Therapeutic effect of Zuojin Pill on chronic atrophic gastritis induced by *Helicobacter pylori* through JMJD2B/COX-2/VEGF

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Research

Keywords: Zuojin Pill, Chronic atrophic gastritis, Helicobacter pylori, JMJD2B, Cyclooxygenase-2, Vascular endothelial growth factor.

Posted Date: June 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-20652/v3>

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Abstract

Background: Zuojin Pill (ZJP), a famous Chinese medicinal formula, is widely accepted for treatment of chronic atrophic gastritis (CAG) in China. This study aimed to explore the therapeutic effects and mechanisms of ZJP in *Helicobacter pylori* (*H. pylori*) - induced chronic atrophic gastritis (CAG) *in vivo* and *in vitro*.

Methods: CAG rat model was induced by *H. pylori*. ZJP (0.63, 1.26, and 2.52 g/kg, respectively) was administered orally for four weeks. Therapeutic effects of ZJP were identified by H&E staining and serum indices. In addition, cell viability, morphology and proliferation were detected by cell counting kit-8 (CCK8) and high-content screening assay (HCS), respectively. Moreover, relative mRNA expression and protein expression related to JMJD2B/COX-2/VEGF axis was detected to investigate the potential mechanisms of ZJP in CAG.

Results: Results showed the symptoms (weight loss and gastric mucosa damage) of CAG were alleviated, and the contents of TNF- α in serum was markedly decreased after treating with ZJP. Moreover, cell viability, proliferation and morphology changes of GES-1 cells were ameliorated by ZJP intervention. In addition, proinflammatory genes and JMJD2B/COX-2/VEGF axis related genes were suppressed by ZJP administration *in vitro* and *in vivo*. Meanwhile, immunohistochemistry (IHC) and western blot confirmed down-regulation of these genes by ZJP intervention.

Conclusion: ZJP treatment can alleviate gastric mucosal damage induced by *H. pylori* via JMJD2B/COX-2/VEGF axis.

Background

Chronic atrophic gastritis (CAG) is a universal disease in digestive system and one of the most continuous health concerns worldwide. *Helicobacter pylori* (*H. pylori*) infection is the strongest risk factor for gastric carcinogenesis [1]. *H. pylori* infection can induce CAG, which develops through the premalignant periods of intestinal metaplasia, and dysplasia, before eventually leading to gastric cancer [2]. At present, there are traditional options against *H. pylori*, including conventional triple therapy, bismuth based quadruple therapy and proton pump inhibitors [3]. Whether it is triple therapy, quadruple therapy or proton pump inhibitors, conventional therapies can cause a series of serious adverse reactions, such as abdominal pain, constipation, decline of eradication rates. Therefore, novel and safe drugs are badly needed to be discovered and used in clinical treatment as soon as possible. Copious evidences from China declare traditional Chinese medicine (TCM) possess unlimited potential in treating *H. pylori* induced CAG. As an alternative therapy, TCM are gaining increasing popularity worldwide for the clinical treatment [4]. Zuojin pill (ZJP)/, recorded in the Danxi's experiential therapy, has been used for the treatment of gastrointestinal diseases for more than 700 years. However, the mechanism underlying the effect of ZJP in the treatment of *H. pylori*-induced CAG remains unclear.

ZJP contains *Coptidis Rhizoma* (CR) and *Euodiae Fructus* (EF) in the ratio of 6: 1 (w/w), which was initially recorded in an ancient medicine treatise, during China's Yuan Dynasty for treating gastrointestinal disorders. CR is the dried rhizome of *Coptis chinensis* Franch., *Coptis deltoidea* C.Y. Cheng et Hsiao, or *Coptis teeta* Wall. (Chinese Pharmacopoeia Commission 2015). In clinical, it is often utilized to treat diarrhea, abdominal fullness, vomiting, jaundice, toothache, high fever coma, diabetes and eczema [5]. EF is the dried and immature fruit of *Euodia rutaecarpa* (Juss.) Benth., *Euodia rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang or *Euodia rutaecarpa* (Juss.) Benth. var. *bodinieri* (Dode) Huang (Chinese Pharmacopoeia Commission 2015). EF is widely applied for the treatment of inflammation, headache, and hypertension [6]. Alkaloids are proved to be the primary compounds of CR and EF, including berberine, coptisine, palmatine, evodiamine and rutaecarpine. ZJP was officially listed in the Chinese Pharmacopoeia (2015 edition) as a common prescription employed in clinical patients, who suffer from esophagitis, gastritis, peptic ulcer, and other disorders. Up to now, ZJP has been well-practiced in clinical application. The mechanism of ZJP acting on CAG is still unclear. In this study, we aimed to elucidate the effects and the molecular mechanisms of ZJP in *H. pylori*-induced CAG.

