Integrating untargeted metabonomics, partial least square regression analysis and MetPA to explore the targeted pathways involved into Huangqi Jiangzhong Tang against chronic atrophic gastritis rats

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ABSTRACT

Huangqi Jiangzhong Tang (HQJZ), a famous traditional Chinese medicine (TCM) formula, has been widely used for treating chronic atrophic gastritis (CAG) in China. However, its action mechanism was still lack of holistic interpretation. Here, an integrated untargeted metabonomics, partial least square regression analysis (PLS-RA) and MetPA was applied to investigate the intervention of HQJZ against CAG. Our metabonomic study revealed that HQJZ showed the potential protection from metabolic perturbation induced by CAG. Eleven altered metabolites and seven involved metabolic pathways were significantly regulated with pretreatment of HQJZ. Regulation of two pathways: glycine, serine and threonine metabolism and taurine and hypotaurine metabolism, were recognized to be the most relevant efficacy of HQJZ against CAG based on PLS-RA and MetPA analysis, which were related to the improvement of the pathological changes including energy imbalance, oxidative stress, alterations of immune system, as well as inflammation. The result suggested that the proposed strategy could decipher the scientific basis of TCM well, and give us new insights into the pathogenesis of CAG and the targets for clinical treatment.

1. Introduction

Numerous studies have revealed that high mortality from gastric cancer results from the prevalence of chronic atrophic gastritis (CAG), especially in Eastern Asia [1]. Recently, the morbidity of CAG has been rapidly increasing in China. The CAG patients’ gastric mucosa atrophies, gastric sinus secret cells reduce, function weakens, gastric acid secretion reduces, and especially pathological changeable epithelium often contains intestine epithelial metaplasia and atypical hyperplasia, which are often seen as precancerous lesions of gastric carcinoma [2]. At present, the clinic treatment for CAG is mainly relied on the usage of antacid, spasmolysant and mucosa protectant. Nevertheless, their effectiveness is limited due to the complexity of the CAG. In recent years, TCM preparations have been widely accepted by patients with CAG, owing to their double-deck functions of the treatment and recuperation, and fewer side effects [3].

Huangqi Jiangzhong Tang (HQJZ), which came from a TCM classic named Synopsis of Golden Chamber, is made up of seven herb materials, including Astragali Radix (HuangQi), Cinnamomi Ramulus (GuiZhi), Paeonie Radix Alba (BaiShao), Jujubae Fructus (DaZao), Rhizoma zingiberis recens (ShengJiang), Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (GanCao) and Saccharum granorum (YiTang). It has been used in the treatment of various gastrointestinal tract diseases, such as gastritis and stomach ulcer [4]. Up to now, evidences from animal test in rat models with spleen-asthenia have showed that HQJZ had potential abilities including anti-inflammation, antioxidative stress, and endothelial and mucosal protection [5,6]. In one word, HQJZ may be a multi-targeting management for the treatment of CAG. However, the mechanism responsible for its beneficial role is poorly understood, especially for its involved metabolic regulation for CAG.

Metabonomics, a new member of omics technologies, considers the human body as a whole system, which is consistent with the TCM concept (“Tian Ren He Yi” in Chinese), and also shows wide application prospects in interpreting the action mechanism and revealing the drug targets of TCM [7]. With its rapid evolution, there is an increasing need for methods to support the processing and analysis of metabonomic data.

Pathway analysis softwares have emerged as an invaluable aid to explore the metabolic network associated with TCM actions in meta-
bonomics, such as Pathway Studio, MetaCore, Ingenuity Pathway Analysis (IPA), etc., which have been originally designed and developed for the analysis of genomic or proteomic data, but not metabolomic data. Additionally, these methods utilized in metabolomic studies were just focused on the assignment of metabolic pathways and the construction of metabolic network, without the pathway enrichment analysis to illustrate the most relevant metabolic pathways involved in TCM metabolomic studies. MetPA, specially designed for metabolomic study, is a user-friendly, web-based tool dedicated to the analysis and visualization of metabolomic data within the biological context of metabolic pathways. It combines several advanced pathway enrichment analysis procedures along with the analysis of pathway topological characteristics to help identify the most relevant metabolic pathways involved in a given metabolomic study [8]. To date, MetPA has been demonstrated as a dedicated pathway analysis and visualization tool to facilitate the use of these relatively new and powerful methods in interpreting the targeted pathways of TCMs against diseases [9]. However, this method was limited based on the qualitative analysis without referring to the metabolic alteration extent of these whole pathways. Especially, few studies have been conducted in the relationship between the metabolic pathway and the relevant physiological outcomes, which might highlight the systemic mechanism exploiting from metabolic pathway and endogenous enzymes.

