Expression of the Matrix Metalloproteinases and the Tissue Inhibitor of Metalloproteinase Factors are Affected by Tetramethylpyrazine Treatment in a Renal Interstitial Fibrosis Rat Model

Jianzhi Li*1), Jiangdong Yu*2), Yuming Liu3), Li Hu1), Bo Yang3), Xiutian Zhou4), Rui Wang1) and Yu Liang4)

1) The Nursing School of University of South China, Hengyang, China
2) Hengyang Central Hospital, Hengyang, China
3) The First Affiliated Hospital of University of South China, Hengyang, China
4) The Medical College of University of South China, Hengyang, China

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Abstract: The purposes of this study were to establish a unilateral ureteral obstruction renal fibrosis model and determine the pathological changes in renal interstitial. Expression of matrix metalloproteinase-2 and tissue inhibitor of matrix metalloproteinase-2 after tetramethylpyrazine treatments were determined. Thirty-two female Sprague Dawley rats were randomly divided into 4 groups: a sham group, model group, tetramethylpyrazine group and valsartan group. The rats in the model group, tetramethylpyrazine group and valsartan group were operated with left ureter ligation to establish the unilateral ureteral obstruction model. Rats in the tetramethylpyrazine group were given intragastric administration 1 day before surgery. The pathological changes of the obstruction renal tissues were examined by hematoxylin-eosin staining and Masson staining. Immunohistochemistry and reverse transcription-polymerase chain reaction were applied to detect the protein and mRNA expression levels of matrix metalloproteinase-2 and tissue inhibitors of matrix metalloproteinase-2. Tetramethylpyrazine could significantly reduce the expansion and contraction of the tubular, the proliferation of renal interstitial fibrous tissue and inflammatory cell infiltration. Compared with sham group, the protein and mRNA expression levels of matrix metalloproteinase-2 and tissue inhibitors of matrix metalloproteinase-2 were significantly increased in model group (p < 0.05). The protein and mRNA expression levels of matrix metalloproteinase-2 and tissue inhibitors of matrix metalloproteinase-2 in tetramethylpyrazine group were lower compared to the model group (p < 0.05). Tetramethylpyrazine could relieve the renal interstitial fibrosis by inhibiting the expression levels of matrix metalloproteinase-2 and tissue inhibitors of matrix metalloproteinase-2.

Key words: MMP, TIMP, Tetramethylpyrazine, Renal interstitial fibrosis

Introduction
Renal interstitial fibrosis (RIF) is a common pathway and main pathological basis for the development of a variety of chronic renal diseases, the process of which involves glomerulosclerosis, renal interstitial fibrosis and renal vascular lesions. The pathogenesis of RIF might be caused by a variety of renal damage factors leading to interstitial inflammatory cellular infiltration, fibroblast activation and phenotype transdifferentiation in renal tubular epithelial cells, which lead to the generation of myofibroblasts (MFB). MFB may produce excessive extracellular matrix (ECM) components and release a variety of soluble cytokines and vasoactive substances, which affect the dynamic balance between matrix synthesis and degradation via numerous ECM is the primary pathophysiological process leading to renal interstitial fibrosis. In the matrix degradation process, matrix metalloproteinase (MMP). Tissue inhibitor of metalloproteinase factor (TIMP) ratio is also a possible explanation for renal interstitial fibrosis. Although the combination treatment of angiotensin converting enzyme inhibitors / angiotensin receptor antagonists provided some positive results, no promising method has been found to cure renal fibrosis.

Tetramethylpyrazine is an alkaloid monomer extracted from Chinese herbal medicine Chuanxiong rhizome, which can promote blood circulation clinically. Its renal protective effect has been demonstrated in a series of clinical and experimental studies. Previous studies have focused on its role in many processes, including antioxidation, improving glycolipid metabolism, inhibiting platelet activation and aggregation, and improving blood rheology. Some of studies showed its delay effect on chronic al-
lograft nephropathy (CAN) of rats \(^{13}\), but few mentioned its effects on RIF. In this study, we established a unilateral ureteral obstruction renal fibrosis model in order to observe the pathological changes of renal interstitial and the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in renal tissue after tetramethylpyrazine treatment. We further discussed the effects of tetramethylpyrazine on renal interstitial fibrosis and its possible mechanism.

