Protective Effect of Zuojin Pill on Helicobacter Pylori-Induced chronic atrophic gastritis in Rats and GES-1 Cells and Mechanisms of Action Exploration

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Shihua Wu
Chengdu University of Traditional Chinese Medicine

1263588540@qq.com

Corresponding Author

Chunmei Bao
The Fifth Medical Center of PLA General Hospital

Ruilin Wang
The Fifth Medical Center of PLA General Hospital

Jianzhong Zhang
National institute for communicable disease control and Prevention

Juling Zhang
The Fifth Medical Center of PLA General Hospital

Ruisheng Li
The Fifth Medical Center of PLA General Hospital

Xing Chen
Chengdu University of Traditional Chinese Medicine

Jiaxia Wen
Chengdu University of Traditional Chinese Medicine

Tao Yang
Chengdu University of Traditional Chinese Medicine

Shizhang Wei
Chengdu University of Traditional Chinese Medicine

Haotian Li
The Fifth Center of PLA General Hospital
Ying Wei
Chengdu University of Traditional Chinese Medicine

Sichen Ren
Chengdu University of Traditional Chinese Medicine

Houlin Xia
Chengdu University of Traditional Chinese Medicine

✉ xhl64@163.com Corresponding Author

Yanling Zhao
The Fifth Medical Center of PLA General Hospital

✉ zhaoyl2855@126.com Corresponding Author

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Abstract
Objective Zuojin Pill (ZJP) containing two Chinese herbal drugs: Coptidis Rhizoma and Euodiae Fructus is a classical formula and is widely accepted as a treatment of chronic atrophic gastritis (CAG) in China. This study aimed to explore the therapeutic effect and mechanism of ZJP which attenuated H. pylori -induced CAG in vivo and in vitro.

Methods: H. pylori (Helicobacter pylori) was used to induce CAG rat model. 0.63, 1.26, and 2.52 g/kg of ZJP (was administered orally for four weeks. Therapeutic effect of ZJP was identified by H & E staining and serum indices. In addition, cell viability, morphology and proliferation were detected by cell counting kit-8 and high-content screening assay. Gene and protein expression related to JMJD2B/COX-2/VEGF axis were detected to further investigate the potential mechanism.

Results Compared with the control group, the ZJP groups showed a significant protection effects on Gastric mucosa, as indicated by the reduced loss of glands and inflammatory cell infiltration. Meanwhile, ZJP could ameliorate cell viability, morphology changes, and proliferation in GES-1 cells. Moreover, the ZJP treatment decreased the amount of IL-8, and TNF-α, indicating that it could reduce the level of inflammation, and decrease stomach damage. The expression of JMJD2B/COX-2/VEGF axis related genes and proteins were measured by real-time quantitative PCR, western blot and immunohistochemistry methods. The ZJP groups were found to decrease relative genes and protein expression level compared with the model group. ZJP could improve gastric mucosa protection and reduce inflammation level by inhibiting the expression level of JMJD2B/COX-2/VEGF axis.

Conclusion Our data confirmed the effective therapy of ZJP in H. pylori -induced CAG, which supports the role of ZJP as an anti-inflammatory and protection of gastric mucosa agent in CAG induced by H. pylori. These results may provide helpful tools for the treatment of CAG.

Background
CAG is a universal disease of the digestive system and one of the most continuous health concerns worldwide. CAG, as a well-known precursor of gastric cancer, it is characterized by chronic inflammatory changes, such as glands lost, atrophy of gastric mucosa, and pathological changeable epithelium along with intestine epithelium metaplasia [1]. Some serious chronic atrophic gastritis
patients even develop into gastric cancer [2]. Although current therapies, including Standard triple therapy, Bismuth based quadruple therapy, proton pump inhibitors as well as antibiotics can alleviate major symptoms of CAG [3], which can trigger a series of serious side effects. Therefore, novel and safe prevention strategies are required. Under this case, it has become increasingly popular for choosing herbal treatment for clinicians and patients [4]. In China, there are a large number of traditional Chinese medicine (TCM) and prescriptions based on the theory of TCM, which are widely used in clinic with good curative effects.

