A metabolomics-binding network pharmacology study of Zuojin Pill intervention in chronic atrophic gastritis induced by *Helicobacter pylori*

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Research

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Abstract

Background Zuojin Pill (ZJP) is widely used for the treatment of gastrointestinal diseases, while its specific mechanism has not been systematically investigated. The aim of this study was to explore the mechanism of intervention of ZJP in chronic atrophic gastritis (CAG) through metabolomics combined with network pharmacology.

Materials and methods Potential metabolites and possible pathways for ZJP treatment of CAG were explored using a UPLC-Q-TOF/MS-based metabolomics technique. The key targeting mechanism of ZJP for CAG was explored by combining the analysis with network pharmacology.

Results ZJP significantly reduced serum levels of IL-1 β , IL-6, IL-10 and iNOS, and improved pathological characteristics. Metabolomic results indicated that the therapeutic effect of ZJP was mainly related to ten metabolites, including choline, L-threonine, hydroxypyruvic acid, creatine, taurine, succinic acid, cis-aconitic acid, citric acid, succinic acid semialdehyde and uric acid. Pathway analysis showed that the treatment of CAG by ZJP was associated with taurine and hypotaurine metabolism, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, glycerophospholipid metabolism, citrate cycle (TCA cycle), alanine, aspartate and glutamate metabolism, butanoate metabolism and purine metabolism. Validation of potential metabolic markers and key targets of network pharmacology by RT-PCR analysis showed that ZJP significantly down-regulated a series of inflammatory markers, such as MAPK1, PKIA, RB1, SCN5A, RXRA, E2F1, PTGS1, IGF2, ADRB1, ADRA1B, PTGS2, and GABRA1.

Conclusion For the first time, a combination of metabolomics and network pharmacology has been used to clarify the therapeutic effects of ZJP on CAG and its relationship to the regulation of multiple metabolic pathways.

Introduction

Helicobacter pylori (H. pylori) was defined as a type 1 carcinogen by the World Health Organization's International Agency for Research and Cancer (IARC) in 1993, and it is widely accepted that H. pylori infection induces chronic atrophic gastritis (CAG) and even the development of gastric cancer, and several studies have now confirmed this association [1-2]. CAG is a common clinical digestive disorder that is the result of the inflammatory process of H. pylori infection and is commonly characterized by thinning of the mucosal epithelium and atrophy and loss of glands. Although triple and quadruple therapies are commonly used in clinical practice to treat CAG, long-term use enhances bacterial resistance and decreases eradication rates and can lead to a number of side effects, such as gastrointestinal reactions and liver dysfunction [3]. Therefore, there is an urgent clinical need for an effective drug with less side effects to treat H. pylori induced CAG.

Zuojin Pill (ZJP) is a famous Chinese herbal formula, first recorded centuries ago in an ancient medical treatise "Danxi New Law". ZJP consists of two traditional Chinese medicines *Coptidis Rhizoma* (CR) and *Euodiae Fructus* (EF) according to a weight ratio of 6:1 (w/w) and has been officially listed in the Chinese

Pharmacopoeia. The main ingredients in CR are alkaloids, including berberine, palmatine, coptisine and epiberberine. Studies have shown that CR has a wide range of pharmacological effects, such as antibacterial, anti-atherosclerotic, antiviral, antioxidant, anti-hepatic steatosis, anti-inflammatory and antitumor effects [4]. EF is the dried near-mature fruit of *Euodia rutaecarpa* (Juss.) Benth., and the main chemical constituents of EF include alkaloids, and terpenes which have antibacterial, hypotensive and anti-hypoxic pharmacological activities. ZJP has been shown to have anti-inflammatory, anti-ulcer, and anti-acid pharmacological effects, including inhibition of *H. pylori* growth and proliferation [5]. Currently, ZJP is clinically used to treat a variety of gastrointestinal disorders such as gastritis, cholecystitis, and peptic ulcers [6]. Previous studies have shown that ZJP and its constituent active components of herbal CR and EF exhibit multiple pharmacological effects on cancer through multiple molecular mechanism [7-8]. ZJP plays a gastroprotective role by modulating the NF- κ B signaling pathway to regulate inflammatory cytokines and thereby reduce the risk of gastric ulcers [9]. ZJP treats gastrointestinal disorders by restoring the rhythm of the stomach as well as improving gastrointestinal peristalsis [10]. The ethanolic extract of ZJP inhibited inflammation by regulating the expression of iNOS, COX-2, IL-6, IL-1 β , and TNF- α [11]. These studies provide a basis for the therapeutic effect of ZJP on gastric ulcers, gastritis and other clinical inflammatory diseases.

