UPLC/MS-Based Metabolomics Investigation of the Protective Effect of Hydrogen Gas Inhalation on Mice with Calcium Oxalate-Induced Renal Injury

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Hydrogen has a significant protective effect on calcium oxalate-induced renal injury, but its effect on metabolic profiles is unknown. This study showed the effects of hydrogen on serum and urine metabolites in a renal injury model. Ultra-HPLC quadrupole time-of-flight-MS-based metabolomics was used to characterise metabolic variations. Twenty-five serum metabolites and 14 urine metabolites showed differences in the the nitrogen and oxygen inhalation (NO), nitrogen and oxygen inhalation combined with calcium oxalate induction (CaOx), and hydrogen inhalation combined with calcium oxalate induction (HO+CaOx) groups. Nineteen serum metabolites and 7 urine metabolites showed significant restoration to normal levels after hydrogen gas (H2) treatment. These metabolites are primarily related to amino acid metabolism, fatty acid metabolism, and phospholipid metabolism. This study showed that a comprehensive metabolomics approach is an effective strategy to elucidate the mechanisms underlying the effects of hydrogen treatment on calcium oxalate-induced renal injury.

Key words calcium oxalate-induced renal injury; hydrogen; metabolomics

Metabolic analysis is an important component of systems biology and has been defined as “qualitative and quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification”. Metabolomics analyses have been used to identify small-molecule metabolites in serum or urine for holistic metabolic profiling of the metabolic status, which can help elucidate the biological network of metabolites in normal or pathological models and can provide information on disease processes and gene function. Calcium crystals could trigger kidney injury by the following steps: firstly, transient non-adhesive contact between crystals and cells would lead to tubular epithelial dedifferentiation, secondly, the crystals may adhere to the dedifferentiated epithelium, thirdly, the accumulation of crystals would obstruct renal tubular and increase mechanical pressure, finally, the tubular epithelial gets injured by the mechanical pressure and dedifferentiation. According to our previous study, inhalation of hydrogen gas (H2) can ameliorate calcium oxalate deposition and renal oxidative stress in mice. Thus, in this study, metabolic analysis of serum was performed to investigate changes in the metabolic regulatory networks in a model of renal injury induced by crystallisation of calcium oxalate. Furthermore, the effects of inhalation of high-dose hydrogen gas were assessed. Our results helped identify potential biomarkers of renal injury induced by crystallisation of calcium oxalate and analyse the potential targets of hydrogen gas.

MATERIALS AND METHODS

Animal Experiments Eighteen wild-type male C57BL/6 mice (7–8 weeks old) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were given water and food (mouse feed, purchased from Shanghai Shilin Biologic Science & Technology Co., Ltd., Shanghai, China) ad libitum and were kept under a controlled 12-h light/dark cycle at 20–25°C with relative humidity at 55–65%. All animal studies were performed at the Facility for Laboratory Animals of the Second Military Medical University (Shanghai, China) and in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All research protocols were approved by the Biomedical Ethical Committee of the Second Military Medical University.

Study Groups and Sample Collection After habituation for one week, eighteen mice were randomly divided into three groups: the nitrogen and oxygen inhalation group (NO), the nitrogen and oxygen inhalation combined with calcium oxalate induction group (CaOx), and the hydrogen inhalation combined with calcium oxalate induction group (HO+CaOx). The mice in the NO group inhaled a mixed gas consisting of 67% N2 and 33% O2 and underwent a daily intra-abdominal injection with saline (20mL/kg/d). The mice in the CaOx and HO+CaOx groups were injected with glyoxylate (100mg/kg/d) obtained from TCI (Tokyo, Japan) to establish the renal injury by crystallisation of calcium oxalate, and the CaOx group received mixed gas consisting of 67% N2 and 33% O2, while the HO+CaOx group received mixed gas consisting of 67% H2 and 33% O2. One day after the intra-abdominal injection, blood samples were collected by retro-orbital puncture and stored at 4°C for 2h. Then, we use a centrufuge (Sigma 3–30K, Germany) to separate the acquired venous blood at 3000rpm (654g) for 10min to obtain the serum for biochemical analysis. We collected the urine of the...
mice for 24 h through a metabolic cage, which was placed in a larger, ventilated chamber during inhalation experiments, and all samples were stored at −80°C before analysis.