It has been reported that histone demethylase JMJD2B in stomach tissues when *H. pylori* infection became increased expression, and Cyclooxygenase-2 (COX-2) upregulated as downstream target protein of JMJD2B in *H. pylori* induced inflammatory process [7]. Histone modification is an epigenetic mechanism, which plays a crucial role in gastric cancer carcinogenesis [8]. Overexpress of JMJD2B in gastric cancer can accelerate cell proliferation, survival, invasion and metastasis of gastric tumor [9]. *H. pylori* infection activates the JMJD2B promoter and upregulates expression at transcriptional level. Recently, evidence has shown that JMJD2B is required for *H. pylori*-induced COX-2 activation. COX-2 is involved in inflammation as a key enzyme in the synthesis of prostaglandin and overexpresses after *H. pylori* infection [10]. The expression level of COX-2 is low under resting conditions in most cells, but can be induced by *H. pylori* in a cag T4SS-dependent manner [11]. In addition, JMJD2B was shown to regulate vascular endothelial growth factor (VEGF), phosphoinositide 3-kinase pathways, and angiogenesis, all of which play important roles in inflammation or tumorigenesis [12]. It is well known that in *H. pylori*-infected gastritis, the concentration of angiogenic factor increases, resulting in the formation of new blood vessels. New angiogenesis will enhance supplement of nutrient and oxygen, and thus promote the development of gastritis. COX-2 is the key target responsible for promoting angiogenesis, which stimulate Vascular endothelial growth factor (VEGF) expression induced by *H. pylori* [13].

In this study, we explored the curative effect of ZJP in *H. pylori* induced CAG *in vivo* and *in vitro*. Moreover, we attempted to conduct a preliminary examination of the roles of JMJD2B/COX-2/VEGF axis in mechanism of ZJP for better understanding protective effects of ZJP in CAG.

Methods

Material

Coptidis Rhizoma (Lot: 18011901) and Euodiae Fructus (Lot: 17021602) were purchased from Beijing Lvye Pharmaceutical Co., Ltd. (Beijing, China). The document codes of the product quality inspection number are CP-18-01-22 (Coptidis Rhizoma) and CP-17-02-11 (Euodiae Fructus). All detection results demonstrated that the quality of Coptidis Rhizoma and Euodiae Fructus was in complete requirement of in the Chinese Pharmacopoeia 2015. Omeprazole (positive drugs) was purchased from Astrazeneca Pharmaceutical Co., Ltd. (batch number: 1906194, Suzhou, China). All the other unspecified chemicals were of analytical grade.

Preparation of ZJP

Coptidis Rhizoma and Euodiae Fructus were soaked in pure water (6/1, *w/w*) for 30 min and were extracted twice (1 hour each time). Then, the extract was collected and evaporated to prepare dried powder under reduced pressure, respectively. Finally, the weight ratio of ZJP was 25.59%. ZJP powder was kept at 4°C until oral administration to rats. ZJP was dissolved in and acted on GES-1 cells. ZJP powder was dissolved in dimethyl sulfoxide (DMSO) to configure as mother liquor and then Dulbecco's modified Eagle's medium (DMEM) was used to dilute to corresponding concentration for using.

Bacterial strain and culture condition

H. pylori isolated strain (ICDC111001) was kindly provided by Dr. Jianzhong Zhang (Chinese Disease Control and Prevention Center, Beijing, China). *H. pylori* strain was maintained and grown on Columbia blood agar (Thermo Fisher Scientific, China), with incubation under micro-aerobic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C. After three to five days' culture, bacteria strain was collected and adjusted to 1.0×10⁸ colony forming units (CFU) /mL.

Animal experiments

Thirty-six male Sprague-Dawley (SD) rats were raised normally until 1 week before the experiments and maintained in the standard laboratory condition of stable temperature (25 ± 0.5°C), continuous humidity (55 ± 5%), alternant lighting (12 hours light: 12 hours dark cycle), and were free access to enough food and water. All specific pathogen free (SPF) male SD rats (170-190g) were purchased from Beijing Sibeifu Animal Breeding Center [Permission No. SCXK-(Jing) 2016-0002]. Firstly, the rats were randomly divided into the control group and model group. The rats in the model group were induced with *H. pylori* (1.5×10⁸ CFU/ml, 1.5 ml each rat) suspension to establish CAG model (4 times a week, at day 1, 3, 5 and 7) and rats in the control group were induced with equal volume saline by oral gavage. All rats were fasted about 12 h before intragastric administration. After 8 weeks, gastric tissues were obtained for rapid urease test to detect the model. Finally, the CAG rats were randomly divided into five different groups with six rats in each, including the model group, ZJP low-dose (0.63 g/kg), medium-dose (1.26 g/kg) and high-dose (2.52 g/kg) groups, and Omeprazole group (1.8 mg/kg). All rats were administered once a day for 4 weeks. After 4 weeks, all rats were executed and gastric mucosa samples were isolated and cut in half along the greater curvature. The serum and half of the gastric tissue samples were collected and stored at -80°C to