As an important branch of systems biology, metabolomics conducts a systematic research of the relationships between, medicine, disease and metabolites. Metabolomics not only characterizes the overall physiological and pathological state after exogenous stimuli, but also systematically identifies endogenous small molecule metabolites. Modern analytical technologies, including nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are combined with multivariate data analysis, such as principal component analysis (PCA), partial least-squares discriminate analysis (PLS-DA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) [12]. In these methods, ultra-high performance liquid chromatography with quadrupole time-of-flight (UPLC-Q-TOF) has the advantages of high selectivity, resolution and accuracy and is particularly suitable for traditional Chinese medicine (TCM) [13]. Due to the easy collection of blood and urine samples and their close association with diseases, choice of these two biological fluids may be a novel and effective methods for metabolomics research [14]. Serum metabolite profiles are considered to be important indicator of physiology and pathology and can help to understand the interventions, metabolic pathways and mechanisms of disease progression. Urine metabolite profiles is important for the discovery of disease biomarkers, particularly excreted metabolites. Serum samples typically represent low-polarity metabolites, while urine samples consist of high-polarity metabolites. These two metabolite profiles complement each other and reflect overall characteristics, demonstrating the mechanism, intervention and metabolic pathways [15]. Nevertheless, the variety of metabolites and their wide range of concentrations limits the ability to understand disease progression using metabolomics alone, metabolomics analysis of interference with metabolic pathways is still far from completely elucidating the mechanism of TCM. In recent years, network pharmacology has been used to investigate and anticipate mechanisms of drug-target interactions in biological metabolism and related pathways [16]. It provides a systems level approach to understanding the interactions between

disease features, physiologically active substances and drug targets [17]. A growing number of studies have shown that network pharmacology, as a holistic and effective tool for studying the effects of TCM, can better understand the complexity of TCM and elucidate its underlying mechanisms through understanding its active chemical components and therapeutic targets [18]. In addition, integrated metabolomics and network pharmacology have been successfully used to explore the interactions between TCM and disease targets, which provide important directions for the therapeutic mechanisms of TCM.

In order to elucidate the intervention of ZJP in the treatment of CAG, this study explored the pathological changes and serum biochemical parameters of ZJP through pharmacological experiments based on metabolomics. A rat model of CAG was established by *H. pylori*, and the success of the model was assessed by a rapid urease kit. UPLC-Q-TOF/MS serum metabolomics and urine metabolomics were used to determine the changes of endogenous metabolites in urine and serum caused by ZJP and to screen relevant targets in combination with network pharmacology techniques to elucidate possible biomarkers and potential metabolic pathways for ZJP treatment of CAG, providing new strategy and feasibility analysis.

Methods

Reagents

Interleukin-6 (IL-6, Lot: 202007), interleukin-10 (IL-10, Lot: 202007), interleukin-1 β (IL-1 β , Lot: 202007) and inducible nitric oxide synthase detection (iNOS, Lot: 202007) ELISA kits were bought from Shanghai MLBIO Biotechnology Co.Ltd (Shanghai, China). RNA-Quick Extraction Kit (No: RN001) was purchased from Beijing Yishan Biotechnology Co.Ltd (Beijing, China).

Preparation of ZJP water extract

CR (Lot: 18011901) and EF (Lot: 17021602) were purchased from Beijing Lyve Pharmaceutical Co., Ltd. (Beijing, China). Firstly, ZJP was extracted with boiling water (1/10, w/w) two times, 1.0 h each time. The aqueous extract of ZJP was then dried to powder in a freeze-vacuum drying oven, and the final yield of ZJP was approximately 25.59%.

Bacterial culture and animal handling

H. pylori strain (strain number: ICDC111001) was supplied by Dr. Jianzhong Zhang (Chinese Disease Control and Prevention Center, Beijing, China). *H. pylori* strains cultured on blood agar (Thermo Fisher Scientific, China) in a 3-5 day cycle.

170-190g male Sprague-Dawley rats were provided by Beijing Sibeifu Animal Breeding Center [Permission No. SCXK-(Jing) 2016-0002] and fed under standard environment (temperature: 25°C $\pm$ 2°C, humidity: 55% $\pm$ 5%, 12: 12 h light: dark cycle) at the Animal Experimental Center of the Fifth Medical Center of the PLA General Hospital. Thirty-six rats were randomly divided into 6 groups. Rats in the control group were

gavaged with regular saline, and rats in the other five groups were gavaged with *H. pylori* suspension (1.5×10^8 colony forming units (CFU)/ml, 1.5mL) four times a week for 8 weeks. Urine was collected from rats 12 hours after the last treatment for 12 hours. Then, except for the control and model groups, ZJP (0.63, 1.26, 2.52 g/kg/day) and omeprazole (1.8 mg/kg/day, as a positive group) were administered by gavage for 4 weeks. Twelve hours after the last treatment, the rats were executed and stomach and blood were collected. The blood was centrifuged at 3,500 rpm for 15 min and the serum was separated. The serum and stomach tissues were stored at -80°C for further experiments.

Serum levels of IL-6, IL-10, IL-1 β and iNOS were measured according to the kit instructions. Stomach tissues were fixed in 10% neutral formalin buffer for 24 hours. The fixed gastric tissues were embedded in paraffin wax and then cut into slices using a slicer. Hematoxylin-eosin (HE) was used to highlight gastric sinus injury and inflammatory cell infiltration.