**Gas Administration** The mixed gas consisting of 67% H₂ and 33% O₂ was produced by an AMS-H-01 hydrogen oxygen nebuliser (Asclepius, Shanghai, China), which extracted the hydrogen and oxygen from water. Mixed gas consisting of 67% N₂ and 33% O₂ was obtained by mixing N₂ and O₂ at a ratio of 2:1. We placed the mice in a closed box (20×18×15 cm, length×width×height), and the mixed gas was let into the box at a rate of 200 mL/min for 30 min to replace the air in the box. The mice then inhaled the mixed gas for 30 min. During each experiment, the concentration of hydrogen gas in the box was monitored.

**Sample Preparation** The serum samples were defrosted at room temperature for 30 min, and a 100 µL aliquot of each serum sample was mixed with 300 µL of methanol. After centrifugation at 3000 rpm (654 g) for 1 min, these samples were centrifuged at 13000 rpm (12281 g) for 15 min at 4°C and incubated for 10 min at 4°C. The urine samples were defrosted at room temperature, and a 100 µL aliquot of each urine sample was mixed with 300 µL acetonitrile to precipitate the proteins. Clear supernatant was transferred to the sampling vial for ultra (U)-HPLC-MS analysis, and an aliquot of 2 µL was injected for LC-MS analysis. UHPLC-quadrupole time-of-flight (Q-TOF)/MS profiling analysis was performed by an Agilent 1290 Infinity LC system equipped with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight mass spectrometer (Agilent Technologies, Santa Clara, CA, U.S.A.). Chromatographic separations were performed at 40°C on an ACQUITY UPLC HSS T3 column (2.1 mm×100 mm, 1.8 µm, Waters, Milford, MA, U.S.A.). The mobile phase consisted of 0.1% formic acid (A) and methyl cyanide modified with 0.1% formic acid (B). The optimised UPLC elution conditions were as follows: 0–2 min, 5% B; 2–10 min, 5–15% B; 10–14 min, 15–30% B; 14–16 min, 30–95% B; 16–18 min, 95% B, and the post time was 6 min to equilibrate the system. The flow rate was set to 0.4 mL/min, the injection volume was 2 µL and the auto-sampler was maintained at 4°C. An electrospray ionisation (ESI) source was used in both positive and negative mode. The experimental conditions were as follows: capillary voltage, 4000 V for positive mode and 3500 V for negative mode; gas temperature, 350°C; drying gas flow, 11 L/min; fragmentor voltage, 120 V; skimmer voltage, 60 V. Data were collected in profile mode from 50 to 1100 m/z, and we chose data from 121.0509 to 922.0098 for correction. These potential biomarkers were further analysed by MS/MS, and the collision energy was set from 10 to 50 eV.

**Detection of Serum Kidney Injury Molecule-1** Serum levels of kidney injury molecule 1 (KIM-1) were detected by enzyme-linked immunosorbent assay (ELISA) kit (Uscn, Wuhan, China), and the experiment was performed according to the manufacturer’s instructions.

**Data Processing and Statistical Analysis** The statistical analysis included pre-processing, pattern recognition, model validation and variable screening. The purpose of the pre-processing was to reduce the error produced in the experiments and analysis after processing the raw data and standardise the data structure. After this process, each sample had a total ion current (TIC) profile, and we used Agilent MassHunter Qualitative software and R software (XCMS package) to normalise the data to reduce the analytical error. Data were introduced into SIMCA-P software (Umetrics, Sweden) to produce multidimensional analysis, which used partial least squares discriminant analysis (PLS-DA). The absicca of the score plots of the principal component analysis represents the first principal component of the multivariate statistical analysis, and the ordinate represents the second principal component. The y-axis of the score dots of the composite diagrams of the PLS-DA scatter plot and variable importance plots represents the VIP value of every metabolite, and the y-axis of triangle dots represents the partial correlation coefficients (Pcorr) value of every metabolite. The relevant parameters, such as R² and Q², were analysed to evaluate the quality of the models. The results are presented as the mean±standard deviation (S.D.) We used one-way factorial ANOVA and Tukey’s test to test for differences among the groups, p<0.05 indicated a statistically significant difference.

**Identification of Potential Biomarkers** Identification of Potential Biomarkers Based on Mass Spectrometry Information and Network Database. The steps were as follows:

1. Confirm the ions based on the TIC and determine the nucleoplasmic relation of these ions.
2. Input the exact masses of molecular ions into online databases, such as the Metlin database (http://metlin.scripps.edu/), Human Metabolome Database (http://www.hmdb.ca/), and Mass Bank (http://www.massbank.jp/), to identify possible metabolites.
3. Compare the MS/MS spectra with the MS/MS information from the above databases to verify the structure of the putative metabolites.
4. Confirm the metabolites using standard samples based on the retention time and the fragment information.