detect expression of gene and protein. The other of the gastric tissue samples were excised and fixed in 4% paraformaldehyde general tissue fixative, and then stained with H&E.

Serum Tumor Necrosis Factor- α (TNF- α) and VEGF measurements

The serum levels of TNF- α and VEGF were measured on a Synergy H1 Hybrid Reader (Biotech, USA). The measurement steps were conducted as per the manual of the ELISA kit (MLBIO biotechnology Co., Ltd., Shanghai, China).

Immunohistochemistry (IHC)

Paraffin-embedded rat stomach tissues were deparaffinized and antigen-repaired, and then samples with primary anti-JMJD2B Ab (Cat No.: ab191434, Abcam, 1:125) and anti-COX-2 Ab (Cat No.: 12375-1-AP, Proteintech, 1:100) incubated at 4°C for the night. Slides were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and the slides were counterstained with hematoxylin. Light microscopy (Olympus, Japan) at 200 $\times$ and 400 $\times$ magnification was applied to photograph images.

Cell viability assay and *H. pylori* infection

The GES-1 cells were obtained from the FuHeng Cell Center, (Shanghai, China), which were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a constant incubator containing 5% CO₂ at 37°C. The GES-1 cells were cultured overnight to reach at least 80% confluency. Cell viability was detected by cell counting kit-8 (CCK-8; Lot. PG658, DOJINDO, Japan). The optical density (OD) value was measured at 450 nm by using a Synergy H1 Hybrid Reader (Biotech, USA).

The *H. pylori* strain was harvested from Columbia blood agar plates, suspended in antibiotic-free DMEM medium complemented with 10% FBS, and then was added to the GES-1 cells culture. The *H. pylori* added to GES-1 cells at a multiplicity of infection (MOI) ratio of 10:1, 20:1, 50:1 and 100:1 for 0, 6, 12 and 24 h. Bacterial counting of *H. pylori* was examined through Synergy H1 Hybrid Reader (Biotech, USA). The measurement of OD value was set at 600 nm to count colony forming units of *H. pylori* (1 OD_{600nm}=1.5 $\times$ 10⁸ CFU/ml). Cocultivation was maintained at 37°C in a 5% CO₂ atmosphere.

High-Content Analysis Experiments (HCS)

Nuclear, cell morphology and the number of dead cells and living cells were detected by Array Scan High-Content System (Thermo Scientific, Massachusetts, USA) [14]. Hoechst 33342 (H3570, Invitrogen), calcein AM (C3099, Invitrogen), and ethidium homodimer-1 (EthD-1) (L3224, Invitrogen) were applied to quantify the GES-1 cells. Cell health profiling assay module was selected in the HCS system, and several different wavelength channels were set to collect fluorescence images. The measured parameters and format were similar to those used previously [15]. Array Scan XTI (The Array Scan software algorithm was used to perform analysis) was used to quantify the mean fluorescence intensity of GES-1 cells.

Real-time quantitative PCR Analysis *in Vivo* and *in Vitro*

Total mRNA of all rats' gastric tissue and GES-1 cells were extracted by TRIzol reagent (Nordic Bioscience, Beijing, China) and transformed into cDNA by reverse transcription kit (Promega, Madison, USA) according to the instructions. RT-qPCR for mRNA of JMJD2B, COX-2, VEGFR1, VEGFR2 and VEGF in rats and GES-1 cells were performed using SYBR Green PCR Master Mix (Nordic Bioscience, Beijing, China). Primer sequences are listed in Table 1. RT-qPCR was conducted on the 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Results were shown and exported in 7500 software (Applied Biosystems for 7500 and 7500 Fast Real-Time PCR Products, version 2.0.5). The relative amounts of mRNA were determined based on $2^{-\Delta\Delta C_t}$ calculations with β -actin as the endogenous reference.