Sample preparation and UPLC-Q-TOF/MS testing

Mix 600 μ L of methanol with 200 μ L of serum. The mixture was allowed to stand at 4°C for 20 minutes and then centrifuged at 12,000 rpm for 10 minutes. Finally, the supernatant was aspirated and filtered through a 0.22 μ m micropore filter. The filtrate was collected and analyzed in the next step, and the urine sample was prepared in the same procedure as the serum sample.

The analysis of serum metabolic profile and urine metabolic profile was performed by Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, USA) system. On a ZORBOX RRHD C₁₈ analytical column (2.1 mm i.d. $\times$ 100 mm, 1.8 μ m i.d., Agilent Technologies, USA), 4 μ L of each sample was taken and injected into the system, and samples were separated at 30°C. Solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) were used as the mobile phase and separated at a flow rate of 0.30 mL/min for 25 min with a linear gradient of 100% A over 0-1.0 min, over 100-60% A over 1.0-9.0 min, 60-10% A over 9.0-19.0 min, 10-0% A over 19.0-21.0 min, and 100% A over 21.0-25.0 min.

Positive and negative mode electrospray source parameters were set as follows: electrospray capillary voltage of 3.5 kV in negative mode and 4 kV in positive mode, mass range of m/z 50-1200, gas temperature and flow rate were 225°C and 13 L/min, atomizer setting of 20 psi, sheath gas temperature and flow rate were 275°C and 12 L/min with a nozzle voltage of 2000 V in both positive and negative ion mode.

Data extraction and multivariate analysis

Data were extracted, peaks detection and comparison performed by using MassHunter Profinder software (Agilent, CA, USA). Full scan mode was applied to the mass range m/z 80-1000 and the initial and final retention times for data collection were set. Data were standardized using the MetaboAnalyst website (<https://www.metaboanalyst.ca>) and then analyzed by principal component analysis (PCA) and

orthogonal-partial least squares-discriminant analysis (OPLS-DA) using SIMCA-P 14.1 software (Umetrics, Umea, Sweden).

Potential biomarker identification and pathway enrichment analysis

Biomarkers were identified by metabolic differential metabolite screening. In the OPLS-DA analysis, the screening conditions for differential metabolites were set to VIP > 1.0, |p(corr)| ≥ 0.58, P < 0.05, and the obtained metabolites were identified as potential biomarkers [19]. Metabolites were identified based on the precise molecular weight of the Human Metabolome Database (HMDB) (molecular weight error < 20 ppm). The identified compounds were resubmitted to MetaboAnalyst to enrich for potential signaling pathways.

ZJP drug target identification and network pharmacology techniques

The chemical composition and associated targets of CR and EF were collected in the TCMSP database (<https://tcmospw.com/tcmosp.php>). Potential metabolite-associated protein targets were collected via the MBROLE 2.0 database (<http://csbg.cnb.csic.es/mbrole2/index.php>). The Genecards database (<https://www.genecards.org/>) is used to collect CAG-associated protein targets. Uniprot ID was used to convert different types of protein IDs, and then a network of component-metabolite-target interactions was established using protein interaction information. Finally, the component-metabolite-target interaction network was visualized and analyzed using Cytoscape 3.7.1 software.

Real-time polymerase chain reaction (RT-PCR) detection

Total RNA was extracted from gastric tissues using the RNA-Quick Extraction Kit and the total RNA was reverse transcribed to cDNA using the PrimerScript RT kit (Promega, Madison, USA). The cDNA was subsequently amplified by PCR by ABI Step One Plus. The cDNA was subsequently PCR amplified by ABI Step One Plus. The data were analyzed by the $2^{-\Delta\Delta CT}$ method, and the list of primers is shown in Table 1.

Statistical Analysis

All data were presented as mean ± standard deviation (SD) and analyzed using the SPSS software program (version 25.0; SPSS Inc., Chicago, IL, USA). The differences were considered to be statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

Results

ZJP reduces H. pylori-induced CAG pathological damage and serum inflammatory factors

As shown in Figure 1, the control group rats had normal gastric histology, and the model group rats showed pathological features of CAG, such as epithelial rupture of the gastric mucosa, atrophy and misarrangement of glands, accompanied by inflammatory cell infiltration. ZJP administration was effective in improving gastric mucosal lesions, glandular loss, and reducing inflammatory infiltration,

especially in the 2.52 g/kg ZJP group. Moreover, the serum levels of IL-1 β , IL-6, IL-10 and iNOS were significantly increased in the model group rats compared with the control group ($P < 0.01$). serum concentrations of IL-1 β , IL-6, IL-10 and iNOS were decreased after ZJP administration. IL-1 β , IL-6, IL-10, and iNOS levels were significantly reduced after administration of 2.52 g/kg ZJP compared to the model group ($P < 0.01$).