PLS-RA has been reported to be a good choice to illustrate both the correlation and changing direction of corresponding metabolite [10]. Confidence intervals for the calculated regression coefficients could create means for understanding the significance of the variables and the interactions between variables on the measured responses. So PLS-RA could provide supplementary for identifying the relevant pathways with quantitative metabolic information. The combination of PLS-RA and MetPA might be a powerful tool for defining the important targeted pathway related to TCMs.

The present study was to address the protective role of HQJZ in CAG rats and its underlying mechanism based on a new strategy integrating untargeted metabolomics, partial least square regression analysis and MetPA. To our knowledge, this is the first study that deeply assessed the metabolic regulation and the targeted pathways of HQJZ against CAG.

2. Materials and methods

2.1. Reagents and materials

Six raw herbal medicines, including Astragalus Radix (HuangQi), Cinnamomi Ramulus (GuiZhi), Paoniae Radix Alba (BaiShao), Jujubae Fructus (DaZao), Rhizoma zingiberis Recens (ShengJiang) and Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (GanCao), were purchased from Beijing Tongren Tang Pharmaceutical Co. Ltd. (Beijing, China), and authenticated by Prof. Xuemei Qin from Modern Research Center for Traditional Chinese Medicine (MRCTCM) of Shanxi University, and then kept in our laboratory at MRCTCM of Shanxi University, and then kept in our laboratory at MRCTCM of Shanxi University, Taiyuan, China. Saccharum granorum (YiTang) was purchased from Jingchun Tianli Bio-tech, Co. Ltd. (Huanggang, China). Vitacoenzyme tablets were purchased from Hubei Greengold pharmaceutical Co. Ltd. (Ezhou, China). Sodium Deoxycholate was provided by Beijing Aoboxing Bio-tech, Co. Ltd. (Beijing, China). Deuteriumoxide with 0.05% 3-trimethylsilyl-(2, 2, 3, 3-H4)-1-propionate (TSP) (D2O, 99.9%) were purchased from Sigma-Alorich (St. Louis, USA). The assay kits for superoxide dismutase (SOD), malondialdehyde (MDA) and lipoprotein (PA) were purchased from Nanjing Jincheng Bioengineering Institute (Nanjing, China). Ultrapure water (18.2 μL) was prepared with a Milli-Q water purification system (Millipore, France). All other used chemicals were of analytical grade.

2.2. Preparation of HQJZ

The mixed crude herbs, including HuangQi (9 g), GuiZhi (9 g), BaiShao (18 g), ShengJiang (12 g) and GanCao (6 g) and 4 fruits of DaZao, were crushed into small pieces, soaked and mixed with water (solvent:sample = 5:1, v/w) for 30 min. The extraction process was refluxed in haven for two times and 2 h for each. The extracted solutions were filtered and added 30 g of Yitang, and then evaporated to 1000 μL of under reduced pressure at 60 °C. The HQJZ solution was stored in a –20 °C refrigerator for subsequent experiments.

2.3. Animal treatment

All procedures and the care of the rats were in accordance with the National Guidelines for Experimental Animal Welfare (MOST, China, 2006) at the Center for Animal Experiments, which has full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International. Maximum effort was exerted to minimize animal suffering and the number of animals necessary for the attainment of reliable data. SPF-grade male Sprague-Dawley (SD) rats (body weight, 180 ± 20 g), were obtained from Vital River Laboratory Animal Technology Co. Ltd. They were maintained at a constant humidity (ca. 60%) and temperature (ca. 23 °C) with a light/dark cycle of 12 h.

After one week of adaptation, the rats were randomly separated into 5 groups according to the body weights (n = 6). The control group had free access to normal chow and water. From the first day, those rats in other group were administrated freely with ammonia solution (0.1%) and deoxycholic acid (20 mmol/L) on alternate days, respectively. Meanwhile, the animals were treated with the hunger disorder method, which rats had free access to normal diet for two days, and then fasted for one day. The cycles were performed during the whole experimental period of 10 weeks. Rats in low-dose group (HQJZ, 9.2 g crude herbs/kg), high-dose group (HQJZ, 36.8 g crude herbs/kg) and positive group (vitacoenzyme, 0.86 g/kg) were received for 10 weeks, respectively. Body weights were measured every 6 day in first month and every 3 day in the followed experimental period.