Western Blot analysis to detect the protein expression *in vivo* and *in vitro*

Total protein was extracted from the GES-1 cells and tissue samples by ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and protease inhibitor. Protein concentration was detected by using a bicinchoninic acid assay (BCA) protein assay kit (Solarbio, Beijing, China). The polyvinylidene difluoride (PVDF) membranes were incubated with the primary antibodies at 4°C overnight, including rabbit anti-JMJD2B monoclonal antibody (ab191434, Abcam, dilution: 1:1,000), rabbit anti-COX-2 antibody (12375-1-AP, Proteintech, dilution: 1:500), rabbit anti-VEGFR1 antibody (ab32152, Abcam, dilution: 1:2,500), rabbit anti-VEGFR2 antibody (ab221679, Abcam, dilution: 1:1,000), and anti-beta actin antibody (bs-0061R, Bioss, dilution: 1: 10,000). Then, membranes were washed three times for 5 min each with TBS-0.1% Tween 20 (TBST) and incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG (H + L) (Zhongshan Golden Bridge Biotechnology; dilution, 1:25000; ZB-2301) for 1 h at room temperature. The antigen-antibody bands were detected by using the solution and visualized by using the X-ray film (Beyotime Institute of Biotechnology). β -actin was served as an internal control.

Statistical Analysis

All results were presented as mean $\pm$ standard deviation (SD) and analyzed with the SPSS software program (version 19.0; 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

Macroscopic pathology and histological examination of gastric mucosa

Firstly, after 8 weeks, the rapid urease test in the model group was positive (Fig.1A). After 4-week ZJP administration, the result of rapid urease test in low dose group was negative (Fig.1B). During 8-week *H. pylori* infection, the rats induced by *H. pylori* exhibited CAG clinical symptoms, such as weight loss, diarrhea and loss of appetite (Fig.1C). The gastric mucosa of rats in model group showed paleness and thinning of gastric mucosa, with disarrayed plicae and small white nodules (Fig.1D).

Histological features were critical evidence for the therapeutic effects of ZJP against *H. pylori*-induced CAG. Rats in the control group showed mucosal intact with tightly, abundant and orderly gastric glands. CAG model rats showed inherent glands was missing, part of the mucosa was stripped, and neutrophils were infiltrated in the mucosa. Conversely, pathological changes in the omeprazole and the ZJP groups showed significantly improved in terms of the degree of edema, hyperemia, erosion and atrophy of gastric mucosa. Administration of high dose ZJP particularly exhibited lower inflammatory cell infiltration and gastric mucosal injury. Results were presented in Fig.1E.

Expression of JMJD2B in GES-1 cells with *H. pylori* infection

CCK8 was used for determination of the optimal concentration for ZJP administration. (Fig.2A). The results showed that ZJP treatment for 24 h potently suppressed cell viability in a concentration-dependent manner with the increasing concentration (0, 10, 20, 30, 40, 60 and 120 µg/ml). The results showed that 120 µg/ml of ZJP could significantly inhibit the cell viability ($P < 0.01$). When 60 µg/mL of ZJP was given, the cell viability was close to 100%. Accordingly, 60 µg/mL of ZJP played a relatively protective role and was used as the optimal concentration to investigate the protective effects. For the *in vitro* study, 30 and 60 µg/mL of ZJP were used as low-dose and high-dose to GES-1 cells, respectively.

It has been reported that the expression of JMJD2B increased with *H. pylori* infection. GES-1 cells were infected with *H. pylori* at different MOI (10:1, 20:1, 50:1, 100:1) through 24 h. As expected, *H. pylori* infection at a lower MOI (10:1, 20:1, 50:1) resulted in a dose-dependent induction (Fig.2B). When MOI=100:1, there were a number of cells floating in the culture medium. Thus, MOI=50:1 was chosen for further study. By co-culture of *H. pylori* and GES-1 cells for 0, 6, 12, 24 h, the mRNA expression of JMJD2B increased in a time-dependent manner, but there was no significant difference between 12 h and 24 h (MOI=50:1) (Fig.2C). Therefore, MOI=50:1 infection was finally chosen for 12 h for further research.

High-Content Analysis Experiments

In order to further determine the influence of ZJP on cell morphology, HCS was used to investigate the effect of ZJP on the morphology of GES-1 cells. Nucleus staining (blue fluorescence), cell cytoplasm labeling (green fluorescence), and dead cells (red fluorescence) were marked by Hoechst 33342, calcein AM, and EthD-1, respectively (Fig. 3A). In the control group, nucleus and cytoplasm of GES-1 cells possessed a homogenous Hoechst and calcein AM fluorescence. After infection of *H. pylori*, there were cell viability, cell number and morphological changes, such as nuclear deformations, cell count decreased, green and red fluorescence reduced and increased, respectively. ZJP could certainly boost the green fluorescence and reduce the red fluorescence of GES-1 cells (Fig.3B-D). These results indicated that ZJP could ameliorate nuclear morphology and cell proliferation in *H. pylori*-induced injury.