Multivariate data analysis

Two analytical methods, PCA and OPLS-DA, are powerful statistical tools that provide insight into the separation between different groups based on the results of different spectra [20]. After Pareto normalization of data from serum and urine samples from the control group, model group and 2.52 g/kg ZJP group, the three groups were distinguished by PCA using SIMCA-P 12.0 software. As shown in Figure 3, there was a good separation between the control group, model group and 2.52 g/kg ZJP group in both ESI+ and ESI- modes, indicating a significant difference in the metabolic distribution of the three groups. As shown in Figure 3B, D, there was a clear separation between the serum and urine samples of the control group and model group in ESI- mode, and the 2.52 g/kg ZJP group was located between the control group and model group, indicating that ZJP was regulating the metabolically abnormal rats to a normal state. The ESI+ model has a poor trend in the 2.52 g/kg ZJP group therefore further analysis is required.

OPLS-DA models were then developed for the ESI+ and ESI- modes of serum and urine samples. Given that OPLS-DA was used to screen for differentially expressed metabolites between the two groups [21], we identified the differential metabolites under OPLS-DA analysis for the control group and model group, and for the model group and 2.52 g/kg ZJP group, respectively. As shown in Figure 4 and 5, in the OPLS-DA mode established from the serum and urine data of the control group and model group for ESI+ and ESI- modes could explain the 0.999, 0.993, 0.991 and 0.999 variance of the response variable (R^2Y) and the cumulative explained variance for modeling in cross-validations (Q^2) were 0.975, 0.938, 0.857 and 0.92, respectively. For serum and urine data of the model group vs 2.52 g/kg ZJP group in ESI+ and ESI- modes, R^2Y values were 0.998, 0.987, 0.98 and 0.989 with Q^2 0.979, 0.81, 0.774 and 0.91, individually. These parameters indicate that the models have good explanatory and predictive capabilities. The S-plot was used to investigate the inherent clustering variables. Variables with an average VIP value above 1 and an absolute $P(\text{corr})$ value above 0.58 in the corresponding S plot can be considered as potential biomarkers. The S-curve plots show the apparent differences between the endogenous metabolites in the control group, model group and 2.52 g/kg ZJP group. The variables located at the ends of the S-curve made an important contribution to the separation of the two groups. The predictive power of the established OPLS-DA model is then verified by a permutation test ($n=100$). The results showed that the R^2 and Q^2 values of serum and urine data were lower than the permutation tests, indicating the superior fit and better predictive ability of the OPLS-DA model.

Identification of potential metabolites in ZJP treatment

For more accurate identification, potential metabolites were selected based on the principles of $|VIP| > 1$ and $|P(\text{corr})| \geq 0.58$ in the S-plots extracted between the control group and model group, model group and 2.52 g/kg ZJP group. At this threshold, variables with significant differences were screened using ANOVA analysis and t-test for multivariate and univariate analyses, respectively. Matching the m/z of metabolite candidates to online databases, including the HMDB database and the Metaboanalyst database, identifies candidates with significant changes as biomarkers. The mass tolerance value and the exact mass of the measured m/z are defined as less than 20 ppm.

For serum samples, a total of six endogenous metabolites were screened in ESI+ mode and ESI- mode (see Table 2 for details). For urine samples, four endogenous metabolites were identified based on the above procedure (please see Table 2). These ten potential biomarkers are choline (C00114), L-threonine (C00188), hydroxypyruvic acid (C00168), creatine (C00300), taurine (C00245), succinic acid (C00042), cis-aconitic acid (C00417), citric acid (C00158), succinic acid semialdehyde (C00232) and uric acid (C00366). The levels of L-threonine, hydroxypyruvic acid, taurine, succinic acid and cis-aconitic acid were significantly lower in the model group compared to the control group ($p < 0.05$ and $p < 0.01$). In addition, levels of choline, creatine, citric acid, succinic acid semialdehyde and uric acid were significantly higher in the model group compared to the control group ($p < 0.01$). The mean peak area of these metabolites tended to normalize in the 2.52 g/kg ZJP group compared with the model group ($P < 0.05$ and $p < 0.01$), showing good therapeutic efficacy. The mean peak area of these metabolites is shown in Figure 6 to visualize the effects of ZJP. Alterations in these parameters indicate that metabolic abnormalities occur in control group rats following gastric mucosal inflammatory injury and that metabolic markers return to normal levels after ZJP treatment.

Metabolic pathway analysis

To explore the potential mechanism of ZJP's effect on CAG, potential metabolites identified from serum and urine samples were introduced into MetaboAnalyst to construct metabolic pathways, and twenty-eight metabolic pathways were obtained. Of these twenty-eight metabolic pathways, seven of them play an important role, including Taurine and hypotaurine metabolism, Glyoxylate and dicarboxylate metabolism, Glycine, serine and threonine metabolism, Glycerophospholipid metabolism, Citrate cycle (TCA cycle), Alanine, aspartate and glutamate metabolism, Butanoate metabolism and Purine metabolism. The match status, P value, $-\log(P)$ and impact of each pathway are listed in Table 3.

Network Pharmacology

To visualize the interactions between potential metabolites, disease targets and ZJP components, components and potential CAG disease targets were collected and combined with potential metabolites via the TCMSP database and Genecards database to construct a potential metabolite-CAG target-components network, as shown in Figure 8. MBROLE was used to extend this potential metabolite to related metabolic pathways to further explore the relationship between metabolite-associated proteins and disease targets. Information of the 12 proteins of relevance in compounds-disease targets-

metabolites is presented in Table 4, which are directly regulated by 124 chemical components and collected from ZJP (Figure 8B).