ZJP contains two herbal drugs: Coptidis Rhizoma (CR) and Euodiae Fructus (EF) in the ratio of 6: 1 (w/w), which was first recorded in an ancient medicine treatise, Danxi's experiential therapy, during China’s Yuan Dynasty for treating gastro-intestinal disorders. 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, as yet, unknown. In this study, we aimed to elucidate the effects and the molecular mechanisms of ZJP in H. pylori-induced CAG.

Histone modification is an epigenetic mechanism, which plays a crucial role in gastric cancer carcinogenesis [5]. Histone demethylase JMJD2B was newly confirmed and characterized as a member of the histone demethylase JMJD2 family. Overexpress of JMJD2B is in gastric cancer can accelerate cell proliferation, survival, invasion and metastasis of gastric tumor [6]. Cyclooxygenase-2 (COX-2) is involved in inflammation as a key enzyme in the synthesis of prostaglandin and overexpresses after H. pylori infection [7]. Moreover, JMJD2B activation and COX-2 upregulation contribute to gastric inflammation and carcinogenesis [8]. 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 [9]. Hence, it's reasonable to believe that inhibition of JMJD2B/COX-2/VEGF axis could improve the progression of inflammation in CAG.

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_ (CR, Lot: 18011901) and _Euodiae Fructus_ (EF, 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 (CR) and CP-17-02-11 (EF). All detection results demonstrated that the quality of CR and EF 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

CR and EF 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 use.

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 were 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 the infection for 8 weeks, gastric tissue was obtained for rapid urease test to detect confirm the model. Finally, the animals with successfully prepared CAG model 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 (1.8mg/kg). Rats in all groups were administered with corresponding drugs once a day as long as 4 weeks including the control group. After 4 weeks, all rats were executed and gastric mucosa samples were isolated and cut in half along the greater curvature, and then were rinsed with Saline. The serum and half of the gastric tissue samples of each rat were collected and stored at -80°C for the detection of mRNA and protein expression. The other of the gastric tissue samples was excised and fixed in 4% paraformaldehyde general tissue fixative, and then stained with hematoxylin and eosin (HE).

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

The serum TNF-α and VEGF levels 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 tissue was 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× and 400× 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 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 OD₆₀₀nm = 1.5×10⁸ CFU/ml). The number of GES-1 cells was obtained through counting slides (Bio-Rad, USA). Cocultivation was maintained at 37°C in a 5% CO₂ atmosphere.

High-Content Analysis Experiments (HCS)

Nuclear, cell morphology and number of dead cells and living cells were detected by using the Array Scan High-Content System (Thermo Scientific, Massachusetts, USA) [10]. 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 [11]. 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 using TRIzol reagent (Nordic Bioscience, Beijing, China) and transformed into cDNA by using 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 $2^{-\Delta\Delta Ct}$ 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 gastric tissue by using the 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) following the manufacturer’s instructions. 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). Quantification of bands was performed by densitometric analysis using Bio-Rad Quantity One. β-Actin was served as an internal control.

**Statistical Analysis**

All results were presented as mean ± 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 $P < 0.01$. 