JMJD2B/COX-2/VEGF axis mRNA and protein expression *in Vivo*

Previous gene ontology analysis revealed that JMJD2B regulates VEGF signaling pathways and angiogenesis which play important roles in inflammation or cancer progression [14]. Blood circulation

disorders significantly influence pathological process of CAG. VEGF is the target gene to closely regulate angiogenesis, which can stimulate the proliferation of epithelial cells, the formation of blood capillaries, and then participating in the defense and repair of gastric mucosa. Widely accepted, COX-2 is a prostaglandin-endoperoxide synthase, which is responsible for the formation of thromboxanes as a key rate-limiting enzyme. In *H. pylori*-infected gastric mucosal cells, COX-2 is involved in the regulation of VEGF expression [16]. Nevertheless, whether ZJP could interfere with CAG through JMJD2B/COX-2/VEGF axis has not been studied. IHC revealed that Control group samples expressed low levels of JMJD2B as evidenced by barely positive staining of JMJD2B in gastric mucosal cells. Correspondingly, model group samples hold higher level of COX-2 compared with non-infected tissues. Interfered by ZJP, the expression of JMJD2B and COX-2 decreased (Fig.4).

Compared with control group, the serum TNF- α level was significantly increased in model group. After administration of ZJP group (0.63, 1.26 and 2.52 g/kg), TNF- α significantly reduced, and the high dose group (2.52 g/kg) and Omeprazole group had lower level of TNF- α compared with group with low dose group and medium dose group (Fig. 5F). Moreover, RT-qPCR and Western Blot were used to explore intervention effect of ZJP on JMJD2B/COX-2/VEGF axis (Fig.5B-K). Compared with control group, JMJD2B, COX-2, VEGF, VEGFR1 and VEGFR2 expressed at a relatively high level in model group. However, the mRNA and protein expression levels of these genes in the ZJP groups were decreased. High dose group of ZJP could significantly reduce the mRNA and protein expression levels, while, the medium and low dose group of ZJP exhibited weaker reduction.

JMJD2B/COX-2/VEGF axis mRNA and protein expression in *vitro*

We previously demonstrated that the mRNA and protein levels of JMJD2B, COX-2, VEGF, VEGFR1 and VEGFR2 were significantly decreased following intervention of ZJP. In order to further explore the effect of ZJP on JMJD2B/COX-2/VEGF axis, the model of *H. pylori* induced (MOI=50:1, 12h) GES-1 cells was established (Fig.6). The IL-8 mRNA level was significantly increased in *H. pylori*-infected cells. ZJP at 30 μ g/mL and 60 μ g/mL could all decrease the IL-8 mRNA level compared to control group (Fig.6G). Evidently, the expression of JMJD2B, COX-2, VEGF, VEGFR1 and VEGFR2 were increased in *H. pylori*-infected cells compared with the control group. Administration of ZJP at high dose (60 μ g/mL) expressed lower level of JMJD2B and its downstream genes noticeably compared with the *H. pylori*-infected group and low dose (30 μ g/mL) group. The results further emphasize that ZJP may relieve *H. pylori*-induced inflammation and gastric mucosa injury via the downregulation of JMJD2B/COX-2/VEGF axis.

Discussion

ZJP is a commonly used traditional Chinese prescription which was first written by Zhu Zhen-heng. ZJP has a wide range of pharmacological action in digestive system, including bacteriostasis, anti-inflammatory and analgesic effects. Studies show that the protection offer by the ZJP against ethanol-induced mucosal lesions is indicative of its outstanding gastroprotective effect, which may be related to regulate the NF- κ B signaling pathway [17]. More and more studies regarding the effects of single herb in

ZJP on gastric diseases is available. ZJP composed of *Coptidis Rhizoma* (CR) and *Euodiae Fructus* (EF) were mixed at the ratio of 6 : 1. Berberine, a natural isoquinoline alkaloid from CR, executes much attention for its multiple therapeutic effects on antitumor, antimicrobial, antidiabetic effects and available for *H. pylori*-induced chronic gastritis [18]. In addition, palmatine, as another protoberberine-type alkaloid, is the main component of CR, which has been shown to have multiple pharmacological activities, including gastroprotective effect [19]. Evodiamine, a botanical alkaloid extracted from the dried unripe fruit of *Evodia rutaecarpa* Benthham, exhibits extensive pharmacological effects including anti-obesity, anti-tumor, vasodilatory, antiinflammatory effects and gastric protection [20]. From a clinical application, ZJP is widely used in gastrointestinal diseases due to strong anti-inflammation, antimicrobial and protection of gastric mucosa [17]. In addition, ZJP and its active ingredients have obvious therapeutic effects against both inflammation and gastric mucosal damage in the stomach [21-22]. Recent study has exhibited the gastrointestinal regulating functions of ZJP by restoring gastric electrical rhythm [23]. Although more and more evidence for ZJP's therapeutic benefits for *H. pylori* induced CAG, the underlying therapeutic mechanism remains poorly understood. The aim of this study was to investigate the mechanism of ZJP on *H. pylori*-induced CAG.