The effect of ZJP on the mRNA expression in gastric tissue of CAG rats

CAG is a critical step in the development of gastric cancer, and with increased inflammation comes a progressively higher chance of cancer. A large amount of clinical evidence suggests that ZJP inhibits inflammatory lesions including gastric mucosal injury, neutrophil infiltration and intestinal epithelial chemosis in CAG patients. Thus, inflammation related genes MAPK1, PKIA, RB1, SCN5A, RXRA, E2F1, PTGS1, IGF2, ADRB1, ADRA1B, PTGS2, and GABRA1 may play a more important role in ZJP treatment of CAG. To verify the validity of the network pharmacology predictions, RT-PCR was used to detect the mRNA expression of these genes in gastric tissues. As shown in Figure 9, the relative mRNA expression levels of MAPK1, PKIA, RB1, SCN5A, RXRA, E2F1, PTGS1, IGF2, ADRB1, ADRA1B, PTGS2, and GABRA1 in the model group were significantly elevated ($P<0.01$). The expression levels of the above genes in the ZJP groups were significantly decreased ($P<0.05$ and $P<0.01$), indicating the effectiveness of ZJP in treating CAG through anti-inflammation.

Discussion

CAG is a common clinical disorder of the gastrointestinal tract and is considered to be a precancerous condition of gastric cancer. Current conventional treatments for CAG are mainly focus on symptomatic treatment, however, symptomatic treatment is often associated with some degree of side effects, poor efficacy and high relapse rates [22]. Metabolomics combined with network pharmacology can effectively overcome these problems by extensively mining data to identify targets of metabolism and disease [23]. Metabolomics analyzes small molecule levels in serum and urine to explore compounds with significant abnormalities and reveal mechanisms of disease development. Network pharmacology reveals the molecular mechanisms of drug treatment for diseases by determining the interactions between chemical components and disease protein targets. Therefore, in this study, rat serum metabolomics and urine metabolomics based on the UPLC-Q-TOF/MS method combined with network pharmacology were used to elucidate the therapeutic mechanism of ZJP on CAG, which also provides a molecular level exploration of the ZJP mechanism.

H. pylori is the most commonly infected bacterium worldwide and is a significant risk factor for the development of gastric cancer. Numerous studies have been published showing that *H. pylori* can colonize and grow in an extremely acidic gastric environment, establishing a persistent infection and decreasing host immune function, leading to the development of gastritis and gastric cancer [24]. Currently, *H. pylori* is widely used to establish rodent models of CAG [25]. Therefore, in the present study, the CAG rat model was established by gavage of *H. pylori*. HE staining confirmed that ZJP was effective in reducing gastric mucosal damage and maintaining epithelial structural integrity, and improving monocyte and lymphocyte infiltration. Serum biochemical parameters showed that ZJP significantly reduced serum levels of IL-1 β , IL-6, IL-10 and iNOS, and restored the integrity of gastric mucosa and

effectively reduced the expression of inflammatory factors. The above results confirm that ZJP shows a therapeutic effect on CAG, which is consistent with clinical efficacy.

Next the changes in serum metabolomic and urine metabolomic characteristics of ZJP in CAG treatment were identified. ZJP protects the normal structure of the gastric mucosa by restoring potential metabolites to normal levels. The screened biomarkers can be effective targets for disease diagnosis, treatment, and prevention [26]. The PCA model revealed significant differences in metabolite distribution between the control group, model group and 2.52 g/kg ZJP group. The distribution of potential metabolic biomarkers was identified in the OPLS-DA model. There was a significant separation of biomarkers between the control group and model group, indicating a significant effect of *H. pylori* on the serum metabolomic profile and the urine metabolomic profile. There were also significant differences between the 2.52 g/kg ZJP group and model group, suggesting a therapeutic effect of ZJP on CAG. Finally, six differential metabolites were screened in serum samples and four metabolic differentials in urine samples to reveal the regulatory mechanism of ZJP in the treatment of CAG. These metabolites interact and regulate the disease process in different ways. The results suggest that the occurrence and development of CAG is regulated by changes in many physiologically and pathologically relevant biomarkers, most of which in the organism are mutually influential. ZJP restores normal levels of expression by modulating these ten potential biomarkers, suggesting that ZJP can treat *H. pylori*-induced CAG through multiple targets and multiple pathways.

