Jianpiyiqi formula ameliorates chronic atrophic gastritis in rats by modulating the Wnt/β-catenin signaling pathway

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Abstract. Jianpiyiqi formula is a Traditional Chinese Medicine (TCM) prescription and is used for the clinical treatment of patients with chronic atrophic gastritis (CAG). The aim of the present study was to examine the underlying mechanisms of Jianpiyiqi formula treatment for CAG via the Wnt/β-catenin signaling pathway. The high-performance liquid chromatography (HPLC) chromatogram of Jianpiyiqi formula was constructed. A CAG rat model induced by N-methyl-N'-nitro-N-nitrosoguanidine and ranitidine was established. The body weight and food intake of the rats was recorded and rat gastric morphology was visually examined. Pathological analysis of rat gastric tissue was also performed. The levels of gastrin (GAS), pepsin (PP), somatostatin (SS) and prostaglandin E_2 (PGE_2) in rat serum were detected using ELISAs. The expression levels of proteins and genes associated with the Wnt/β-catenin signaling pathway were measured via immunohistochemistry and reverse transcription-quantitative PCR. The HPLC chromatogram of Jianpiyiqi formula was determined and as active components, liquiritin and hesperidin were identified from the chromatogram. Compared with the blank group, the body weight and feed intake of the rats were decreased, and gastric mucosal atrophy and inflammation appeared in the model group. Treatment with Jianpiyiqi formula increased the body weight and feed intake of the rats, as well as relieved the gastric atrophy and inflammation. The contents of GAS, PP, SS and PGE_2 were significantly reduced in the model group compared with the blank group. Jianpiyiqi formula significantly increased GAS, PP, SS and PGE_2 levels in serum of rats with CAG. In the model group, Wnt1, β-catenin and cyclin D1 protein expression levels were increased, and glycogen synthase kinase-3β (GSK-3β) protein expression levels were decreased. Jianpiyiqi formula decreased the protein expression levels of Wnt1, β-catenin and cyclin D1 and increased the protein expression levels of GSK-3β. Compared with the blank group, the mRNA expression levels of Wnt1, Wnt5a, β-catenin, cyclin D1 and MMP7 were upregulated, and the mRNA expression levels of GSK-3β were downregulated in the model group. Treatment with Jianpiyiqi formula downregulated the mRNA expression levels of Wnt1, Wnt5a, β-catenin, cyclin D1 and MMP7 and upregulated the mRNA expression levels of GSK-3β. All of the experimental results indicated that Jianpiyiqi formula exerted a therapeutic effect on rats with CAG and inhibited the activation of the Wnt/β-catenin signaling pathway. Thus, Jianpiyiqi formula, as an effective TCM prescription for treating patients with CAG, may be more widely used in the clinic.

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Abbreviations: CAG, chronic atrophic gastritis; HPLC, high-performance liquid chromatography; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; GSK-3β, glycogen synthase kinase-3β; GC, gastric cancer; TCM, Traditional Chinese Medicine; IHC, immunohistochemistry; qPCR, quantitative PCR; GAS, gastrin; PP, pepsin; SS, somatostatin; PGE_2, prostaglandin E2; PLGC, precancerous lesion of gastric cancer

Introduction

Chronic atrophic gastritis (CAG) is a common digestive-tract disease characterized by gastric gland atrophy, decreased number of gastric glands and chronic inflammation (1). The typical clinical symptoms of patients with CAG include reduced appetite, nausea and acid reflux (2). *Helicobacter pylori* (*H. pylori*) infection, smoking, drinking and immune abnormalities may lead to the development of CAG (3). Furthermore, CAG is a precancerous state of gastric cancer (GC). Therefore, effective and timely treatment of CAG may help prevent the occurrence of GC (4,5). Proton pump inhibitors, antibiotics, folic acid, vitamins and gastric mucosal protective agents are frequently used as conventional treatments for patients with CAG (6). However, there is a lack of specific drugs that may reverse the atrophy of patients with CAG in clinical practice.

Jianpiyiqi formula, also called Weiwei No. 1, is a type of Traditional Chinese Medicine (TCM) formula and is used to

treat CAG in clinical settings (7). Jianpiyiqi formula is not a commercial product and there is currently a lack of effective commercial formulas for treating CAG in the clinic. Jianpiyiqi formula is composed of 12 Chinese medicinal herbal components/ingredients: Dangshen, Baizhu, Fuling, Gancao, Chenpi, Banxia, Muxiang, Sharen, Ezhu, Baihuasheshecao, Yunmushi and Yujin (Table I). It is mainly used for patients with CAG with a weak spleen and stomach, qi stagnation and blood stasis according to the theory of TCM (8). However, the pharmacological mechanisms of action of the Jianpiyiqi formula for improving CAG remained elusive. In the present study, a CAG rat model induced by N-methyl-N'-nitro-N-nitrosoguanidine and ranitidine was constructed to assess the molecular pharmacology of the Jianpiyiqi formula.

The gastric mucosa of patients with CAG is continuously damaged by various pathogenic factors and it cannot be repaired within a sufficient time period (1). It has been reported that the damage repair and proliferation of gastric epithelial cells are closely associated with the Wnt/β-catenin signaling pathway (9-12). *H. pylori* infection is associated with the expression of β-catenin in CAG with intestinal metaplasia (13). GC has been associated with increased expression of the Wnt/β-catenin signaling pathway, which may promote abnormal proliferation of gastric gland cells and then induce tumors (14). To date, a limited number of studies have investigated the association between CAG and the Wnt/β-catenin signaling pathway (15). Therefore, the aim of the present study was to clarify the pharmacological mechanisms of action of Jianpiyiqi formula in the treatment of CAG via the Wnt/β-catenin signaling pathway.