2.4. Sample collection

After the last body weight determination, rats were anesthetized with 10% urethane. Blood samples were collected, and centrifuged at 3000 rpm for 15 min at 4 °C. The resultant plasma samples were stored at –80 °C until analysis. The gastric tissues were immediately removed and washed with physiological saline. One part of gastric tissues was cut and put into a tube containing 10% buffered formalin solution for the histopathology analysis.

2.5. Biochemistry assays and histopathology

Biochemical indexes of plasma SOD, MDA and gastric PA were measured according to the instructions of enzymatic kits. Gastric tissues were fixed with 10% formaldehyde solution for 48 h, embedded in paraffin, 5 mm sectioned, and stained with hematoxylin-eosin (HE). Images were obtained and studied under light microscopy (Olympus, BX53, Japan).

2.6. NMR analysis

300 μL of plasma and 300 μL of 0.9% NaCl (saline) in D2O (20%) were pipetted individually and mixed together. Following centrifugation (13,000 rpm, 10 min, 4 °C), 550 μL of supernant was removed in a 5 mm NMR tube for NMR analysis.

The one dimensional (1D) NMR spectra and two-dimensional (2D) NMR spectra were recorded at 298 K on a Bruker 600-MHz AVANCE III NMR spectrometer (Bruker BioSpin, Bremen, Germany) equipped with a Bruker 5 mm PA BBO probe operated at 600.13 MHz 1H frequency. Samples were analyzed using one-dimensional Carr-Purcell-Meibom-Gill (CPMG) NMR spectra with water suppression. Each 1H NMR
spectra of plasma consisted of 64 scans requiring a 2.654 s acquisition time with the following parameters: spectral width of 12,345.7 Hz, spectral size of 65,536 points, and a relaxation delay (RD) of 1.0 s.

For spectral assignment purposes, two-dimensional (2D) NMR spectra including $^1$H-$^1$H correlation spectroscopy (COSY), $^1$H-$^13$C heteronuclear single-quantum correlation spectroscopy (HSQC) were recorded. 2D $^1$H-$^1$H COSY spectra were analyzed using the noesy gpp rqp pulse sequence for plasma samples and following parameters: 1.5 s relaxation delay and 6602.1 Hz spectral width in F2 and 6601.5 Hz in F1. 2D $^1$H-$^13$C HSQC spectra were analyzed using the hsqcetgpsisp pulse sequence for plasma samples and following parameters: 1.2 s relaxation delay and 6602.1 Hz spectral width in F2 and 36,220.3 Hz in F1.

2.7. Data processing and multivariate pattern analysis

All NMR spectra were then corrected for phase and baseline distortions using MestReNova (version 8.0.1, Mestrelab Research, Santiago de Compostela, Spain). The peaks of plasma spectra were referenced internally to the chemical shift of creatinine at δ 3.04 ppm. The plasma spectra were divided and the signal integral computed in 0.001 ppm intervals across the region δ 0.50–9.00 ppm. The region of δ 4.68–5.19 ppm was excluded from the analysis to eliminate the effects of imperfect water saturation.

Prior to further data analysis, the integral values of each spectrum were normalized to a total sum of the spectrum with decreasing any significant differences among samples. Multivariate statistical analysis was performed to process the acquired NMR data using SIMCA-P 13.0 (Umetrics, Sweden). Import data was mean-centered and pare to-scaled prior to multivariate analyses. Partial least squares discriminate analysis (PLS-DA) was utilized to reveal the differences among different groups, which were necessary to eliminate outliers. Potential biomarkers were extracted from S-plot constructed with the orthogonal partial least squares discriminate analysis (OPLS-DA), and the biomarkers were chosen based on their contribution to the differences.

Metabolite peaks were interpreted with available biochemical databases, such as HMDB (http://www.hmdb.ca/) and KEGG (http://www.kegg.com/). Further validations were achieved by extensive analysis of 2D NMR spectra (COSY and HSQC), and the cross peaks in HSQC were input in COLMAR $^{13}$C-$^1$H Query server (http://spin.cccr.ohio-state.edu/index.php/hsqc/). The cutoff value for $^1$H and $^{13}$C were set as 0.06 and 0.6 ppm respectively. These results were manually checked by interactive user interface using the “Show Me” button, as well as the parameter of Matching ratio and Uniqueness.

2.8. Metabolic pathway analysis

The construction, interaction and pathway analysis of these identified potential biomarkers were performed with MetPA (http://metpa.metabolomics.ca) [8], and database sources, including the KEGG, the Human Metabolome database (HMDB), METLIN and the related literatures.