In total, ten differential metabolites were identified and were involved in seven major metabolic pathways. These metabolites involve seven major metabolic pathways, including taurine and hypotaurine metabolism, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, glycerophospholipid metabolism, TCA cycle, alanine, aspartate and glutamate metabolism, butanoate metabolism and purine metabolism. Taurine and hypotaurine metabolism is important metabolic pathways. Taurine is involved in multiple biological pathways and has a variety of pharmacological activities, such as eye and brain development, immune function, osmoregulation, as well as antioxidant and anti-inflammatory activities. Previous studies have shown that it is protective against oxidative stress-induced gastrointestinal injury [27]. Taurine is one of the most abundant free amino acids in animal tissues and plays an important role in a variety of important physiological processes. Upregulation of taurine in serum by ZJP treatment may be a marker of inflammatory response. Elevated amino acid levels are frequently observed in patients with gastritis, which helps to distinguish CAG patients from normal people [28]. In addition, metabolic pathway analysis identified several amino acid-related pathways in CAG, including the metabolism of glycine, serine, and threonine and the metabolism of alanine, aspartate, and glutamate. The results of the present study showed that the expression of choline, creatine, and succinic acid semialdehyde associated with amino acid metabolism was elevated and L-threonine expression was decreased in the model group, and this trend was ameliorated by ZJP administration. The involvement of glyoxylate and dicarboxylate metabolism further supports the involvement of ZJP in energy metabolism. Gastric wall cells contain a large number of mitochondria [29]. Mitochondria provide energy to the cells through oxidative phosphorylation, which plays an important role in maintaining the steady state and function of the gastric mucosa. ZJP further elevates

hydroxypyruvate expression by restoring energy metabolism. Purines are essential components of nucleotides in cell proliferation, thus impaired purine metabolism is associated with inflammation and the development of cancer [30]. The expression of uric acid was elevated in CAG rats, which is consistent with what has been reported in the research [31]. The TCA cycle is the final metabolic pathway for carbohydrates, lipids, and amino acids and is the most important metabolic pathway for energy supply. Succinic acid and cis-aconitic acid belong to the TCA cycle and mediate the formation and metabolism of intracellular ATP [32]. The citric acid is a key intermediate of the TCA cycle [33]. The results showed that succinic acid and cis-aconitic acid contents were decreased and citric acid contents were significantly increased in CAG rats. These results indicate the importance of the TCA cycle in the pathological process of CAG.

In an attempt to further understand the mechanism of *H. pylori*-induced CAG by ZJP treatment and the association between chemical composition and metabolites, we established a “compounds-disease targets-metabolites” interaction network by combining network pharmacology. The results showed that 124 chemical components originating from ZJP acted directly with 12 potential protein targets, suggesting the advantages of multiple components in ZJP in regulating endogenous metabolic changes in the body, showing the advantages of ZJP in the clinical treatment of CAG. Twelve targets were strongly associated with inflammation, including MAPK1, PTGS2, PTGS1, RXRA, ADRA1B, RB1, ADRB1, E2F1, SCN5A, PKAIGF2, and GABRA1 [34-37]. Therefore, to verify the accuracy of the network pharmacology predictions and to further elucidate the potential mechanism of ZJP, we further tested the expression of these genes in gastric tissues of CAG rats. The expression of MAPK1, PTGS2, PTGS1, RXRA, ADRA1B, RB1, ADRB1, E2F1, SCN5A, PKAIGF2, and GABRA1 was significantly elevated in the CAG model group. However, ZJP significantly reduced the relative mRNA expression of these genes. These results suggest that ZJP may play a role in the treatment of CAG by further ameliorating gastric mucosal inflammatory lesions through anti-inflammation.

Conclusion

This study systematically investigated the efficacy of ZJP for the treatment of *H. pylori*-induced CAG and its molecular mechanisms through a combination of metabolomics and network pharmacology. This study can lay a foundation for further understanding of the interactions between components and targets of TCM and provide a theoretical basis for guiding TCM clinical practice.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Design of the study: Honghong Liu, Ruilin Wang and Jianyu Li; data collection and analysis: Shihua Wu, Xing Chen and Min Wang; drafting the manuscript: Jianxia Wen, Tao Yang, Ying Wei, Sichen Ren, Shizhang Wei, Manyi Jing and Haotian Li; supervising and providing Funding acquisition: HouLin Xia, Yanling Zhao. All authors participated in amending the manuscript before submission of the mutually agreed final version.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were performed under the approval of the Ethics Committee of the Ethics of Animal Experiments of the Fifth Medical Center of PLA General Hospital (Approval ID: IACUC-2018-010).

Consent for publication

All authors agree to publish this paper.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

ZJP: Zuojin Pill; CAG: Chronic atrophic gastritis; *Helicobacter pylori*: *H. pylori*; TCA cycle: citrate cycle; IARC: International Agency for Research and Cancer; CR: *Coptidis Rhizoma*; EF: *Euodiae Fructus*; NMR: nuclear magnetic resonance; GC-MS: gas chromatography-mass spectrometry; LC-MS: liquid chromatography-mass spectrometry; PCA: principal component analysis; PLS-DA: partial least-squares discriminate analysis; OPLS-DA: orthogonal projection to latent structures-discriminant analysis; UPLC-Q-TOF: ultra-high performance liquid chromatography with quadrupole time-of-flight; TCM: traditional Chinese medicine.

