent a
sham operation were used as normal control (sham group). Rats in the four treatment groups were orally administered 10, 20, 40 and 80 mg/kg•d TAES by intubation, respectively. The positive group received losartan (20 mg/kg•d). The same volume of distilled water was given to the sham and control groups. All rats were sacrificed after eight weeks of successive treatments. Overnight (24 h) urine samples of 8 randomly selected rats from each group were collected in metabolic cages at week 0 (pre-dose), and at 2, 4, 6 and 8 weeks after 5/6 nephrectomy. All urine samples were stored at −80°C. The animals were anesthetized with chloral hydrate and blood was obtained from the abdominal aorta for renal function analysis. The kidneys were isolated and fixed with 10% buffered formalin for histological study.

2.5 Assays for Serum creatinine (Scr) and Blood urea nitrogen (BUN)
Levels of Scr and BUN were measured using an Automatic Biochemical Analyzer (HITACHI 7080, JAP).

2.6 Histological Study
A portion of the kidney tissue was trimmed, fixed with 10% buffered formalin, and embedded in paraffin for light microscopy analysis. Sections with a thickness of 3 µm were stained with haematoxylin and eosin stain.

2.7 Urine sample preparation and GC/MS assay
All the urine samples were thawed in ice water bath and vortex-mixed before analysis. Each 600 µL aliquot of standard mixture or urine sample was added to a screw tube. After adding 100 µL of 12-chlorophenylalanine (0.1 mgmL⁻¹), 400 µL of anhydrous ethanol, and 100 µL of pyridine to the urine sample, 50 µL of ECF were added for first derivatization at 20.0 ± 0.1°C. The pooled mixtures were sonicated at 40 kHz for 60 s. Then, extraction was performed using 300 µL of chloroform, with the aqueous layer pH was carefully adjusted to 9-10 using 100 µL of NaOH (7 mol L⁻¹). The derivatization procedure was repeated with the addition of 50 µL ECF into the aforementioned products. After the two successive derivatization steps, the overall mixtures were vortexed for 30 s and centrifuged for 3 min at 3000 rpm. The aqueous layer was aspirated off, and the remaining chloroform layer containing derivatives was isolated and dried with anhydrous
sodium sulfate and subsequently subjected to GC-MS analysis.

Sample analysis by GC/MS was performed according to our previously published work with minor modification [15]. All GC-MS analyses were performed by a mass spectrometer 5975B (Agilent technologies, USA) coupled with an Agilent 6890 (Agilent technologies, USA) gas chromatography instrument. In the gas chromatographic system, a capillary column (Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm, film thickness 0.25 μm) was used. Helium carrier gas was injected at a constant flow rate of 1.0 mL × min\(^{-1}\). Derivatized samples of 1 μL each were injected into the GC/MS instrument in splitless injection mode. A programmed column temperature was optimized for successful separation (Table 1). The temperatures of the injection port, interface, and the source were set at 260°C, 280°C and 230°C, respectively. The measurements were collected using electron impact ionization (70 eV) in full scan mode (m/z 30 – 550). The solvent post time was set to 5 min.

<table>
<thead>
<tr>
<th>Rate (°C/min)</th>
<th>Temperature (°C)</th>
<th>Hold time (min)</th>
</tr>
</thead>
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<tr>
<td>70</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>210</td>
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<tr>
<td>10</td>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>290</td>
<td>0</td>
</tr>
</tbody>
</table>

2.8 Data analysis

All results were presented as mean ± SD. Data were analyzed using SPSS 13.0 statistical package. Data for multiple comparisons were performed by one-way ANOVA followed by Dunnett’s test. A value of \( P < 0.05 \) was considered statistically significant.