Under MetPA analysis, Rattus norvegicus (rat) was selected as the source organism. And the names of the identified metabolites were imputed to obtain the linked pathways in which these metabolites are involved, along with hyperlinks to their pathway images. All results are linked to the HMDB where we can obtain more detailed information for each metabolite or pathway. Meanwhile, the software was applied to identify metabolic pathways that are most likely to be associated with the efficacy of HQJZ against CAG, which combined functional enrichment analysis and network topology analysis. A global test was selected for enrichment analysis, once time in combination with a between ness centrality test and one time in combination with degree centrality tests, which are tests of topological analysis. The result is a list of pathways in the KEGG database with corresponding p-values from enrichment analysis and a measure of the impact of each pathway based on topological analysis. Associated pathways can be visualized and explored via a Google-map style interactive visualization framework [11].

Meanwhile, the underlying relationship between differential metabolic pathways and PA was evaluated by PLS-RA. PLS-RA is a multivariate calibration model to reveal the relationship between two matrices (X and Y) based on the two-block predictive PLS model, which could afford the latent structures of observations by regression [12]. Predicted variations (Q$^2$Y) and significance of the model (p value) are its main diagnostic parameters. In this study, plasma PA level acted as Y-variables to characterize the gastric injury, and the integrating peak integrals of differential metabolites involved into the same metabolic pathways acted as X-variables.

At last, the metabolic networks associated with CAG and HQJZ mediations were constructed with a web-free tool Cytoscape (v3.2.1) [13].

2.9. Statistical analysis

All values were expressed as mean ± S.D. A two-tailed unpaired t-test by SPSS 16.0 (Chicago, IL, USA) was applied to analyze those significant differences between two groups, and the significance threshold was considered at p < 0.05.

3. Results

3.1. Effect of HQJZ on body weight of CAG rats

The loss of body weight is one typical feature in the pathogenesis of CAG. As showed in Fig. 1, rat body weights were measured during the whole experiment in our study. Compared with control group, their weight were more significant loss in model group, indicated that the CAG model was successfully established. After pretreatment of HQJZ at low and high doses, this obvious symptom of CAG was both inhibited significantly. Meanwhile, the weight improvement was also ameliorated by positive drug, which was less than HQJZ.

3.2. Effects of HQJZ on biochemical indexes of CAG rats

Table 1 depicted that the biochemical indexes changes in all the experimental groups. Significant reduction of gastric PA in model groups showed a clear evidence of the pathogenesis of gastric damage (p < 0.01). HQJZ at both doses restored this alteration induced by CAG (p < 0.05 and p < 0.01), while the positive drug vitacoenzyme showed a potential tendency to inhibit it with no significance. Meanwhile, SOD and MDA levels were used to characterize the oxidative pathogenesis associated with CAG. A significant decrease of plasma SOD was observed in the CAG group, while MDA level increased markedly. Administration of HQJZ and vitacoenzyme could effectively ameliorate these abnormalities (p < 0.05), while HQJZ at low-dosed significantly decreased the MDA concentration near to normal levels (p < 0.01).

![Fig. 1. Plot of weight trend in all the experimental groups. Values are expressed as mean ± SD (n=6). (C) control group, (M) model group, (C) control group, (L) HQJZ at low dose treated group, (H) HQJZ at high dose treated group, (P) positive group.](image-url)
3.3. Effects of HQJZ on gastric pathological injury of CAG rats

HE staining was applied to analyze the gastric histopathological changes in the five experimental groups (Fig. 2), where thickness of gastric mucosa was also labeled. The normal architecture of gastric mucosa was showed in control rat with inerratic gland shape. Gastric changes in model rats showed typical pathological features of CAG, including the thinned and defluium appearance of gastric mucosa, eosinophilic infiltration, significant gland atrophy, and watery degeneration of epithelial cells. The changes of gastric damages were moderated obviously after drug treatments. All pathological abnormalities in CAG rats were markedly ameliorated by pretreatment of them. The protective effect of HQJZ at low dose exerted the best appearance than that of vitacoenzyme.

3.4. Plasma metabolic profiles of CAG rats associated with HQJZ treatment

The plasma metabolic profiles were acquired using NMR technology. Their $^1$H NMR and 2D NMR spectra from the five experimental groups were showed in Figs. S1 and S2. Metabolite peaks were identified according to the literature and available biochemical databases, such as HMDB (http://www.hmdb.ca/) and KEGG (http://www.kegg.com/).