### RESULTS

**The Level of Serum KIM-1** Serum KIM-1 levels were significantly increased in mice after the intraperitoneal injection of glyoxylate. At the same time, hydrogen inhalation effectively alleviated the elevation of serum KIM-1 induced by glyoxylate (Fig. 1).

**Multivariate Statistical Analysis** Based on the visual processing of the detection system, TIC profiles of typical serum samples from each group are shown in Fig. 2. Nor-

![Fig. 1. Changes in Serum KIM-1 Levels in the NO, CaOx, and HO+CaOx Groups](image-url)

Serum KIM-1 levels in the CaOx group were significantly higher than those in the NO group. Hydrogen reduced the increase of serum KIM-1 level induced by calcium oxalate crystal. *p<0.05 versus the NO group, #p<0.05 versus the CaOx group.
normalised and centralised processes and PLS-DA were performed to eliminate the experimental and analytical errors and show the distribution and differences between each set of samples. The positive and negative modes in serum and urine samples of PLS-DA score plots (Figs. 3A, B and Figs. 4A, B) revealed the general distribution of the three groups. The score plots showed the distribution in the same groups, and the three groups showed obvious differences. To screen differences among variables and identify significant information from the data we obtained, we used variable importance in projection (VIP) and Pcorr to assess differential variables. The PLS-DA plot (Figs. 3A, B and Figs. 4A, B) and VIPs (Figs. 3C, D and Figs. 4C, D) can be used to define the important differential metabolites for distinguishing the two groups.

**Potential Biomarkers and Classification**

In the present study, the ions with VIPs greater than 1.0 and p values greater than 0.05 were considered important differential metabolites. Based on this, we used statistical analysis to compare the differences in serum and urine of the CaOx group and NO group and identified 25 metabolites in serum (Table 1) and 14 metabolites in urine (Table 2) as potential biomarkers.

**Evaluation of the Protective Mechanism of H$_2$**

In the first part, we obtained 25 potential serum biomarkers and 14 potential urine biomarkers in the CaOx and NO groups. Based on these 25 potential serum biomarkers and 14 potential urine biomarkers, we used statistical analysis to evaluate the
Table 1. Potential Serum Biomarkers Related to Crystal-Induced Kidney Injury and Their Metabolic Pathways