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Results
ZJP ameliorates macro performance of H. pylori-induced chronic atrophic gastritis in SD rats
Firstly, through 8 weeks of H. pylori induction, the rapid urease test reaction was used to confirm the
CAG rat model. The rapid urease reaction in the gastric antrum of rats in the model group was
positive with red light, while the control group was negative with yellow color (Fig. 1A). After a 4-week
administration, the reaction in the ZJP low-dose group was negative compared with the model group
(Fig. 1B). During the 8 = week H. pylori induction, the weight of rats in the model group showed a
downward trend. After a 4-week administration, the weight of the rats gradually increased (Fig. 1C).
The gastric mucosa of rats in model group showed paleness and thinning of gastric mucosa, with
disarrayed plicae and small white nodules, while was pink, moistened and smooth in the control
group. The rats in the ZJP low-dose group exhibited good elasticity, mild mucus conjugation and
edema on the mucosal surface, dark color and regular folds (Fig. 1D).
ZJP ameliorates H. pylori-induced Histological examination of gastric damage in SD rats
Histological features of gastric tissue were the critical evidence for the therapeutic effect of ZJP
against H. pylori-induced CAG. In this study, H & E staining was used to evaluate the loss of glands
and inflammatory cell infiltration in gastric tissue of rats (Fig. 2). Rats in the control group showed
Mucosal intact with tightly, abundant and orderly gastric glands. In the model group, rats showed
inherent glands and that in the gastric tissue was missing, part of the mucosa was stripped, and
lymphocytes and neutrophils were infiltrated in the mucosa. Conversely, gastric tissue in the
omeprazole and the ZJP high-dose group, pathological changes were significantly lower than in the
model group in terms of the degree of edema, hyperemia, erosion and atrophy of gastric mucosa was
significantly alleviated Administration of ZJP medium-does group and low-does group exhibited a
moderately reduced severity of inflammatory cell infiltration and other histological injuries.
Effects of ZJP on the proliferation of GES-1 cells
Cell viability was detected by using CCK-8 kit to determine the appropriate concentration of ZJP to
GES-1 cells (Fig. 3). The results showed that ZJP treatment for 24 h potently suppressed cell viability
in a concentration-dependent manner with increasing the concentration (0, 10, 20, 30, 40, 60 and
120 µg/ml), among which, 120 µg/ml of ZJP could significantly inhibit the cell viability compared with
the control group (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 on GES-1 cells. 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.

Induction of JMJD2B mRNA expression by H. pylori in GES-1 cells

GES-1 cells were infected with H. pylori at different MOI (10, 20, 50, 100) through 24 h [40]. As was expected, H. pylori infection at a lower MOI (10, 20, 50) resulted in a dose-dependent induction of JMJD2B mRNA expression, and that at the highest stimulation MOI (MOI = 100), a slight decrease in JMJD2B expression was found (T = 24 h, MOI = 10, 20, 50, 100) (Fig. 4A). When MOI was set at 100, H. pylori was observed to induce cell death in the meantime. Thus, MOI = 50 was chosen to further determine the infection time. By co-culture of H. pylori and GES-1 cells for 0, 6, 12, 24 h, the mRNA expression level of JMJD2B increased in a time-dependent manner, but there was no significant difference between 12 h and 24 h (MOI = 50, T = 0, 6, 12, 24 h) (Fig. 4B). Therefore, MOI = 50 infection was finally chosen for 12 h for further research.