PLS-DA, a technique that transforms variables in a data set into a smaller number of new latent variables, was firstly carried out on the preprocessing of raw data from the spectra to find out their metabolic distinction. As a result, clear separation of the five groups was observed from the PLS-DA (Fig. 3A). The established model of PLS-DA presented satisfied explanation and prediction according to the cross-validations analysis, where the $R^2X$ (cum) (0.429), $R^2Y$ (cum) (0.999) and $Q^2$ (cum) (0.92) worked well. Among them, control and model group could be classified, indicating that there was significant difference between the two groups. The metabolic profile of low-dose group fairly differed from the model group, which was most closed to the control, indicating the deviations induced by CAG were significantly improved after pretreatment of HQJZ at the low dose. While vitacoenzyme treated group was more near the model group without apparent classification.

3.5. Potential biomarkers responsible for CAG rats associated with HQJZ treatment

The OPLS-DA analysis is a better and supervised method to pick out discriminating variables contributing to the classification. In the OPLS-DA model, the classification between two groups was visualized in the forms of score plot and S-plot, where the group clusters and those variables contributing to the classification were displayed, respectively. To search the potential biomarkers, a parameter VIP (Variable Importance in the Projection) was employed to reflect the variable importance. It is considered that the metabolites with large VIP (VIP > 1) are the most relevant for explaining the classification. The

<table>
<thead>
<tr>
<th>Biochemical parameters measured in experimental animals. (mean ± S.D.)</th>
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<tbody>
<tr>
<td><strong>SOD (U/mL)</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>L</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>P</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01 compared with control group. *p < 0.05, **p < 0.01 compared with model group.

Fig. 2. Histological examination of gastric tissues from all the experimental groups. (C) control group, (M) model group, (L) HQJZ at low dose treated group, (H) HQJZ at high dose treated group, (P) positive group.
score plots of OPLS-DA showed metabolic profiles of the control and CAG group were separated clearly (Fig. 3B). Those metabolites contributed to the observed separation in S-plots were selected as potential biomarkers (Fig. 3C). Among them, 19 varied metabolites were selected as the potential biomarkers contributing to the classification (VIP > 1). Their detailed information was showed in Table 2, including their moieties, chemical shifts and VIP values.

3.6. Metabolic change of the potential biomarkers in CAG rats associated with HQJZ treatment

To further analyze the metabolic changes of these potential biomarkers, we performed the comparison of the plasma metabolites using Student's t-test among the experimental groups. As showed in Fig. 4, levels of 3-hydroxybutyrate, lactate, acetate, succinate, glycerophosphocholine (GPC) and valine significantly increased in CAG group compared with control group, whereas the contents of choline, creatinine, trimethylamine oxide (TMAO), proline, betaine, arginine, taurine, citruline, glycine, \( \beta \)-glucose, \( \alpha \)-glucose and glycogen decreased significantly. By further comparing the levels of these identified biomarkers among CAG, HQJZ and vitacoenzyme-treated group, HQJZ can remarkably reverse the abnormalities of succinate, choline, TMAO, GPC, valine, betaine, taurine, citruline, glycine, \( \alpha \)-glucose and glycogen. While the positive drug just showed regulative effects on some metabolites (betaine, taurine, citruline, glycerol and \( \alpha \)-glucose). (Fig. 5).

3.7. Metabolic pathway analysis in response to CAG rats associated with HQJZ treatment

According to MetPA, KEGG PATHWAY database and the related literatures, the metabolic pathways involved in CAG and regulation of HQJZ were assigned and constructed based on the identified potential biomarkers. As showed in Table 3, 19 potential metabolites were involved into eight different metabolic pathways, including fatty acid...
metabolism (3-hydroxybutyrate and acetate), glucose metabolism (lactate, \(\beta\)-glucose, \(\alpha\)-glucose and glycogen), TCA cycle (succinate), arginine and proline metabolism (creatinine, proline, arginine and citrulline), valine, leucine and isoleucine biosynthesis (valine), taurine and hypotaurine metabolism (taurine), glycerolipid metabolism (glycerol), glycine, serine and threonine metabolism (choline, TMAO, GPC, betaine and glycine).