| No. | $t_r$/min | $m/z$ | Ion Formula | Identification | Related pathway |
|-----|-----------|-------|-------------|----------------|----------------|
| 1   | 0.70      | 175.119 | $[M+H]^+$ | C$_6$H$_{11}$N$_2$O$_2$ $\delta$-Arginine | 0.017 0.63 0.000 2.15 Arginine metabolism |
| 2   | 0.80      | 203.052 | $[M+H]^+$ | C$_6$H$_8$O$_7$ $\delta$-Inositol | 0.001 0.60 0.000 1.83 Fatty acid metabolism |
| 3   | 0.85      | 204.123 | $[M+H]^+$ | C$_6$H$_8$O$_7$ Acetyl carnitine | 0.000 2.55 0.001 0.61 Fatty acid metabolism |
| 4   | 0.98      | 150.058 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ $\delta$-Methionine | 0.041 0.45 0.000 1.90 Metionine metabolism |
| 5   | 1.20      | 204.123 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Tryptophanamide | 0.000 2.27 0.000 0.47 Tryptophan metabolism |
| 6   | 1.40      | 130.088 | $[M-H]^-$ | C$_6$H$_8$NO$_3$ $\delta$-Leucine | 0.009 0.63 0.000 2.20 Leucine metabolism |
| 7   | 2.21      | 209.092 | $[M+H]^+$ | C$_6$H$_8$O$_7$ Kynurenine | 0.001 2.50 0.317 0.80 Tryptophan metabolism |
| 8   | 4.99      | 178.051 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Hippuric acid | 0.000 0.18 0.212 1.67 Glycine metabolism |
| 9   | 5.61      | 283.083 | $[M-H]^-$ | C$_6$H$_8$NO$_3$ p-Cresol glucuronide | 0.000 15.20 0.494 0.89 Tyrosine metabolism |
| 10  | 5.69      | 260.186 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ $\delta$-Hexanoyl carnitine | 0.000 2.80 0.000 0.89 Fatty acid metabolism |
| 11  | 5.85      | 187.007 | $[M+H]^+$ | C$_6$H$_8$O$_7$ p-Cresol sulfate | 0.000 26.67 0.964 0.99 Tyrosine metabolism |
| 12  | 5.93      | 174.112 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Isovaleryl alanine | 0.000 10.00 0.000 0.12 Alanine metabolism |
| 13  | 7.05      | 201.113 | $[M-H]^-$ | C$_6$H$_8$O$_7$ Sebacic acid | 0.000 6.00 0.000 0.39 Fatty acid metabolism |
| 14  | 8.89      | 344.28  | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Dodecanoyl carnitine | 0.000 2.71 0.000 0.42 Fatty acid metabolism |
| 15  | 9.29      | 370.295 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Tetradecanoyl carnitine | 0.000 3.44 0.000 0.36 Fatty acid metabolism |
| 16  | 9.53      | 396.31  | $[M+H]^+$ | C$_6$H$_8$NO$_3$ 9,12-Hexadecadienoyl carnitine | 0.000 8.57 0.000 0.17 Fatty acid metabolism |
| 17  | 9.71      | 372.311 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Tetracosanoyl carnitine | 0.000 4.22 0.001 0.26 Fatty acid metabolism |
| 18  | 9.96      | 398.326 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ trans-Hexadec-2-enoyl carnitine | 0.000 3.75 0.000 0.23 Fatty acid metabolism |
| 19  | 10.20     | 424.342 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Oleoyl carnitine | 0.000 3.20 0.000 0.38 Fatty acid metabolism |
| 20  | 10.30     | 380.257 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Sphinganine phosphate | 0.005 1.47 0.002 0.64 Phospholipid metabolism |
| 21  | 10.40     | 518.324 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ LysoPC (18:3) | 0.032 0.75 0.006 1.47 Phospholipid metabolism |
| 22  | 10.66     | 426.358 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ Vaccenoyl carnitine | 0.000 3.50 0.000 0.36 Fatty acid metabolism |
| 23  | 10.81     | 478.293 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ LysoPE (18:2:0:0) | 0.000 0.57 0.000 1.88 Phospholipid metabolism |
| 24  | 11.90     | 522.354 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ PC (18:1:0:0) | 0.024 0.69 0.233 1.22 Phospholipid metabolism |
| 25  | 11.82     | 319.227 | $[M+H]^+$ | C$_6$H$_8$NO$_3$ HETE | 0.018 1.40 0.096 0.80 Fatty acid metabolism |

\(a\) Indicates the comparison of the CaOx group and NO group; \(b\) indicates the comparison of the HO+CaOx group and CaOx group.
### Table 2. Potential Urine Biomarkers Related to Crystal-Induced Kidney Injury and Their Metabolic Pathways

| No. | tₜ/min | m/z | Ion Formula       | Identification              | CaOx groupᵃ | Ho+CaOx groupᵇ | Related pathway                                             |
|-----|--------|-----|-------------------|------------------------------|--------------|-----------------|------------------------------------------------------------|
| 1   | 0.70   | 146.165 | [M+H]⁺ C₆H₈N₂ | Spermidine                   | 0.015 0.58   | 0.595 0.85      | Phenylpropanoid biosynthesis                               |
| 2   | 0.84   | 215.035 | [M+H]⁺ C₆H₁₀O₃P | Deoxyribose 5-phosphate      | 0.017 1.66   | 0.001 0.37      | Pentose phosphate pathway                                  |
| 3   | 1.61   | 221.091 | [M+H]⁺ C₆H₁₂N₂O₂ | 5-Hydroxy-γ-tryptophan       | 0.000 1.82   | 0.046 0.76      | Tryptophan metabolism                                     |
| 4   | 3.88   | 298.097 | [M+H]⁺ C₆H₁₄N₂O₄S | 5'-Deoxy-5'-(methythio) adenosine | 0.001 0.35   | 0.971 1.02      | Cysteine and methionine metabolism                         |
| 5   | 4.53   | 144.046 | [M−H]⁻ C₆H₁₄NO | Indole-3-carboxaldehyde      | 0.000 2.47   | 0.033 0.73      | Tryptophan metabolism                                     |
| 6   | 6.06   | 192.066 | [M−H]⁻ C₆H₁₄NO₃ | Phenylacetlyglycine          | 0.037 1.18   | 0.697 1.03      | Phenylalanine metabolism                                  |
| 7   | 6.32   | 190.052 | [M−H]⁻ C₆H₁₂NO₂ | 5-Hydroxyindoleacetic acid   | 0.034 1.57   | 0.259 1.19      | Fatty acid metabolism                                     |
| 8   | 8.53   | 229.141 | [M−H]⁻ C₆H₉O₃ | Tyramine                     | 0.020 0.60   | 0.108 0.56      | Fatty acid metabolism                                     |
| 9   | 8.91   | 200.128 | [M−H]⁻ C₆H₁₁NO₃ | N-Hexanoyl-γ-homoserine lactone | 0.008 1.58   | 0.171 0.82      | Serine metabolism                                          |
| 10  | 9.84   | 239.164 | [M−H]⁻ C₆H₁₂O₃ | 7-αx-Tetradecadienoic acid   | 0.002 3.10   | 0.002 0.33      | Fatty acid metabolism                                     |
| 11  | 10.42  | 288.290 | [M−H]⁻ C₆H₁₄NO₂ | C17 Sphinagane               | 0.029 1.68   | 0.010 0.51      | Phospholipid metabolism                                   |
| 12  | 10.61  | 243.161 | [M−H]⁻ C₆H₁₄O₃ | Undecanedicarboxylic acid    | 0.000 2.73   | 0.001 0.16      | Fatty acid metabolism                                     |
| 13  | 11.04  | 241.180 | [M−H]⁻ C₆H₁₄O₃ | 7-αx-Tetradecenoic acid      | 0.011 0.43   | 0.705 0.82      | Fatty acid metabolism                                     |
| 14  | 13.07  | 279.159 | [M−H]⁻ C₆H₁₈O₄ | α-CEHC                      | 0.000 1.90   | 0.000 0.36      | Vitamin E metabolism                                      |