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Tables

Table 1 Primers used for real-time PCR

Primers	Sequence-Forward	Sequence-Reverse
PTGS1	TCTGGACCTGGCTTCGGAGTTC	TGGACCGCACCGTGAGTACC
PTGS2	AGGTCATCGGTGGAGAGGTGTATC	CGGCACCAGACCAAAGACTTCC
MAPK1	TGAAGACACAGCACCTCAGCAATG	GGTGTTTCAGCAGGAGGTTGGAAG
GABRA1	AGTGCCAGAAATTCCCTCCCAAAG	CAATCAGAGCCGAGAACACGAAGG
IGF2	CAGCCGTGGCATCGTGGAAG	CGGGGTATCTGGGGAAGTCGTC
PKIA	TTCCTCTGCAAGTGGCAACAGC	TGCTTCTCCCTGGGCTTCGC
SCN5A	GGAAGGTCTGGTGGCGATTGC	TTCCGCTCCTCCAGGTAGATGTC
E2F1	TGGTGGCTGCTGACTCACTCC	TGGGGTGGGGAAAGGCTGATG
ADRB1	CTCATCGTGCTGCTCATCGTAGTG	GCCAGGGACATGATGAAGAGGTTG
RB1	CAAGCAGCCTCAGCCTTCCATAC	TGTGTTCTGGCTCTGGGTGGTC
ADRA1B	ATCGTGGGCAACATCTTGGTCATC	ACTCAACAGCAGGTCAGCAATGG
RXRA	AACTGGGCTTCGGGACTGG	GGAACCTTGAGGACGCCATTGAG
β-Actin	CCCGCGAGTACAACCTTCTTG	TCATCCATGGCGAACTGGTGG

Table 2 Identification of serum and urinary metabolites in different groups.

No.	RT(min)	mass	Metabolites	Formula	KEGG	Pathway
1	17.65	104.1069	Choline	C ₅ H ₁₄ NO	C00114	Glycine, serine and threonine metabolism
2	2.33	120.0658	L-Threonine	C ₄ H ₉ NO ₃	C00188	Glycine, serine and threonine metabolism
3	7.58	126.9998	Hydroxypyruvic acid	C ₃ H ₄ O ₄	C00168	Glyoxylate and dicarboxylate metabolism
4	0.90	132.0771	Creatine	C ₄ H ₉ N ₃ O ₂	C00300	Glycine, serine and threonine metabolism
5	0.89	124.0073	Taurine	C ₂ H ₇ NO ₃ S	C00245	Taurine and hypotaurine metabolism
6	14.66	119.034	Succinic acid	C ₄ H ₆ O ₄	C00042	TCA cycle
7	0.78	173.0087	cis-Aconitic acid	C ₆ H ₆ O ₆	C00417	TCA cycle
8	1.22	215.0165	Citric acid	C ₆ H ₈ O ₇	C00158	TCA cycle
9	0.79	101.0239	Succinic acid semialdehyde	C ₄ H ₆ O ₃	C00232	Alanine, aspartate and glutamate metabolism
10	0.87	167.0202	Uric acid	C ₅ H ₄ N ₄ O ₃	C00366	Purine metabolism

Table 3 Results of integrating enrichment analysis of biomarkers with MetaboAnalyst

	No.	Pathway name	Match status	<i>P</i> value	-log(p)	Impact
serum	1	Taurine and hypotaurine metabolism	1/8	0.05192	2.9581	0.42857
	2	Glyoxylate and dicarboxylate metabolism	1/32	0.19345	1.6427	0.21958
	3	Glycine, serine and threonine metabolism	4/34	4.1098×10 ⁻⁵	10.1	0.06662
	4	Glycerophospholipid metabolism	2/36	0.022085	3.8128	0.04318
	5	Citrate cycle (TCA cycle)	1/20	0.12526	2.0774	0.03273
urine	6	Citrate cycle (TCA cycle)	2/20	0.0085481	4.762	0.14041
	7	Glycerophospholipid metabolism	1/36	0.23395	1.4527	0.09446
	8	Glyoxylate and dicarboxylate metabolism	2/32	0.021272	3.8503	0.05556
	9	Alanine, aspartate and glutamate metabolism	2/28	0.016472	4.1061	0.04808
	10	Butanoate metabolism	1/15	0.1044	0.1044	0.03175
	11	Purine metabolism	1/66	0.38959	0.94266	0.94266

Table 4 Participating in the "Component-Target-Potential Metabolite" interactive network for ZJP treatment of CAG

Uniprot ID	Gene name	Protein name
P28482	MAPK1	Mitogen-activated protein kinase 1
Q6ZYK7	PTGS2	Prostaglandin G/H synthase 2
X6RJD6	PTGS1	Prostaglandin G/H synthase 1
P19793	RXRA	etinoic acid receptor RXR-alpha
P35368	ADRA1B	Alpha-1B adrenergic receptor
P06400	RB1	Retinoblastoma-associated protein
P08588	ADRB1	Beta-1 adrenergic receptor
Q01094	E2F1	Transcription factor E2F1
Q14524	SCN5A	Sodium channel protein type 5 subunit alpha
P61925	PKIA	cAMP-dependent protein kinase inhibitor alpha
P01344	IGF2	Insulin-like growth factor II
A0A0U1RRB2	GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1

Figures

Figure 1

ZJP affects gastric histopathology in CAG rats. (A) Control group; (B) Model group; (C) 0.63 g/kg ZJP group; (D) 1.26 g/kg ZJP group; (E) 2.52 g/kg ZJP group; (F) omeprazole group (HE staining, ×200 magnification); (G) Control group; (H) Model group; (I) 0.63 g/kg ZJP group; (J) 1.26 g/kg ZJP group; (K) 2.52 g/kg ZJP group; (L) omeprazole group (HE staining, ×400 magnification).