High-Content Analysis Experiments

The number, morphology and viability of GES-1 cells were detected by high-content live-cell imaging assays to directly 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. 5A). In the control and ZJP groups, nucleus and cytoplasm of GES-1 cells possessed a homogenous Hoechst and calcein AM fluorescence. However, there were Nuclear deformations after infecting MOI = 50:1 of H. pylori to GES-1 cells, compared with the control group, especially, 60 µg/mL ZJP could improve morphological alterations. Compared with the control group, cell count in the H. pylori group was significantly decreased (after treatment with MOI = 50:1 of H. pylori for 12 h). Furthermore, the green and red fluorescence of the H. pylori group, compared with the control group, was significantly reduced and increased, respectively, which manifested that the number of living cells was decreased and dead cells were increased. ZJP could certainly boost the green fluorescence and reduce the red fluorescence of GES-1 cells (Fig. 5B-C). These results indicated that ZJP could ameliorate nuclear
morphology and cell proliferation in H. pylori-induced injury and cytotoxicity in GES-1 cells.
ZJP reduced the Serum TNF-a Level in CAG rats and IL-8 mRNA expression in H. pylori infected cells
The serum supernatant of TNF-α was measured to elucidate the expression level of TNF-α in H. pylori-induced CAG rats. Compared with control group, the serum TNF-a level was significantly increased in H. pylori-infected rats. ZJP at 0.63, 1.26 and 2.52 g/kg could all decrease the serum TNF-a level in a dose-dependent manner (Fig. 6A). Omeprazole could also obviously decrease the TNF-a level. The IL-8 mRNA level was significantly increased in H. pylori-infected GES-1 cells. ZJP at 30 µg/mL and 60 µg/mL could all decrease the IL-8 mRNA level compared to control group (Fig. 6B).
ZJP induce JMJD2B and COX-2 expression in H. pylori-infected animal models
Recently, emerging evidence has shown that H. pylori could promote the integration of JMJD2B with COX-2 promoter and then recruit NF-κB to bind on COX-2 promoter, and further to improve COX-2 induction [8]. Here, we explored whether ZJP could reduce gastric mucosa injury via regulating protein expression of JMJD2B and COX-2. IHC was used to determine those expression levels (Fig. 7A-B). The model group showed boosted levels of JMJD2B and COX-2, compared with expression levels in the control group. However, ZJP treatment can obviously decrease those protein expressions. This phenomenon provided the first evidence that ZJP may relieve gastric mucosa injury via the downregulation of JMJD2B and COX-2 activity.
ZJP induced JMJD2B/COX-2/VEGF axis mRNA and protein expression in Vivo
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 [12]. Nevertheless, whether ZJP could interfere with CAG through JMJD2B/COX-2/VEGF axis has not been studied. In the present study, the mRNA and protein expression levels of JMJD2B, COX-2, VEGF, VEGFR1, and VEGFR2 in the model group were significantly elevated compared with the control group (Fig. 8). Compared with the model group, the mRNA and protein expression levels of these genes in the ZJP groups were decreased. High-dose
group of ZJP could reduce the mRNA and protein expression levels, while, the medium- and low-dose group of ZJP exhibited weaker reduction.

ZJP inhibited JMJDB2/COX-2/VEGF axis mRNA and protein expression in vitro

The model of H. pylori induced (MOI = 50:1, 12 h) GES-1 cells was established to further confirm the role of ZJP for relieving H. pylori infected gastric epithelial cell damage through JMJDB2/COX-2/VEGF axis in vitro (Fig. 9). Firstly, the mRNA expressions of JMJDB2, COX-2, VEGF, VEGFR1 and VEGFR2 in GES-1 cells were measured. Consistent with the expression of mRNA and protein in rats, the mRNA and protein levels of JMJDB2, COX-2, VEGF, VEGFR1, and VEGFR2 were significantly increased after the infection of H. pylori and could decrease after treating with ZJP to some degree. This phenomenon further strengthened that ZJP may relieve H. pylori-induced inflammation and gastric mucosa injury via the downregulation of JMJDB2/COX-2/VEGF axis activity.

Discussion

The clinical features of CAG are focused on satiety, belching, abdominal pain and nausea, and weight loss. CAG is a proverbial signal of precancerous lesions of gastric cancer, which has currently been the second most common cause of cancer-related deaths worldwide [13]. In rodent models, H. pylori gavage causes a series of inflammatory reactions in the gastric mucosa, such as inflammatory cell infiltration [14]. Thus, in this study, 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.

In the CAG rat model group, the weight of rats, H. pylori colonization and inflammatory factor, as well as gastric pathological features were ameliorated after treatment with ZJP. Histological analysis showed that loss of glands and inflammatory cell infiltration were reduced to a certain extent after ZJP treatment. In addition, ZJP ameliorated cell viability, morphology changes and proliferation in GES-1 cells. On the basis of the above results, ZJP had the potent possibility to prevent the development of CAG.