ᵃ) Indicates the comparison of the CaOx group and NO group; b) indicates the comparison of the Ho+CaOx group and CaOx group.

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**Fig. 5.** The Serum Metabolic Pathway Network of the Effect of Hydrogen Treatment on Renal Tubular Epithelial Cells Injury Caused by Crystallisation of Calcium Oxalate

The levels of potential biomarkers in the Ho+CaOx group compared to the CaOx group are labelled in red (up-regulated after hydrogen gas inhalation) and green (down-regulated after hydrogen gas inhalation), and black represents metabolites not found in this experiment. Solid arrows indicate the direct production of downstream metabolites, and dotted arrows indicate the indirect production of downstream metabolites. (Color figure can be accessed in the online version.)
These findings confirmed the protective effect of H2, and the effects of N2 and H2 changes in the metabolic processes of the crystal renal injury. The serum metabolite pathway (Fig. 5) of the kidney injury by crystallisation of calcium oxalate was assessed by searching the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY Database (http://www.genome.jp/kegg/) to reflect the effects of H2.

DISCUSSION

In our experiment, we established a glyoxylate-induced crystal-related kidney injury model, and we used a metabolomics approach based on UHPLC-Q-TOF/MS to study the changes in the metabolic processes of the crystal renal injury and the effects of N2 and H2. Twenty-five serum metabolites and 14 urine metabolites changed significantly in the CaOx group; these molecules were primarily related to amino acid metabolism, fatty acid metabolism and phospholipid metabolism. Nineteen serum metabolites and 7 urine metabolites were significantly restored to normal levels after H2 treatment. These findings confirmed the protective effect of H2, and we found that metabolomic changes were closely related to the damage of renal tubular epithelial cells. Renal tubular epithelial cells become apoptotic and necrotic following oxidative stress, a key element in renal tubular epithelial cell damage. This process contributes to renal crystal deposition and the incidence of renal stones, resulting from the production of reactive oxygen species (ROS). ROS are considered cytotoxic and can injure multiple macromolecules, an early event in the damage of renal tubular epithelium and the formation of calcium oxalate crystal stones.