**Material and Methods**

**Rat model of experiments**

Female Sprague Dawley rats of healthy and clean grade from 4 to 6-week were provided from Department for Animal Testing of University of South China. Permit number: SCXK (Xiang) 2011-0012, body weight 220 to 250 g. Thirty-two female rats were randomly divided into 4 groups (8 in each group): sham group, model group, tetramethylpyrazine group and valsartan group. Except sham group, the rats in the other three groups were operated with left ureter ligation to establish the unilateral ureteral obstruction model under sterile conditions \(^{14}\). Rats in the sham group were performed with intragastric administration of tetramethylpyrazine at 1 day before surgery, 40 mg/(kg.d) once per day and lasted for 2 weeks. Rats in the valsartan group were performed with intragastric administration of valsartan (1.5 mg/100g). Valsartan Capsules (80 mg / pellet, Lot No. 101203) was purchased from Hainan AoMeiHua Pharmaceutical Co., Ltd (Give City, Nation). Rats in the sham group and model group were performed with intragastric administration of the same volume of saline. All rats were sacrificed 14 days after surgery and their left renals were collected as specimens.

**Measurement of Scr and BUN**

To evaluate renal health, we measured two important indicators, serum creatinine (Scr) and blood urea nitrogen (BUN) using OlymusAU21000 fully automatic chemistry analyzer. All four groups of rats in this experiments were intraperitoneal injected with 2 % Amobarbital 5ml/kg on the 14th postoperative day, blood samples were obtained from abdominal aorta. This experiment was completed by the biochemical laboratory of First Affiliated Hospital in University of South China.

**Renal histopathological examination**

Renal tissues were fixed, dehydrated, embedded in paraffin, performed by routine histopathological section with 3 im of thickness, and stained with Hematoxylin-Eosin (HE) and Masson. We assessed tubulointerstitial pathological lesion by observing the degree of tubulointerstitial fibrosis of each group using Masson staining section. Semiquantitative score was evaluated according to the method described in literature \(^{16}\). Under 400 × light microscope with 10 renal cortical visions and no glomeruli, Great vessels from each slice were selected randomly. Each parameter was assessed from 0 to 3 points (0 = normal, 1 = mild impairment, 2 = moderate impairment, 3 = severe damage), using three parameters: protein casts and tubular expansion, necrosis, atrophy; inflammatory cellular infiltration; the degree of interstitial fibrosis. The score of tubulointerstitial in each microscopic field was 0-9, and the mean value was calculated as tubulointerstitial injury index.

**Immunohistochemistry assay of nephridial tissue**

We chose MMP-2 (1:160), MMP-9 (1:160), TIMP-1 (1:160), TIMP-2 (1:160) as primary antibodies, biotinylated goat anti-mouse IgG as secondary antibody. Rabbits anti-mouse MMP-2 polyclonal antibody, rabbit anti-mouse MMP-9 polyclonal antibody, rabbit anti-mouse TIMP-1 polyclonal antibody, rabbit anti-mouse TIMP-2 polyclonal antibody, SABC immunohistochemical kit and DAB chromogenic agent were purchased from Wuhan Boster Biotechnology Co., Ltd (Give City, Nation).

All samples were colored with DAB, counterstained with hematoxylin and mounted with neutral gum. PBS was used as a negative control instead of primary antibody. True color pathological image analysis system was applied for analysis of MMP-2, MMP-9, TIMP-1, TIMP-2, five sections of visual field with same area were selected randomly under 400 times magnification, and integral optical density image automatic measurements were analyzed for immunohistochemistry positive signals.

**Reverse transcription-polymerase chain reaction**

Trizol total RNA extraction reagent was purchased from Shanghai Hua Shun Biological Engineering Co., Ltd (Give City, Nation). Reverse transcriptase kit was purchased from the United States Fermentas Co (Give City, Nation). Design and synthesis of primer was purchased from Shanghai Biological Engineering Co., Ltd (Give City, Nation). RNA concentration meter, polymerase chain reaction instrument we purchased from Eppendorf Co., (Give City, Germany).