All the GC/MS raw files were converted to NetCDF format using Data Bridge software (Perkin-Elmer Inc., USA), and were subsequently processed using XCMS toolbox (http://metlin.scripps.edu/download/) with default settings for baseline correction, peak discrimination and alignment. The resulting data were exported into Microsoft Excel, and the
peaks were normalized to the total sum of spectrum prior to multivariate analyses. The data were analyzed by principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA) and orthogonal partial least squares (OPLS) using SIMCA-P 11.5 software (Umetrics, Umea, Sweden) after undertaking a unit variance procedure. The concentrations of potential biomarkers were represented as relative areas using the internal standard areas as reference. For GC/MS data, significant variables (markers) are selected based on a threshold of a multivariate statistical parameter, such as variable importance in the projection (VIP) value from an OPLS model. The higher the VIP values, the greater influence the variables have on the discrimination between the two groups. Variables with VIP values exceeding 1 are first selected. In a second step, those differential metabolites are validated at a univariate level with Mann-Whitney U test. The threshold of p value is usually set to 0.05. These variables, then, were identified by searching in NIST database and verified by standards.

3. Results

3.1 Effects of TAES on BUN and Scr levels. Fig. 1 shows the effects of TAES on parameters indicative of renal function. Compared with the sham group, the levels of BUN (A) and Scr (B) were dramatically increased in the control group after eight weeks of water treatment. However, the BUN and Scr levels were decreased after eight weeks treatment with TAES (10, 20, 40 and 80 mg/kg•d) in comparison with those of the control group.

3.2 Histological Findings. Histological examination further confirmed renal dysfunction in 5/6 nephrectomy animals (Fig. 2). B shows features of renal tissues from the model group compared with those from the sham group (A). These features include disordered glomerular structure, hyperemia, interstitial cell hyperplasia, severe inflammatory cell infiltration, and fibrous tissue hyperplasia. In contrast, these changes were significantly reversed after eight consecutive weeks of TAES treatment.

3.3 Metabonomics analysis

Urine data of sham group and model group before operation were analyzed by PCA and PLS-DA. Automatic modeling parameters indicated the poor explanation and predication of the models as
shown in Table 2, meaning that there was no difference in urine metabolism between these two groups (Table 2). The time-related metabolic pattern of PLS-DA scores is shown in Fig. 3. In both the model and the sham groups, distinct metabolic changes were apparent from week 0 onwards, suggesting that age might have an influence on urine metabolism in rats. For more reliable comparison, urine metabolism analysis should base on samples collected at the same time across different treatment groups.

Table 2 Automatic modeling parameters for the classification of sham operation group versus model group before model establishment.

<table>
<thead>
<tr>
<th>Model</th>
<th>Amount of components</th>
<th>$R^2_X$</th>
<th>$R^2_Y$</th>
<th>$Q^2_Y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA-X</td>
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<td>0.674</td>
<td>0.352</td>
<td></td>
</tr>
<tr>
<td>PLS-DA</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$R^2_X$ and $R^2_Y$ represent the cumulative sum of squares (SS) of all the X’s and Y’s explained by all extracted components. $Q^2_Y$ is an estimate of how well the model predicts the Y’s.

3.3.1 Analysis of metabolic profiles and identification of potential biomarkers. Urine data of sham group and model group at the 8th week after operation, with the greatest metabolism changes, were chosen to OPLS analysis. Based on the metabolic profiles, metabolites, which related to the group separation with the parameter VIP (Variable Importance in the Projection$^{16}$) $>$ 1, were selected as potential biomarkers ($p < 0.05$ Student’s t-test) (Table 3). Each of these potential biomarkers was further identified using the available reference compounds and the commercial compound libraries NIST.

Table 3 Potential biomarkers related to CRF.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Model/Sham</th>
<th>TAES40/Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cysteine</td>
<td>↑</td>
<td>↓Δ</td>
</tr>
<tr>
<td>Malate</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Uracil</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Metabolite</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Retinotic acid</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Creatinine</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Ribitol</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>D-galactonic acid</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Xylitol</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

The up and down arrows represent the relative increasing or decreasing trend of the metabolites in the model group compared to those in the sham group or in the TAES40 group compared to the model group. △ represents reverse trend compared to that in model/sham group.

3.3.2 Influence of losartan on the urinary metabolic profiles

Sham operation group, model group and losartan group were distinguished by PLS-DA analysis (Fig. 4). Sham group can be separated completely from the model group in 3D-PLS-DA score plot with the losartan group between them (Fig. 4A). This result indicated that losartan might improve kidney function to a certain degree. Samples plotted in one dimension are showed in Fig. 4B. It is evident that the principle component (PC) 3 accounted mainly for the treatment efficacy of losartan on CRF (Fig. 4B (c)). In the score plot of PC 1, CRF rats (including those in the model group and losartan treatment group) were distinctly separated from those in the sham operation group (Fig. 4B (a)), which might be implicative of disease formation. PC 2 suggested possible undesirable effects of losartan on CFR rats (Fig. 4 B (b)), as the urine metabolic profile of losartan treated group was different from that of the non-administrated groups (including both the model and the sham group).