CAG is considered to be a pre-malignant gastric lesion at a high risk of progression to gastric cancer. In recent years, the rising incidence of CAG has exhibited a trend towards younger age onset, CAG seriously affects the health and quality of life of more and more patients [24]. The most common clinical presentations of CAG are abdominal belching, abdominal pain, anorexia and weight loss [25]. Since the *H. pylori* was discovered in the human stomach, infection by the bacteria has been shown to be strongly related to CAG [26]. In a rodent model, *H. pylori* gavage induces a series of diseases associated with persistent inflammation [27]. Thus, *H. pylori* was used for preparing the CAG model in rats in this study to investigate the intervention effect and mechanism of ZJP in *vivo* and in *vitro*. The present study revealed that ZJP showed anti-inflammation effect in *H. pylori*-infected rats. Our results indicate that rats exhibited typical CAG-like clinical symptoms after administration of *H. pylori* for 8 weeks, such as weight loss, diarrhea, loss of appetite, positive results of rapid urease test and pathological changes of gastric mucosa, including arrangement of gastric glands, infiltration of lymphocytes and plasma cells. Meanwhile, the gastric mucosal injury and inflammation induced by *H. pylori* were improved by ZJP. At the cellular level, ZJP significantly enhanced the cell protection against *H. pylori* infection. Cell viability, morphological identification of GES-1 cells was directly ameliorated following ZJP administration. Then, further studies are required to clarify the mechanism between ZJP and anti-inflammation effect.

IL-8 plays an important role in the response of gastric epithelial cells to *H. pylori* infection, and shows significantly higher level in the gastric mucosal tissues of *H. pylori*-positive patients [28]. IL-8 can induce proinflammatory cytokines, such as IL-1 β , IL-6, IFN- γ and TNF- α , which further regulate the inflammatory reaction. Histone modification, an epigenetic mechanism, plays a crucial role in gastric cancer carcinogenesis [29]. JMJD2B, also known as KDM4B, is newly discovered and characterized as a member of the histone demethylase JMJD2 family. JMJD2B specifically catalyzes the removal of di- and trimethylated H3K9 (H3K9me₂/me₃), converting both histone marks to the monomethylated state [30]. JMJD2B promotes COX-2 expression via cooperating with NF- κ B in *H. pylori*-infected gastric epithelial

cells [7]. We found that ZJP treatment improved the *H. pylori*-induced gastric epithelial cells viability and pathological changes observed in the gastric mucosa through regulating the activation of JMJD2B and reducing the expression of COX-2. Besides, in our study, we found that ZJP suppressed *H. pylori*-induced expression of IL-8 and TNF- α , and this is consistent with these studies.

Besides, the current work provides evidence that both COX-2 and VEGF mRNA and protein were decreased in the gastric tissue after ZJP treatment in *H. pylori*-induced rats. COX-2 associated with pain, inflammatory reaction, tumorigenesis and so on. The expression of COX-2 is known to be increased in the gastric mucosa of *H. pylori*-infected gastritis patients [31]. In *H. pylori*-infected gastritis, there is an increase in angiogenic factors, and subsequently a formation of new blood vessels. New angiogenesis will enhance supply of nutrient and oxygen, and promote the development of gastritis [32]. Gastritis induced by *H. pylori* is related to VEGF, and the overexpression of VEGF is parallel to the increase in gastric mucosal vascularization [33]. VEGF and its receptor VEGF receptor 1 (VEGFR-1), receptor VEGF receptor 2 (VEGFR-2) are the most important pro-angiogenic factors. In this context, our study has shown that *H. pylori* gavage in rats could not only lead to colonization with *H. pylori* but also improve the COX-2, VEGF, VEGFR-1 and VEGFR-2 expression. For further verification with the effect of ZJP on the expression level of JMJD2B/COX-2/VEGF axis *in vitro*, we conducted a cell model of GES-1 cells co-culture to infection with *H. pylori* and treatment with ZJP. Accordingly, the expression of JMJD2B, COX-2 and downstream genes including VEGF, VEGFR1 and VEGFR2 were increased significantly in *H. pylori*-infected cells. Similar to the results of animal experiment, trend of the ZJP groups at a lower level, as the RT-qPCR and western blot shown. Thus, our findings indicate that ZJP could play a role of anti-inflammation and protection effects on gastric mucosa by regulating the JMJD2B/COX-2/VEGF axis.