Primers of the polymerase chain reaction (PCR) were designed using Primer Premier Software. MMP-2 primer: the positive strand 5’-ATCTGGTGTCCTC CCT-TACGG-3’ , reverse strand 5’-GTGCAGTGTGTCGGACACAC-3’, product of 150 bp; TIMP-2 primers: positive-strand 5’-AGGCGCCAAGAAGTGGGCGAGAA-3’, the counter chain 5’-CCGCCTTCCC TGCA A TTA GATATTC-3’, product of 214 bp. β-actin mRNA positive strand: 5’-TGACGAGGCCAGCCAGAACAGA-3’, reverse strand: 5’-ATG GCCACAGTGTTGGTGCAC3’, product length of 330 bp. Gray scale scanning was applied with computer image analysis software (TotalLabV 1.01). Grey ratio of strip from the target gene to PCR product of β-actin was used as a relative indicator to reflect the expression level of target genes.
Statistical analysis

The SPSS 11.5 software was used for statistical analysis. Data were shown as $x(\_\_)+s$. The variable means of samples were compared by one-way ANOVA. A P-value of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

Results

Tetramethylpyrazine has positive effects on renal function

32 Sprague Dawley rats were used in this study and divided into four groups. To assess the renal function of rats in those four groups, we measured Scr and BUN using Olymus AU21000 fully automatic chemistry analyzer (Give Company, City, Nation). Our results showed that BUN and Scr of model group were significantly bigger than those of sham group ($p < 0.05$), suggesting that the unilateral ureteral obstruction (UUO) as a convenient model of obstructive nephropathy was successfully applied in this experiment. We further compared the two indicators of tetramethylpyrazine group and valsartan group to those of model group. Our results showed significant reduction of BUN and Scr in tetramethylpyrazine and valsartan groups compared to model group, revealing both drugs have impacts on renal protection ($p < 0.05$). Compare the two groups with different drugs, no statistically significant difference was observed, suggesting tetramethylpyrazine has similar effects to valsartan ($p > 0.05$, Table 1).

To observe the morphological changes in the four groups, we had nephridial tissue fixed, dehydrated, embedded in paraffin, treated by routine histopathological section ($3 \mu m$ thickness), and stained with Hematoxylin-Eosin and Masson. Under the light microscope, the structure of the renal tubule of rats in the sham group was normal: no interstitial widened or inflammatory cell infiltration, and smooth and intact basement membrane of tubule. Renal tubular epithelial cell of rats of the model group diffused and vacuolar degenerated. Most of the renal tubules dilated and atrophied with multifocal inflammatory cell infiltration and fibrosis, which reflected in the renal interstitial damage index showing a significant difference compared to the sham group ($p < 0.05$, Table 2). Compared to the model group, renal interstitial inflammatory cells in the tetramethylpyrazine group and valsartan group showed small focal infiltration, a little epithelial cell swelling, a little renal tubules expanding mildly and no obvious fibrosis of renal interstitium ($p < 0.05$, Table 2).

Masson staining and blue collagen staining were applied to the four groups. Our results suggested that in sham group, the glomerular basement membrane, bowman capsule, the mesangial area and the areas around the tubular capillary showed dark staining, but the peritubular interstitial part has relatively less staining (Fig. 1). In the model group, hyperplasia of renal interstitial collagenous fiber was observed with blue staining, the distribution of interstitial fibrosis was focal, glomerular lesions was less severe, and occasionally segmental glomerular sclerosis was observed (Fig. 1). The lesions in the tetramethylpyrazine group and valsartan group were reduced (Fig. 1).

The expression of MMPs and TIMPs are influenced by tetramethylpyrazine

Because of the importance of MMPs/ TIMPs in renal function, we chose four members (MMP-2, MMP-9, TIMP-1 and TIMP-2) with known functions to further study. The expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2 of Renal tissue were detected by immunohistochemistry (SABC method). In the sham group, little expression of MMP-2, MMP-9, TIMP-1, TIMP-2 in renal tubular epithelial cells of rats could be detected, but rarely in glomerular or renal interstitial cells. In the model group, the expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2 increased

### Table 1. Measures of Scr and BUN in Four Groups ($x \pm s$, n = 8).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Scr (μmol/l)</th>
<th>BUN (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>48.00±5.10</td>
<td>5.31±0.75</td>
</tr>
<tr>
<td>Model group</td>
<td>79.50±3.87$^a$</td>
<td>7.75±0.61$^a$</td>
</tr>
<tr>
<td>Tetramethylpyrazine group</td>
<td>72.00±2.94$^{ab}$</td>
<td>6.78±0.43$^{ab}$</td>
</tr>
<tr>
<td>Valsartan group</td>
<td>70.75±2.50$^{ab}$</td>
<td>6.88±0.22$^{ab}$</td>
</tr>
</tbody>
</table>

Note: Compared with group sham-operation group, we marked those group with statistically significant difference with “a”, indicating $p$-value < 0.05. Compared with model group, we marked those with statistically significant difference with “b”, indicating $p$-value < 0.05.