3.3.3 Influence of TAES on urinary metabolic profiles
PLS-DA analyses of sham, model and treatment groups were shown in Fig. 5. In the score plot of PC 1, TAES treatment groups were separated from the sham and the model groups (Fig. 5A). Nevertheless, the sham and the model groups were clearly separated from each other in the score plot of PC 2. In addition, the treatment groups showed a trend of reversing to the sham group (Fig. 5B). The results demonstrated that TAES might have other effects on rats in addition to providing protection against CRF.

3.3.4 Time-dependent changes of metabolic profile

The time-related metabolic pattern of the score plot of PC 2 was shown in Fig. 6. In the TAES treatment group, the metabolic pattern was indicative of recovery toward the baseline state from week 2 onwards, suggesting that TAES might potentially reverse the 5/6 nephrectomy-induced CRF changes in rats.

4. Discussion

The 5/6 nephrectomy rats are a well-characterized model for studying CRF. It shows features of glomerulosclerosis and tubulointerstitial fibrosis, which result in kidney dysfunction and a significant increase in Scr and BUN levels [2]. Glomerulosclerosis and tubulointerstitial fibrosis are the common pathological changes typically observed at the final stage of progression to CRF. In the current study, 5/6 nephrectomy rats showed significant increase in Scr, BUN and fibrous tissue hyperplasia. RAAS inhibitors, such as angiotensin-converting enzyme inhibitors and ARBs, are the first-line drugs for treatment of renal fibrosis [17]. In this study, the therapeutic effect of TAES was compared with that of losartan, an ARB used as positive control. TAES showed similar therapeutic effects as losartan, with regard to improving kidney dysfunction and inhibiting fibrosis in 5/6 nephrectomy rats.

BUN and Scr are two main diagnostic markers for CRF. In the present study, the dramatical increase of BUN and Scr in the model group indicated that the CRF animal model was successfully established. However, the sensitivity of these markers may be relatively low in early CRF diagnosis and accurate therapeutic effect evaluation. Hence, novel approaches for the detection of CRF are urgently needed. The nontarget metabolomics provides a global view of the
organism and can be used to monitor metabolic alterations that occur in different pathological processes. Metabolites biomarkers may have the potential to improve diagnostic, prognostication, and therapy of interest.

Metabonomics is becoming widely popular among research studies that focus on evaluation of drug efficacy and safety due to its ability to identify specific changes in the overall metabolic spectrum. It is worth noting that, besides its therapeutic potential, losartan, as indicated by the PLS-DA analyses of the sham, model and losartan treatment groups, might also have some undesirable effects on CFR rats. Meanwhile, disease phenotypes of CRF, rather than the treatment effect of losartan, are the main contributor to the classifications of sham, model and losartan treatment groups. This indicates that CRF symptoms were not improved completely after 8 consecutive weeks of losartan treatment. However, the inference of the multi-effect of losartan on CRF rats was based on the understanding of single-dimensional mapping of the overall metabolic spectrum, and therefore, should be verified by future studies.

Metabonomics not only allows for the study of a static physical state at a particular time point, but also reflects the body’s dynamic response to medical intervention. In the current study, TAES exhibited a time-effect relationship in the treatment of CRF, as the metabolic pattern of the TAES 40 treatment group followed a time-dependent recovery trend toward the baseline state. Identifying the metabolic indices that change over time in response to a pharmacological intervention and investigating their biological significance are extremely helpful in evaluating drug-target effect.