Figure 2

ZJP affects serum biochemical parameters in CAG rats (mean $\pm$ SD, $n = 6$). (A) IL-1 β content; (B) IL-6 content; (C) IL-10 content; (D) iNOS content. ## $P < 0.01$, compared with the control group. * $P < 0.05$, ** $P < 0.01$, compared with the model group.

Figure 3

PCA score plot of the control group, model group and 2.52 g/kg ZJP group. (A) ESI+ mode of PCA in serum samples between the three groups; (B) ESI- mode of PCA in serum samples between the three groups; (C) ESI+ mode of PCA in urine samples between the three groups; (D) ESI- mode of PCA in urine samples between the three groups.

Figure 4

The OPLS-DA score plots, S-plots and 100-permutation test generated of serum samples between the control group, model group and 2.52 g/kg ZJP group. OPLS-DA score plots were the pair-wise comparisons in ESI+ mode, control group vs model group (A), model group vs 2.52 g/kg ZJP group (D) as well as in ESI- mode, control group vs model group (G), model group vs 2.52 g/kg ZJP group (J); S-plots of the OPLS-DA model in ESI+ mode, control group vs model group (B), model group vs 2.52 g/kg ZJP group (E) as well as in ESI- mode, control group vs model group (H), model group vs 2.52 g/kg ZJP group (K); The 100-permutation test of the OPLS-DA model in ESI+ mode, control group vs model group (C),

model group vs 2.52 g/kg ZJP group (F) as well as in ESI- mode, control group vs model group (I), model group vs 2.52 g/kg ZJP group (L).

Figure 5

The OPLS-DA score plots, S-plots and 100-permutation test generated of urine samples between the control group, model group and 2.52 g/kg ZJP group. OPLS-DA score plots were the pair-wise comparisons in ESI+ mode, control group vs model group (A), model group vs 2.52 g/kg ZJP group (D) as well as in ESI- mode, control group vs model group (G), model group vs 2.52 g/kg ZJP group (J); S-plots of the OPLS-DA model in ESI+ mode, control group vs model group (B), model group vs 2.52 g/kg ZJP

group (E) as well as in ESI- mode, control group vs model group (H), model group vs 2.52 g/kg ZJP group (K); The 100-permutation test of the OPLS-DA model in ESI+ mode, control group vs model group (C), model group vs 2.52 g/kg ZJP group (F) as well as in ESI- mode, control group vs model group (I), model group vs 2.52 g/kg ZJP group (L).

Figure 6

Potential metabolites changes in *H. pylori*-induced CAG with ZJP treatment. (A) Choline; (B) cis-Aconitic acid; (C) Citric acid; (D) Creatine; (E) Hydroxypyruvic acid; (F) L-Threonine; (G) Succinic acid semialdehyde; (H) Citric acid; (I) Taurine; (J) Uric acid. #P < 0.05 and ##P < 0.01, compared with the control group. *P < 0.05, **P < 0.01, compared with the model group.

Figure 7

Summary of metabolic pathway analysis of potential metabolites. (A) Summary of metabolic pathway analysis of potential metabolites in serum samples; 1. Taurine and hypotaurine metabolism; 2. Glyoxylate and dicarboxylate metabolism; 3. Glycine, serine and threonine metabolism; 4. Glycerophospholipid metabolism; 5. TCA cycle. (B) Summary of metabolic pathway analysis of potential metabolites in urine samples. 1. TCA cycle; 2. Glycerophospholipid metabolism; 3. Glyoxylate and dicarboxylate metabolism; 4. Alanine, aspartate and glutamate metabolism; 5. Butanoate metabolism; 6. Purine metabolism.

Figure 8

The crucial compound-CAG target-metabolite network. (A) All target information participating in the treatment of CAG by ZJP; (B) key target information participating in the treatment of CAG by ZJP. The red triangle represents the active chemical composition of ZJP; the blue dots represent the protein targets of ZJP; the yellow square represents the common target for ZJP and disease; purple dots represent targets associated with potential metabolites.

Figure 9

Effect of ZJP on the relative mRNA expressions of MAPK1, PKIA, RB1, SCN5A, RXRA, E2F1, PTGS1, IGF2, ADRB1, ADRA1B, PTGS2, and GABRA1 in rats with CAG. (A) MAPK1; (B) PKIA; (C) RB1; (D) SCN5A; (E) RXRA; (F) E2F1; (G) PTGS1; (H) IGF2; (I) ADRB1; (J) ADRA1B; (K) PTGS2; (L) GABRA1. ##P < 0.01, compared with the control group. *P < 0.05, **P < 0.01, compared with the model group.