### Table 2. Semiquantitative Results of Renal Interstitial Damage Index in All Groups ($x(\_\_)\pm s$, n=8).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Renal Interstitial Damage Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>0.26±0.10</td>
</tr>
<tr>
<td>Model group</td>
<td>6.58±0.25$^a$</td>
</tr>
<tr>
<td>Tetramethylpyrazine group</td>
<td>4.68±0.10$^b$</td>
</tr>
<tr>
<td>Valsartan group</td>
<td>4.48±0.20$^b$</td>
</tr>
</tbody>
</table>

Note: Compared with sham-operation group, we marked those group with statistically significant difference with “a”, indicating $p$-value < 0.05. Compared with model group, we marked those with statistically significant difference with “b”, indicating $p$-value < 0.05.
significantly compared with sham group, widely expressed in the renal tubular interstitial area of cortex and medulla, and the renal tubular epithelial cells. In the tetramethylpyrazine group and valsartan group, those genes were expressed in fewer tissues at lower levels compared to the model group (Fig. 2). Not much difference was observed between the tetramethylpyrazine group and valsartan group, suggesting similar effects of the two drugs.

To further measure the expression level of renal MMP-2, MMP-9, TIMP-1, TIMP-2, we applied semi-quantitative analysis of immunohistochemistry in four different groups. Our results showed that the expression levels of the four genes were lower in sham group compared to the other three (p < 0.05). Both tetramethylpyrazine and valsartan showed significant negative effects on the expression of the four genes compared with model group (p < 0.05). Compared with the sham group, the ratios of MMP-9/TIMP-1, MMP-2/TIMP-2 in other groups significantly
to the development of chronic renal diseases.

To test whether the expression shifts also revealed at mRNA level, we applied reverse transcription - polymerase chain reaction to test the expression of MMP-2, TIMP-2 mRNA from obstructed renal. Our results suggested that both MMP-2 and TIMP-2 were expressed at low level in the sham group and significantly higher in the model group ($P < 0.05$). In the tetramethylpyrazine group, the expression levels of the two genes were lower than those in the model group ($P < 0.05$), but still higher than those in the sham group (Fig. 3 and Table 4).

### Table 3. Integral absorbance value of MMP-2 MMP-9, IMP-1 and TIMP-2 in all groups ($\bar{x}(\pm s, n=8)$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MMP-9 (nm)</th>
<th>TIMP-1 (nm)</th>
<th>MMP-9/ TIMP-1</th>
<th>MMP-2 (nm)</th>
<th>TIMP-2/ TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>sham group</td>
<td>0.25±0.12</td>
<td>0.22±0.19</td>
<td>1.13±0.18</td>
<td>0.27±0.06</td>
<td>1.23±0.08</td>
</tr>
<tr>
<td>model group</td>
<td>0.36±0.10</td>
<td>0.39±0.20</td>
<td>0.93±0.19</td>
<td>0.46±0.10</td>
<td>0.51±0.10</td>
</tr>
<tr>
<td>tetramethylpyrazine</td>
<td>0.29±0.23</td>
<td>0.31±0.24</td>
<td>0.94±0.21</td>
<td>0.36±0.07</td>
<td>0.38±0.08</td>
</tr>
<tr>
<td>valsartan group</td>
<td>0.29±0.14</td>
<td>0.31±0.19</td>
<td>0.95±0.18</td>
<td>0.34±0.08</td>
<td>0.36±0.09</td>
</tr>
</tbody>
</table>

Note: Compared with sham-operation group, we marked those group with statistically significant difference with “a”, indicating $P$-value $< 0.05$. Compared with model group, we marked those with statistically significant difference with “b”, indicating $P$-value $< 0.05$.

### Table 4. Expression of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in all groups ($\bar{x}(\pm s, n=8)$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MMP-2 mRNA</th>
<th>TIMP-2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>sham group</td>
<td>0.55±0.05</td>
<td>0.45±0.05</td>
</tr>
<tr>
<td>model group</td>
<td>1.02±0.04</td>
<td>1.06±0.04</td>
</tr>
<tr>
<td>tetramethylpyrazine</td>
<td>0.78±0.05</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td>valsartan group</td>
<td>0.80±0.06</td>
<td>0.86±0.04</td>
</tr>
</tbody>
</table>

Note: Compared with sham-operation group, we marked those group with statistically significant difference with “a”, indicating $P$-value $< 0.05$. Compared with model group, we marked those with statistically significant difference with “b”, indicating $P$-value $< 0.05$.