Based on the analysis of MetPA, five disturbed metabolic pathways with Impact > 0.1, including arginine and proline metabolism, valine, leucine and isoleucine biosynthesis, taurine and hypotaurine metabolism, glycerolipid metabolism, glycine, serine and threonine metabolism, were considered as the most relevant pathways to the development of CAG (Table S1). Ten involved metabolites in these five key pathways may denote their potential as targeted biomarkers related to CAG. In our study, pretreatment of HQJZ could effectively protect from CAG via its improvement of taurine and hypotaurine metabolism, valine, leucine and isoleucine biosynthesis, glycine, serine and threonine metabolism, which might be the most influencing target pathways related to the therapeutic intervention of HQJZ against CAG.

Fig. 4. Comparison of the relative intensity of putative potential biomarkers in the CAG rats associated with HQJZ treatment. *\(p < 0.05\), **\(p < 0.01\) compared with control group; *\(p < 0.05\), **\(p < 0.01\) compared with model group. (C) Control group, (M) Model group, (L) HQJZ at low dose treated group, (H) HQJZ at high dose treated group, (P) Positive group.
index, according to their other metabolic pathways were poorly associated with the biochemical values of 8.32 \times 10^{-5}. Metabolic pathways were regulated significantly in model group, while concentration of lactate decreased significantly in control group. In this study, regulation of HQJZ on two disturbed metabolic pathways, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, contributed to the protection of HQJZ against CAG, and they were considered as the targeted metabolic pathways acted on CAG rats.

4. Discussions

Up to now, HQJZ was one of the most widely used TCM preparations for treatment of CAG. In our work, pharmacodynamic results demonstrated that HQJZ possessed beneficial activities in treating CAG, which partially ascribed to the improvement of gastric PA and antioxidant system in vivo. Additionally, our metabolomic analysis also revealed the metabolic regulation of HQJZ against CAG. Thus, 11 potential biomarkers, including succinate, choline, TMAO, GPC, valine, betaine, taurine, citrulline, glycine, α-glucose and glycogen, were screened to characterize the potential drug targets of HQJZ, which primary involved into glucose metabolism, TCA cycle, arginine and proline metabolism, valine, leucine and isoleucine biosynthesis and taurine and hypotaurine metabolism and glycine, serine and threonine metabolism. Among them, three metabolic pathways, including arginine and proline metabolism, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, were demonstrated to have the most impacts in the analysis of metabolomic approach.

In this study, regulation of HQJZ on two disturbed metabolic pathways, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, were all demonstrated to have the most impacts in the analysis of metabolic pathway, which were recognized as the important metabolic pathways in the development and progression of CAG (Fig. 6). Correspondingly, ten metabolites (choline), creatinine, TMAO, GPC, proline, betaine, arginine, taurine, citruline and glycine were considered as the most relevant compounds involved in CAG, which could be serve as potential drug targets.

In this study, regulation of HQJZ on two disturbed metabolic pathways, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, contributed to the protection of HQJZ against CAG, and they were considered as the targeted metabolic pathways acted on CAG rats.

3.8. PLS-RA analysis of different metabolic pathways involved into CAG rats with pepsin activity

PLS-RA was performed to gain possible relationships between different metabolic pathways and the clinical parameter in experimental animals, which could illustrate the targeted pathways of HQJZ against CAG. PLS-RA models were subsequently performed and detailed diagnostic parameters of the models. As a result, three pathways, including arginine and proline metabolism, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, were well associated with PA with Q² values of 0.764, 0.574 and 0.898, and p values of 8.32 \times 10^{-5}, 0 and 3.59 \times 10^{-7}, respectively (Table 3). The other metabolic pathways were poorly associated with the biochemical index, according to their p values (p > 0.05). Among them, glycine, serine and threonine metabolism possessed the highest Q² value, indicating that this pathway played the more important role in the pathological of CAG. With the treatment of HQJZ, these three altered metabolic pathways were regulated significantly, indicated that HQJZ exerted the potential protection on them to maintain the homeostasis.

3.9. Identification of the targeted metabolic pathways associated with HQJZ treatment against CAG rats

Integrating the analysis of MetPA and PLS-RA, three disturbed metabolic pathways, arginine and proline metabolism, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, were all demonstrated to have the most impacts in the analysis of metabolic pathway, which were recognized as the important metabolic pathways in the development and progression of CAG. Correspondingly, ten metabolites (choline), creatinine, TMAO, GPC, proline, betaine, arginine, taurine, citruline and glycine were considered as the most relevant compounds involved in CAG, which could be serve as potential drug targets.

In this study, regulation of HQJZ on two disturbed metabolic pathways, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, contributed to the protection of HQJZ against CAG, and they were considered as the targeted metabolic pathways acted on CAG rats.