Epigenetics plays the vital role in the development and progression of gastric cancer [15]. H. pylori infection induces epigenetic changes, like DNA methylation and histone modification, which plays important roles in oncogenic transformation [16]. JMJDB2 can promote the occurrence and
development of gastric cancer and serves as a potential biomarker in gastric cancer[17]. Recently, emerging evidence has shown that H. pylori could promote the integration of JMJD2B with COX-2 promoter and then recruit NF-κB to bind on COX-2 promoter, and further to improve COX-2 induction [8]. Here, we next explored whether ZJP could reduce gastric mucosa injury via regulating protein expression of JMJD2B and COX-2. 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 prostaglandin-endoperoxide synthase, which 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 [12]. Therefore, controlling JMJD2B/COX-2/VEGF axis might be effective in the treatment of CAG. COX, as is a key rate-limiting enzyme, can promote arachidonic acid convert to prostanoids and thromboxanes in two forms, COX-1 and COX-2 [18]. COX-1 maintains normal function in most tissues. In contrast, COX-2 associated with pain, inflammatory reaction, tumorigenesis and so on. Besides, the expression of COX-2 is known to be increased in the gastric mucosa of H. pylori-infected gastritis patients [19]. In H. pylori-infected gastritis, there is an increased 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 [20]. H. pylori-induced gastritis is associated with VEGF, whose overexpression parallels the increased formation of blood vessels in the gastric mucosa [21]. COX-2 could induce overexpression of VEGF in the gastric tissue colonized by H. pylori. H. pylori infection might be able to induce the expression of COX-2 in gastric tissue, which in turn upregulates the expression of VEGF [22]. In this study, it is found that the expressions of VEGF and its receptor VEGFR1 and VEGFR2 were decreased remarkably after treatment with ZJP both at mRNA and protein level, suggesting that ZJP could decrease the expression of VEGF/VEGFR1/VEGFR2. Furthermore, the level of JMJD2B was higher in H. pylori group than control group, while was reduced notably after treatment with ZJP. In H. pylori-infected cells, the expression of JMJD2B gene was increased with increasing the infection time and plural number in a certain range. In this examination, Proinflammatory genes, IL-8 mRNA expression and TNF-a protein levels were elevated significantly,
showed that, compared with the H. pylori group, ZJP could reduce the expression of IL-8 and TNF-α. In conclusion, ZJP might improve inflammation in CAG rats by inhibiting JMJD2B/COX-2/VEGF axis. Based on the above data, ZJP was confirmed to improve CAG and gastric mucosal injury induced by H. pylori. In addition, the results of the present study suggested that JMJD2B/COX-2/VEGF axis is closely related to the therapeutic and anti-inflammatory effects of ZJP on CAG. JMJD2B/COX-2/VEGF axis plays an important role in H. pylori induced inflammation. These results of this study laid a theoretical foundation for the further study of ZJP in the treatment of chronic atrophic gastritis.

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-mediated inflammation via JMJD2B/COX-2/VEGF axis. The results from this study suggest a potential role of ZJP in treatment of CAG, which need to be further investigated.

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: dissolved in dimethyl sulfoxide; DMEM: Dulbecco’s modified Eagle’s medium; CFU: colony forming units; SD: Sprague-Dawley; SPF: specific pathogen free; HE: 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

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Authors’ contributions
Design of the study: Shihua Wu, Ruilin Wang, Chunmei Bao and Jianzhong Zhang; data collection and analysis: Juling Zhang, Ruisheng Li, Xing Chen, Jiaxia Wen and Tao Yang; drafting the manuscript: Shizhang Wei, Haotian Li, Ying Wei, Sichen Ren; supervising and providing Funding acquisition: Yanling Zhao, HouLin Xia. 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**

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.