Collectively, our findings suggested that ZJP could alleviate the CAG symptoms induced by *H. pylori*, which might be related to the regulated JMJD2B/COX-2/VEGF axis. Hence, our results may provide a new perspective into the potential of TCM in therapeutic intervention for CAG. Our study set the foundation to explore the mechanism and pharmacological effects of ZJP in the treatment of *H. pylori*-induced CAG.

Conclusions

Taken together, our study confirmed the therapeutic effect of ZJP in *H. pylori*-induced CAG model. We also found that Histone demethylase played a vital role in CAG model. Importantly, ZJP prevented gastric mucosal injury by inhibiting the *H. pylori*-induced inflammation via JMJD2B/COX-2/VEGF axis. The results of this study suggest a potential role of ZJP in treatment of CAG, which need to further investigate.

Abbreviations

ZJP: Zuojin Pill; CAG: Chronic atrophic gastritis; *Helicobacter pylori*: *H. pylori*; COX-2: Cyclooxygenase-2; VEGF: Vascular endothelial growth factor; TCM: Traditional Chinese medicine; CR: Coptidis Rhizoma; EF: Euodiae Fructus; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; CFU: Colony

forming units; SD: Sprague-Dawley; SPF: Specific pathogen free; H&E: Hematoxylin and eosin; CCK-8: Cell counting kit-8; OD: Optical density; MOI: Multiplicity of infection; HCS: High-Content Analysis Experiments ; IHC: Immunohistochemical staining.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Design of the study: Shihua Wu, Ruilin Wang, Chunmei Bao, Xiaomei Zhang and Jianzhong Zhang; data collection and analysis: Sijia Gao, Weihang Qin, Juling Zhang, Ruisheng Li, Xing Chen, Jianxia Wen and Tao Yang; drafting the manuscript: Shizhang Wei, Haotian Li, Ying Wei, Sichen Ren; supervising and providing Funding acquisition: HouLin Xia, Yanling Zhao. All authors participated in amending the manuscript before submission of the mutually agreed final version.

Funding

This study was supported by the National key R & D Program of China [No. 2018YFC1704500].

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

The experimental protocols were approved by 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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Table

Table 1 Primers used for real-time PCR

	Primers	Sequence-Forward	Sequence-Reverse
Cells	JMJD2B	CCTTCCTGCGGCATAAGATGAC	GGTGGCGAAGTTGGTAGATTCTG
	COX-2	AATCTGGCTGCGGGAACACAAC	TGTCTGGAACAACCTGCTCATCACC
	VEGF	GCCTTGCCTTGCTGCTCTACC	CTTCGTGATGATTCTGCCCTCCTC
	VEGFR1	TGGTGAGTAAGGAAAGCGAAAGGC	GTGTGGTTTGCTTGAGCTGTGTTC
	VEGFR2	AGGGAGTCTGTGGCATCTGAAGG	GTGGTGTCTGTGTCATCGGAGTG
	IL-8	GCTCTGTGTGAAGGTGCAGT	TTTCTGTGTTGGCGCAGTGT
	β -actin	GGCACCACACCTTCTACAATGAGC	GATAGCACAGCCTGGATAGCAACG
Rats	JMJD2B	CTACTACCGCTGCCGTGTCATTG	CTCTGCTTGTGATGCTCTCTGGATAC
	COX-2	AGGTCATCGGTGGAGAGGTGTATC	CGGCACCAGACCAAAGACTTCC
	VEGF	CACGACAGAAGGGGAGCAGAAAG	GGCACACAGGACGGCTTGAAG
	VEGFR1	GAGCATCTATCAGGCAGCGGATTG	CGACCCACTCTTCACACGACAAG
	VEGFR2	TGGCAATTCCCGTCCTCAAAGC	CCTTGGTCACTCTTGGTCACACTG
	β -actin	CCCGCGAGTACAACCTTCTTG	TCATCCATGGCGAACTGGTGG

Figures

Figure 1

Macro performance of CAG in rats. (A) Rapid urease test of stomach tissues. (B) Rapid urease test of stomach tissues. (C)Weight of rats. (D) Morphology of gastric mucosa stomach tissue (n=3). (F) H & E staining of chronic atrophic gastritis in rats (×200, ×400). Data were shown as mean ± SD;

Figure 2

Cell activity and model determination. (A) Effect of ZJP on cell viability (10µg/mL-120µg/mL). (B) JMJD2B mRNA expression in different MOI of *H. pylori* (MOI=10:1, 20:1, 50:1 and 100:1). (C) JMJD2B mRNA expression in different infection time of *H. pylori*. (T=0, 6, 12 and 24 hours). ## $P < 0.01$ versus control group, # $P < 0.05$ versus control group. Data were shown as mean $\pm$ SD (n=3).