Table 3
Summary of the PLS-RA models diagnostic parameters.

<table>
<thead>
<tr>
<th>NO.</th>
<th>Pathway</th>
<th>R²Xc</th>
<th>R²Yc</th>
<th>Q²Yc</th>
<th>p valuec</th>
<th>Metabolites</th>
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<td>1</td>
<td>Fatty acid metabolism</td>
<td>0.736</td>
<td>0.392</td>
<td>0.335</td>
<td>0.07</td>
<td>3-hydroxybutyrate,</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acetate</td>
</tr>
<tr>
<td>2</td>
<td>Glucose metabolism</td>
<td>0.884</td>
<td>0.394</td>
<td>0.328</td>
<td>0.08</td>
<td>Lactate, β-Glucose, α-Glucose, Glycogen</td>
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<tr>
<td>3</td>
<td>TCA cycle</td>
<td>1</td>
<td>0.217</td>
<td>0.19</td>
<td>0.25</td>
<td>Succinate, Creatinine, Proline, Arginine, Citrulline</td>
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<td>4</td>
<td>Arginine and proline metabolism</td>
<td>0.81</td>
<td>0.798</td>
<td>0.764</td>
<td>8.32 \times 10^{-5}</td>
<td>Valine</td>
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<tr>
<td>5</td>
<td>Valine, leucine and isoleucine biosynthesis</td>
<td>1</td>
<td>0.365</td>
<td>0.33</td>
<td>0.07</td>
<td>Taurine</td>
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<tr>
<td>6</td>
<td>Taurine and hypotaurine metabolism</td>
<td>1</td>
<td>0.613</td>
<td>0.574</td>
<td>0</td>
<td>Glycerol</td>
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<tr>
<td>7</td>
<td>Glycerolipid metabolism</td>
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<td>0.229</td>
<td>0.18</td>
<td>Choline, TMAO, GPC, Betaine, Glycine</td>
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<tr>
<td>8</td>
<td>Glycine, serine and threonine metabolism</td>
<td>0.839</td>
<td>0.916</td>
<td>0.898</td>
<td>3.59 \times 10^{-7}</td>
<td></td>
</tr>
</tbody>
</table>

a R²X and R²Y are cumulative modeled variation in the X and the Y matrix, respectively.
b Q²Y is the cumulative predicted variation in the Y matrix.
c p-value, obtained from cross validation ANOVA of the PLS-RA models.
acetate is another important product of fatty acid oxidation and its was also con
hydroxybutyrate on programmed cell death and oxidative stress in vivo
fi
CAG rats, suggesting the de
betaine and glycine, were involved in the disturbances of this pathways
[19]. Five important metabolites, including choline, TMAO, GPC,
important energy metabolism precursors to enter into the citrate cycle
imbalance might contribute to the antagonism of HQJZ against CAG.
Additionally, TMAO, a bio-
synthesized product from trimethylamine derived from choline, has also been found to act as an indicator of the alteration of intestinal flora
[23]. The increment level of TMAO was observed in our study, indicated the homeostasis of bacterial was impaired by CAG. Mean-
while, the reduction of arginine in CAG plasma sample were also supported the disturbance of gut flora, which diet supplemented with arginine may reduced bacterial growth in chemoradiation-induced gastrointestinal injury rats [24]. HQJZ were observed to prevent the decreased tendency of the five involved metabolites (choline, TMAO, GPC, betaine and glycine) under CAG condition, indicating therapeutic effects of HQJZ may base on the modulations of energy imbalance, alterations of immune system, inflammation, as well as alteration of intestinal flora. We found that this pathway possessed the highest association with PA in PLS-RA, which suggests that they play key roles in the progression of CAG. Ameliorating of them might be the important targets for HQJZ against CAG.

According to the PLS-RA and MetPA results, taurine and hypotaur-
ine metabolism was another important targeted pathway related to the development of CAG and regulation of HQJZ. It is demonstrated that taurine can improve drug induced-gastric damage by its antioxidant and/or membrane-stabilizing effects [25]. Additionally, it has been reported that the protective effect of taurine against indomethacin-induced gastric mucosal injury was due to its inhibitory effects on lipid peroxidation and neutrophil activation [26]. Reduced plasma taurine level in our study might contribute to the widely operates lipid peroxidation and inflammation in the pathogenesis of CAG. Pretreatment of HQJZ and vitacoenzyme may maintain the concentration of taurine at near normal levels through significantly inhibiting the lipid peroxidation and inflammation induced by CAG.