In fatty acid metabolism, mitochondria and peroxisomes are the major site for β-oxidation. Previous studies have indicated that in acute kidney injury (AKI), fatty acid β oxidase in the mitochondria and peroxisomes decreased significantly. In our study, the CaOx group showed significant increases in 9 of 25 serum metabolites, including acetylcarnitine, t-hexanoylcarnitine, dodecanoylcarnitine, tetradecanoylcarnitine, 9,12-hexadecadienoylcarnitine, tetradecanoylcarnitine, trans-hexadec-2-enoyl carnitine, linoelaidylcarnitine and vaccenyl carnitine. Most of these metabolites were long-chain carnitine molecules. Sun et al. found that the concentrations of serum acylcarnitine and medium-chain carnitine in AKI patients were higher than those in healthy individuals. Urine fatty acid metabolites were primarily short-chain fatty acid-based products. Interestingly, 5-hydroxyindoleacetic acid, 7-oxo-tetradecadienoic acid, and undecanedicarboxylic acid in urine were increased in the CaOx group, and traumatic acid and 7-oxo-tetradecenoic acid were decreased. Furthermore, 5-hydroxyindoleacetic acid was shown to be increased in AKI, and traumatic acid could reduce oxidative stress. Acylcarnitine levels are significantly higher in patients with chronic kidney disease and maintenance than in healthy individuals. The serum metabolomics results from our study and other scholars showed that increased levels of acylcarnitine and long-chain carnitine in renal injury indicated fatty acid metabolic disorders.

We compared the HO+CaOx and CaOx groups and found that 9 serum fatty acyl carnitine showed normal levels after inhalation of H2, which indicated that H2 could improve the fatty acid metabolism. Sebacic acid is the product of α oxidation of fatty acids, and in normal circumstances, sebacic acid is converted into acyl-CoA and transferred to the mitochondria for β-oxidation. When the mitochondria are dysfunctional, sebacic acid accumulates in the body and is unavailable for β-oxidation. In our study, the sebacic acid concentration was higher in the CaOx group than the NO group and was at normal levels in the HO+CaOx group.

Renal tubular epithelial cell membranes mainly comprise phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinerse (PS) and phosphatidlyinositol (PI). PS is an acidic phospholipid and is considered to be the key phospholipid in the formation of renal stones. Previous studies have shown that when the cell membrane was exposed to oxalic acid or calcium oxalate crystals, the polarity of the cell membrane changed, and PS was exposed to the outside, which provided sites for crystal adhesion and aggregation. In our study, 5 metabolites related to phospholipid metabolism changed significantly in the CaOx group, indicating that phospholipid metabolism in the CaOx group was disordered. After treatment with H2, 4 metabolites were restored to normal levels, suggesting that H2 can improve the disordered phospholipid metabolism and maintain the stability of cell membranes to protect renal tubular epithelial cells.

Proteins are an important component of the cell membrane, and when renal tubular epithelial cells are damaged, amino acids also show dysfunction. Our study showed that in serum, 6 amino acid metabolites were dysfunctional, and 8 amino acid metabolites were significantly changed in the CaOx group. We found that tyrosine metabolites, such as p-cresol glucuronide and p-cresol sulphate, which are uraemia toxins and inflammation biomarkers, and renal injury caused by oxalic acid and calcium oxalate crystals showed significant improvement. Kynurenine is a metabolite of tryptophan metabolism and a uraemia toxin. Moreover, 5-hydroxy-L-tryptophan and indole-3-carboxaldehyde, which are tryptophan metabolites, were found in urine and increased in renal dysfunction. α-Carboxyethyl hydrochroman is a urinary metabolite of vitamin E that was increased in the CaOx group and may be related to the heavy consumption of vitamin E as an antioxidant. Previous studies have shown that the kynurenine concentration in the blood is proportional to the degree of renal dysfunction. Similar results were observed in our study, and the kynurenine level improved significantly in the CaOx group and slightly decreased after H2 treatment. These findings may be related to the effect or acting time of H2 and should be studied in the future.

Serum metabolomics studies in mice showed that H2 was protective against renal injury caused by crystallisation of calcium oxalate, which may be related to changes in fatty acid metabolism, phospholipid metabolism and amino acid metabolism. In our study, we found the H2 could significantly improve metabolic disorders in mice.

CONCLUSION

In this study, we used a sensitive metabolomics method, which is based on LC and MS, to analyse the complex internal
environment of calcium oxalate-induced renal injury and the potential pathways affected by hydrogen gas. We identified 25 serum metabolites and 14 urine metabolites as potential biomarkers. These biomarkers are related to energy metabolism, amino acid metabolism, fatty acid metabolism and phospholipid metabolism. The biomarkers also play a critical role in other metabolic pathways. Although the complex mechanism of renal injury and the treatment mechanism of hydrogen gas were not clearly elucidated, our study might provide new insights into this disease and suggest new therapeutic methods. However, further studies are needed for a better understanding of the molecular mechanisms of calcium oxalate-induced renal injury and the therapeutic effects of hydrogen gas.

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Conflict of Interest The authors declare no conflict of interest.

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