Figure 3

High-Content Analysis Experiments in GES-1 cells. (A) Green fluorescence, red fluorescence and blue fluorescence reflect living cells, dead cells and nuclei respectively. Scale bar=50 µm. (B) Valid cell counts of HCS analysis for GES-1 cells (% of control). (C) Living cell counts of GES-1 cells (MEAN_TargetAvgIntenCh2). (D) Dead cell counts of GES-1 cells (MEAN_TargetAvgIntenCh3). ##P < 0.01 versus control group. #P < 0.05 versus control group. **P < 0.01 versus H. pylori infection group. The

results are expressed as percentages of control group. Data were shown as mean $\pm$ SD. Control, control group; ZJP, Zuojin Pill. *H. pylori*, *Helicobacter pylori* (n=3).

Figure 4

Effect of ZJP at different doses on JMJD2B and COX-2 expression in rats with *H. pylori*-induced CAG. (A) Expression of JMJD2B in different groups. (B) Expression of COX-2 in different groups.Effect of ZJP at different doses on JMJD2B and COX-2 expression in rats with *H. pylori*-induced CAG. (A) Expression of JMJD2B in different groups. (B) Expression of COX-2 in different groups.

Figure 5

Effects of ZJP on the JMJD2B/COX-2/VEGF axis mRNA and protein expression in vivo with *H. pylori* infection. (A-E) The mRNA expression of JMJD2B/COX-2/VEGF axis. (F) The expression level of TNF-α in rat serum. (G-K) The protein expression of JMJD2B/COX-2/VEGF axis. All data are presented as mean ± SD. ## $P < 0.01$ versus control group; * $P < 0.05$ versus model group; ** $P < 0.01$ versus model group ($n=3$).

Figure 6

Effects of ZJP on the JMJD2B/COX-2/VEGF axis mRNA and protein expression in vitro with *H. pylori* infection. (A-E) The mRNA expression of JMJD2B/COX-2/VEGF axis. (F) IL-8 mRNA expression in GES-1 cells. (G-J) The protein expression of JMJD2B, COX-2 and VEGFR2. All data are presented as mean $\pm$ SD. $###P < 0.01$ versus control group; $*P < 0.05$ versus model group; $**P < 0.01$ versus model group ($n=3$). Effects of ZJP on the JMJD2B/COX-2/VEGF axis mRNA and protein expression in vitro with *H. pylori* infection. (A-E) The mRNA expression of JMJD2B/COX-2/VEGF axis. (F) IL-8 mRNA expression in GES-1

cells. (G-J) The protein expression of JMJD2B, COX-2 and VEGFR2. All data are presented as mean $\pm$ SD. ##P < 0.01 versus control group; *P < 0.05 versus model group; **P < 0.01 versus model group(n=3).

Figure 7

Effect of ZJP at different doses on JMJD2B and COX-2 expression in rats with H. pylori-induced CAG. (A) Expression of JMJD2B in different groups. (B) Expression of COX-2 in different groups.

Figure 8

Effects of ZJP on the JMJD2B/COX-2/VEGF axis mRNA and protein expression in vivo with *H. pylori* infection. (A-E) The mRNA expression of JMJD2B/COX-2/VEGF axis. (F-G) The protein expression of JMJD2B/COX-2/VEGF axis. All data are presented as mean $\pm$ SD and analyzed by one-way ANOVA followed by t-test. $###P < 0.01$ versus control group; $*P < 0.05$ versus model group; $**P < 0.01$ versus model group ($n=3$).

Figure 9

Effects of ZJP on the JMJD2B/COX-2/VEGF axis mRNA and protein expression in vitro with *H. pylori* infection. (A-E) The mRNA expression of JMJD2B/COX-2/VEGF axis. (F) The protein expression of JMJD2B, COX-2 and VEGFR2. All data are presented as mean $\pm$ SD and analyzed by one-way ANOVA followed by t-test. ## $P < 0.01$ versus control group; * $P < 0.05$ versus model group; ** $P < 0.01$ versus model group($n=3$).