It is common knowledge that oxidative stress played a key role in the pathogenesis of CAG. Reports have also found that the metabolism of arginine and proline was closely related to the progression of oxidative stress. Arginine could exert its potential protection from the gastric mucosal damage through inhibition of oxidative stress derived via xanthine-XO [27]. Meanwhile, proline and citrulline, two metab-
lites of arginine, were also involved into the progression. Metabolism of proline could generate electrons to produce ROS and initiate a variety of downstream effects, including blockade of the cell cycle, autophagy and apoptosis. The electrons can also enter the electron transport chain to produce adenosine triphosphate (ATP) for survival under nutrient

Fig. 6. The metabolic network of HQJZ regulation of CAG progress in terms of the PLS-RA and MetPA research.
stress [28]. Citrulline could prevent the oxidative damage and the decrease of nitric oxide content as well as the increase of the myeloperoxidase activity in ethanol-induced gastric ulcer. In the present work, the reduction of proline, arginine and citrulline, were observed in model group, indicating that oxidative stress might be involved into the pathogenesis of CAG. Additionally, Creatinine is a breakdown product of creatine, which was an essential substrate for muscle energy metabolism [29]. The decrease of creatinine was observed compared with control group in our study, matched with the previous study [30]. The analysis of PLS-RA and MetPA both proved the importance of arginine and proline metabolism involved into the formation and development of CAG. After the treatment of HQJZ, alteration of citrulline was effectively restored, suggesting that HQJZ could partially protect from oxidative damage in CAG.

The most relevant pathways of CAG and the intervention of HQJZ were identified firstly by MetPA in our study, which was based on pathway topological analysis and pathway enrichment analysis. As a result, ten metabolites significantly varied following the development of CAG. Five significant metabolic pathways out of a total of eight pathways were found to be uniquely affected in the CAG rats, including arginine and proline metabolism, valine, leucine and isoleucine biosynthesis, taurine and hypotaurine metabolism, glycero-lipid metabolism, glycine, serine and threonine metabolism. However, at this stage, these metabolic pathways were just analyzed as individual pathways in the previous studies. They were involved in the current metabolomes based on the identified potential biomarkers, which were not linked to the pathological characterizes of CAG. This results of pathway analysis need to supplement the practical significance for the comprehensive assessment of biochemical indexes and the related specific metabolic alterations.

Here, PLS-RA was introduced to elucidate the relationships among different metabolic pathways and the biochemical indexes. In our study, gastric PA, a marker of gastric mucosa secretion, was selected to characterize the injury of CAG. Analysis of them could be used to discover the most relevant metabolic pathways associated with CAG and the metabolic targets for HQJZ. Three pathways, including arginine and proline metabolism, taurine and hypotaurine metabolism and glycine, serine and threonine metabolism, were well associated with PA. The results were accordance with the analysis of MetPA, further connecting the pathological change of CAG and its disturbances of metabolic pathways, and demonstrating that these three metabolic pathways were the important pathways related to the development of CAG.

Integrating the two analyses, three pathways were all considered as the most important pathways related to the development of CAG. The results also showed that the two analyses were mutual authentication for each other in the discovery of the most relevant metabolic pathway. With treatment of HQJZ, several metabolites were markedly regulated, which indicated that the drug could effectively ameliorate the abnormal change of these two metabolic pathways, suggested that the gastric protection of HQJZ might be attributed to the regulation of the two targeted pathways.

5. Conclusions

Here, we proposed an integrated strategy to identify the metabolic pathways involved into HQJZ against CAG coupled with untargeted metabolomics, PLS-RA and MetPA. This strategy was successfully exemplified in an investigation of the regulation of HQJZ on the metabolic perturbation induced by CAG. 19 plasma metabolites were used to construct the metabolic profiles of CAG rats from control rats. With the treatment of HQJZ, 11 abnormal metabolites could be significantly adjusted, suggested that its improvement of the pathological changes contributed to its protection, including energy imbalance, excessive oxidative stress, alterations of immune system, as well as inflammation. The further PLS-RA revealed that glycine, serine and threonine metabolism and taurine and hypotaurine metabolism were the most relevant drug targets of HQJZ against CAG, which was in agreement with the MetPA result. The result suggested that the proposed strategy meets the technical demands for the discovery of the drug targets of TCM formulas, and therefore could present a powerful tool for deciphering the scientific basis of TCMs. Additional studies are still necessary to validate the drug targets identified in the current study on the basis of the related proteins and genes.

Conflict of interest

We have no conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemolab.2017.03.005.

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