### Discussion

Unilateral ureteral obstruction (UUO) is widely recognized as the classical model to study the pathological changes of renal interstitial fibrosis and the efficacy of the drugs on renal interstitial fibrosis\(^{16}\). In this experiment, renal atrophy, hard texture of kidney, expansion and cystic of the renal pelvis were observed in the model group, which suggested successful modeling. In the tetramethylpyrazine group, the expansion and contraction of tubular, the proliferation of renal interstitial fibrous tissue and inflammatory cell infiltration of rats reduced significantly.

The results of our experiment showed that: in the 2nd postoperative week, the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the UUO model group significantly increased...
compared with the sham group, but the ratios of MMP-9/TIMP-1 and MMP-2/TIMP-2 reduced. Consistent with previous results from RQ Chen, the expression of TIMP-1, TIMP-2, MMP-2 and MMP-9 of rats with UUO model significantly increased after surgery but the proteolytic activity of MMP-2 and MMP-9 gradually decreased. Similar trend was observed in the activity of TIMP-1 and TIMP-2, Zhang X18 also found that overexpression of TIMP-1 may increase the chance of renal interstitial fibrosis in aging rats. Some studies further showed that increased TIMPs and reduced ratio of MMPs/TIMP-1 are associated with glomerular disease19 and nephrogenic systemic fibrosis20.

MMPs can be secreted into the ECM expressed in the form of non-active latent enzyme or zymogen,21, propeptide was released and MMPs with biological activity was produced to play a biological effectiveness by activators. Gelatinases, including gelatinase A (MMP-2) and gelatinase B (MMP-9), allow gelatin hydrolysis. TIMP-1 and TIMP-2 are specific inhibitors of MMP-9 and MMP-2, respectively. TIMP-1 and proMMP-9 can be formed as stable complexes to hinder self-activation of proMMP-9; Meanwhile, TIMP-1 inhibits the vitality of activated MMP-9, and combines irreversibly to Zn2+ active center of activated MMP-9 in the proportion of 1:1, blocks or inhibits the activity of MMP-9. TIMP-2 can specifically bind to zinc ion in catalytic active center of MMP-2, the close of catalytic center, in order to prevent it from degrading ECM.

Proteins of MMP-2 and MMP-9 and proteins of TIMP-1 and TIMP-2 play opposite roles in the degradation of extracellular matrix. However, in this study we found that the expression of MMPs and TIMPs were synergistic. Our results were consistent with some previous reports. Lu et al.22 reported that extracellular matrix was degraded only when the level of activated MMP-2 in the tissue was higher than that of TIMPs. They also found that although the expression level of MMP-2 and MMP-9 was increased, their hydrolyase activity to degrade gelatin was decreased23. And the elevated levels of TIMPs might inhibit the activity of MMPs24. These data suggest that the synergistic expression of TIMPs with MMPs may help regulate the activity of MMPs. However, other study showed that higher TIMP-1 level might also occur without MMP reduction in protein-overload proteinuria and cyclosporine induced RIF model25. Considering nephrosclerosis26, diabetic nephropathy27 and other renal diseases associated with ECM, which have increased TIMP-1 or MMPs/TIMP-1 ratio decreased, we proposed that both high expression of TIMP-1 and lower ratio of MMPs/TIMP-1 might cause RIF. TIMP-1 can accelerate the development of renal interstitial fibrosis via inflammatory pathways28. In the experiments of renal fibrosis, despite of different methods, it was observed that TIMPs are locally expressed, the activities of MMPs are decreased29. Therefore, how to maintain the balance of MMP-2/TIMP-2 and MMP-9/TIMP-1 becomes a hotspot of renal interstitial fibrosis study.

Few effects of Tetramethylpyrazine on renal interstitial fibrosis have been studied20,29. Our results showed that the therapeutic effects between tetramethylpyrazine and receptor antagonist angiotensin II, and may inhibit extracellular matrix synthesis of collagen and activate its degradation by adjusting the balance between MMPs/TIMPs, which affects renal interstitial fibrosis.

In summary, the expressions of MMP-2, MMP-9 and TIMP-1, TIMP-2 renal are associated with interstitial fibrosis, tetramethylpyrazine can suspend the development and progression by negatively regulating the expressions of MMP-2, MMP-9 and TIMP-1, TIMP-2, which provides a possible approach for the prevention and treatment of renal interstitial fibrosis clinically.

Acknowledgements

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