Metabolite profiling focuses on the analysis of a group of metabolites that are related to a specific metabolic pathway in certain biological states [18]. It has contributed greatly to understanding the pathogenesis of diseases and their pharmacodynamics mechanism in a holistic way. The complex nature of the pathogenesis of CRF has limited our understanding of the disease. As the development of most kidney diseases often manifests as changes in metabolite composition [19], metabonomics is a powerful tool for the study of CRF pathogenesis. Based on pattern recognition analysis of metabolites, a clear separation of the model and the control group was achieved, and
18 differential metabolites related to group separation were found. In order to identify possible pathways that are affected in CRF, metabolites contributing to the separation of the sham and the model animals were analyzed using MetPA (Fig. 7). It is generally accepted that changes occurring at the critical positions within a network would trigger a more severe impact on the pathway than changes at marginal or relatively isolated positions [20]. In this study, the impact-value threshold was set to 0.10. Any pathways that scored above this threshold were categorized as potential target pathways. Metabolic pathway analysis using MetPA revealed that metabolites that are important for the host response to CRF, are those responsible for galactose metabolism (Fig. 7B (a)), cysteine and methionine metabolism (Fig. 7B (b)), retinol metabolism (Fig. 7B (c)), alanine, aspartate and glutamate metabolism (Fig. 7B (d)) and inositol phosphate metabolism (Fig. 7B (e)).

Drug target is usually identified as the key molecule involved in a particular metabolic or signaling pathway that is specific to a disease [21]. In the current study, changes in metabolites related to CRF in the TAES 40 group were analyzed. Compared with the model group, 8 different metabolites in TAES 40 treatment group followed a reversing trend to the levels in the sham group (Table 3). Based on MetPA analysis (Fig. 8), retinol, cysteine and methionine metabolisms are potential targets for CRF drug design. Although, the key metabolites in these two pathways were restored to levels observed in the sham group after TAES treatment, 10 other metabolites were not affected by treatment with TAES. This may account for the failure in getting better curative effect and will be followed up by further studies in our laboratory.

, Evaluating metabolites changes at a higher level, from pathway to network, allows for understanding the biological significance of metabolites affecting the state of an organism. Metabolites with a dramatic impact on the relevant pathways (Fig. 7 B) always play important roles in the pathogenesis and possible complication of the disease. D-Galactose, a reducing sugar, can be converted to aldose and hydroperoxide under the catalysis of galactose oxidase, resulting in generation of a superoxide anion and oxygen-derived free radicals [22]. The excessive levels of D-galactose have been reported to increase the free radical production in renal tissues [23], which is associated with the oxidative renal injury and AGE/ALE renal accumulation in rats. Methionine (Met) is an essential amino acid that is derived primarily from the diet. Homocysteine (Hcy)
(KEGG: C00155) is formed as a primary intermediate during the metabolism of Met, and it is the critical intersection of two metabolizing pathways: remethylation and transsulfuration, which are involved in the salvaging of Met and synthesis of cysteine (Cys), respectively (Fig. 7 B(b)). The increase of Cys and Met caused by CRF leads to abnormal increase of Hcy. Hcy is a toxic non-protein forming sulfur-containing amino acid, which contributes to generation of ROS, RNS, and reactive thiol species, thereby decreases the bioavailability of NO. These processes activate the latent MMPs, and inactive the TIMP, leading to adverse cardiovascular remodeling [24]. It is well-known that people with CKD have a remarkably elevated risk for cardiovascular disease (CVD) [25,26], and hyperhomocysteinaemia was found to be highly prevalent and significantly related to cardiovascular morbidity and mortality in patients with renal disease [27]. Under state of renal disease, the reduction of transport function may result in a decreased content of Retinoic acid (RA) in renal [28]. As such, we drew a hypothesis that the levels of RA in the renal of UUO rats are low while the high levels of RA in urine may associate with the reduction of renal transport function caused by disease. RA is an active metabolite of vitamin A, which is involved in various physiological processes. Vitamin A deficiency can lead to increased expression of FN, LN and collagen IV. Various studies reported that RA regulates the expression of ECM and plays a critical role in fibrotic diseases [29,30]. A protective role of RA against renal fibrosis in UUO rats was reported [31]. Alanine (Ala) is one of the major amino acids present in proteins, and catabolism of Ala yields pyruvate and ammonia, thus, Ala provides a source of carbon for nitrogen transamination [32]. It was shown that Ala promotes insulin secretion from the clonal β-cell line BRIN-BD11 at a substantially greater rate than all other amino acids [33], and the reduction of Ala may decrease transamination of the body and aggravate metabolism disorder of glucose and energy. Inositol is a key metabolite of inositol phosphate metabolism (Fig. 7 B(e)). Phosphoinositides have been investigated as an important agonist-dependent second messenger in the regulation of diverse physiological events depending upon the phosphorylation status of their inositol group [34]. Dysregulation of phosphoinositides formation as well as their metabolism are associated with various pathophysiological disorders [35]. The relative intensity of inositol was upregulated in the model group which may associate with glucose and lipid metabolic disorders and exaggerated inflammatory response.
5. Conclusion