**Author details**

1 College of pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China.

2 Department of Pharmacy, The Fifth Medical Center of PLA General Hospital, Beijing, China. 3 Division of Clinical Microbiology, The Fifth Medical Center of PLA General Hospital, Beijing, China. 4 Integrative Medical Center, The Fifth Medical Center of PLA General Hospital, Beijing, China. 5 Center of Disease Control and Prevention, National Institute for Communicable Disease Control and Prevention, Beijing, China. 6 Research Center for Clinical and Translational Medicine, The Fifth Medical Center of PLA General Hospital, Beijing, China.

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Tables

Table 1 Primers used for real-time PCR

| Cells | Primers | Sequence-Forward | Sequence-Reverse |
|-------|---------|------------------|------------------|
| JMJD2B | CCTTCCTGCGGCTAAGATGAC | GGTGGCGAAGTTGGTAGATTCTG |
| COX-2 | AATCTGGCTGCAGGAACACAAC | TGTCTGGAACACTGCTCATCACC |
| VEGF | GCCTTGCCCTTGGCTGCTCTACC | CTTGGTATGATTCTGCCCTCCTC |
| VEGFR1 | TGGTGAGTAAGGAAACGCAMGACGGGC | GTGTGGTTTGGCTGAGCTGTGTTTC |
| VEGFR2 | AAGGAGTCTGTGGCATCTGAAGG | GTGGTGCTCTGTGCTACGAGGTG |
| IL-8 | GCTCTGTGATGAGGTGACGT | TTTCTGTGTGGCAGCAGTGT |
| β-actin | GCCACACACCTTCTACAATGACG | GATAGCACAGCTGGAAGCAACG |

| Rats | Primers | Sequence-Forward | Sequence-Reverse |
|------|---------|------------------|------------------|
| JMJD2B | CTACTACCAGCTGCCGAGGCATTG | CTCTGGCTTAGCTCTCTGGGATAC |
| COX-2 | AGGCTCAGGTGGAGGTGTATC | CGGCACAGACAAAGACTCTTC |
| VEGF | CACGACAAGGAGGAGACGAGAA | GCCACACAGGACGGCTTCTGAG |
| VEGFR1 | GAGCATTCTAGGAGACGGAGATTG | CGACCACACTCTTCACAGACAGAAG |
| VEGFR2 | TGGCAATTCCTCCTCAACAGC | CCTGGTCACAGTCTGGTCACAGT |
| β-actin | CCCCGGAGTACACCTTCTTGG | TCATCCATGGCGAACTGGTGG |

Figures
Macro performance of CAG in rats. (A) Rapid urease test of stomach tissues. (B) Rapid urease test of stomach tissues. (C) Weight of rats, data were shown as mean ± SD; (D) Morphology of rats stomach tissue (n=3).
Figure 2

H & E staining of chronic atrophic gastritis in rats. (A) HE×200; (B) HE×200.
Effect of ZJP on cell viability (10μg/mL-120μg/mL). ##P < 0.01 versus control group, #P < 0.05 versus control group. Data were shown as mean ± SD (n=3).
JMJD2B is induced by *H. pylori* infection. (A) JMJD2B mRNA expression in different MOI of *H. pylori* (MOI=10:1, 20:1, 50:1 and 100:1). (B) JMJD2B mRNA expression in different infection time of *H. pylori*. (T=0, 6, 12 and 24 hours). ##P < 0.01 versus control group. Data were shown as mean ± SD (n=3).
Figure 5

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 ± SD. Control, control group; ZJP, Zuojin Pill. H. pylori, Helicobacter pylori (n=3).
Figure 6

TNF-α and IL-8 expression level in vivo and in vitro. (A) The expression level of TNF-α in rat serum. (B) IL-8 mRNA expression in GES-1 cells. ##P < 0.01 versus control group. **P < 0.01 versus H. pylori infection 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.
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 ± 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).
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 ± 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).