In this study, 5/6 nephrectomy-induced CRF rat model was used to investigate the effects of TAES on CRF. According to pattern recognition analysis after eight weeks of TAES treatment, our results indicate that TAES can improve renal function and reverse the metabolic perturbations induced by 5/6 nephrectomy in CRF rats. Meanwhile, 18 potential biomarkers associated with CRF were identified and the disturbed pathways in 5/6 nephrectomy rats were extracted based on the differential metabolites. Our findings suggest that TAES have positive effects on 5/6 nephrectomy-induced CRF in rats and show therapeutic potentials in CRF treatment. Our findings also indicate that metabonomics analysis based on GC/MS is a useful tool for studying the effect of drugs on the whole body (including therapeutic and side effects), exploring biomarkers involved in CRF and elucidating the potential therapeutic mechanisms of TCM.

Acknowledgments

This study was financially supported by Shanghai science and technology achievements transformation and industrialization project (13401900306), National Natural Science Foundation of China (NSFC, 81373519), Technology Innovation Supporting Project for Top-grade Discipline Construction (058ZY1206) and Shanghai Interdisciplinary Cultivation Platform of Outstanding and Innovative Postgraduates and Shanghai “085” Science.


Fig. 1. TAES reduces the levels of BUN and Scr in 5/6 nephrectomy rats. After 5/6 nephrectomy rats were treated with TAES at the doses of 10, 20, 40 and 80 mg/kg•d, respectively, for eight successive weeks, BUN (A) and Scr (B) levels were analyzed. * P < 0.05, ** P < 0.01 compared with the sham group. # P < 0.05, ## P < 0.01 compared with the control group. Data are expressed as mean ± SD. n = 15.
Fig. 2 Histological characteristics of renal tissue sections. 5/6 nephrectomy elicited features typical of CRF renal tissue in rats. However, these changes were evidently attenuated by TAES and losartan treatment for eight successive weeks. Figure C, D, E and F show renal tissues from groups administered with TAES at doses of 10, 20, 40 and 80 mg/kg-d, respectively. Figure G shows histological changes in renal tissue after losartan intervention. Original magnification × 100.
Fig. 3 Score plot of PLS-DA derived from the GC/MS profiles of urine samples obtained from A: Control group, B: Model group. In both the model and the sham group, distinct metabolic changes were apparent from Week 0 onwards.
Fig. 4 PLS-DA analyses of sham, model and losartan treatment group. A. Score plot of 3D-PLS-DA model. B. Samples plotted in one dimension, (a) Score plot of PC 1, (b) Score plot of PC 2, (c) Score plot of PC 3.
**Fig. 5** Score plots of PLS-DA model classifying the sham, model and TAES treatment group (10, 20, 40 and 80 mg/kg•d). A. Score plot of PC 1. B. Score plot of PC 2.

**Fig. 6** The trajectory of time-dependent changes in urinary metabolite profile (score plot of PC2) in the TAES 40 mg/kg•d treatment group (the second, fourth, sixth, eighth week of treatment with TAES at a dosage of 40 mg/kg•d).
Fig. 7 Summary of pathway analysis. (A). a. galactose metabolism, b. cysteine and methionine metabolism, c. retinol metabolism, d. alanine, aspartate and glutamate metabolism, e. inositol phosphate metabolism. Identification of network pathway by MetPA software (B). Galactose metabolism (a), Cysteine and methionine metabolism (b), Retinol metabolism (c), Alanine, aspartate and glutamate metabolism (d), Inositol phosphate metabolism (e). Maps were generated using the reference map by KEGG (http://www.genome.jp/kegg/).
Fig. 8. Summary of pathway analysis. b. cysteine and methionine metabolism, c. retinol metabolism.