Materials and methods

Reagents. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from Tokyo Chemical Industry Co., Ltd. Ranitidine hydrochloride was purchased from Shanghai Hengshan Pharmaceutical Co., Ltd. Liquiritin, hesperidin, lobetyolin, curdione, costunolide and atractylenolide II were purchased from Chengdu Must Bio-Technology Co., Ltd. The H&E staining kit and prostaglandin E_2 (PGE_2) ELISA kit (cat. no. H099-1) were purchased from Nanjing Jiancheng Bioengineering Institute. Methanol and acetonitrile were purchased from EMD Millipore. Ethanol, acetic acid, xylene and formaldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd. The gastrin (GAS), pepsin (PP), and somatostatin (SS) ELISA kits were purchased from Elabscience, Inc. Wnt1 was purchased from Abcam Co., Ltd. The β-catenin, glycogen synthase kinase-3β (GSK-3β) and cyclin D1 antibodies and the goat anti-rabbit and anti-mouse IgG secondary antibodies were purchased from Cell Signaling Technology, Inc. The PCR primers for Wnt1, Wnt5a, β-catenin, GSK-3β, cyclin D1, MMP7 and GAPDH were produced by Nanjing Genscript Biological Technology Co., Ltd. TRIzol® reagent, Maxima First Strand cDNA Synthesis Reverse transcription PCR kit and Maxima SYBR Green/ROX quantitative PCR kit were obtained from Thermo Fisher Scientific, Inc.

Preparation of Jianpiyiqi formula water extract. All of the medicinal herbs used to prepare Jianpiyiqi formula were purchased from Jiangsu Province Integrated Chinese and

Western Medicine Hospital (Nanjing, China). All had their identity confirmed by Professor Shihui Qian (Chinese Medicine Plant Resources Laboratory, Jiangsu Province Institute of Traditional Chinese Medicine) and stored in a dry and ventilated laboratory cabinet. One batch weighed 146 g and consisted of 12 Chinese medicinal herbs/ingredients: Dangshen, Baizhu, Fuling, Gancao, Chenpi, Banxia, Muxiang, Sharen, Ezhu, Baihuashshecao, Yunmushi and Yujin (Table I). The 146-g herbal mixture was placed in a round-bottomed flask and soaked for 0.5 h in 1.46 liter water. The water containing the herbs was boiled for 0.5 h continuously. The water extract was naturally cooled to room temperature was then filtered and collected for the first time. Subsequently, 0.73 liter water was added to the round-bottomed flask, followed by continuous boiling for 0.5 h. The water extract was naturally cooled to room temperature was filtered and collected for a second time. The twice collected water extracts were concentrated to 48.7 ml (equivalent to a drug concentration of 3 g/ml) using a vacuum rotary evaporator and stored at -80°C in an ultra-low temperature refrigerator.

Animals. A total of 20 male Sprague Dawley rats [body weight, 200±20 g; age, 6-8 weeks; animal quality certificate no. SCXK (Su) 2019-0001] were purchased from the Laboratory Animal Center of Nantong University. All rats were kept under a 12-h light/dark cycle and housed at the Laboratory Animal Center of Jiangsu Province Institute of Traditional Chinese Medicine (Nanjing, China). The animals had *ad libitum* access to food and water in an environment with a temperature of 20°C and a humidity of 50%. Animal experimental operations complied with the Guidelines of Welfare and Ethics of Laboratory Animals (issued by the General Administration of Quality Supervision, Inspection and Quarantine of the P.R. China) and the Guide for the Care and Use of Laboratory Animals (issued by the US National Institutes of Health). The animal experiment of the present study was approved by the Ethics Committee of Jiangsu Province Integrated Chinese and Western Medicine Hospital (Nanjing, China; approval no. AEWC-20160810-12).

Establishment of CAG rat model. The 20 male Sprague Dawley rats were randomly divided into a blank group, model group, positive control drug group (2.7 mg/kg folate and 13.5 mg/kg teprenone) and Jianpiyiqi formula group (13.2 g/kg crude drug). A total of 5 rats were in each group. The rats in the CAG model groups were freely provided with drinking water containing 150 μg/ml MNNG and with food containing 0.03% ranitidine. The construction of the CAG rat model lasted 24 weeks. Subsequently, the rats were administered the drugs via gavage in positive control drug group and Jianpiyiqi formula group. Rats in the blank group and model group were gavaged with sterile water. The experiment was terminated after 8 weeks of treatment. Body weight and daily food intake were recorded. All rats were anesthetized by intraperitoneal injection of 30 mg/kg (body weight) sodium pentobarbital. Subsequently, 20 ml blood was collected from the abdominal aorta of the live anesthetized rats. After the blood was collected completely, the rats died naturally due to the exsanguination.

High-performance liquid chromatography (HPLC) analysis. Liquiritin, hesperidin, lobetyolin, curdione, costunolide and

Table I. Components of Jianpiyiqi formula.

Chinese name	Latin or English name	Plant parts	Amount (g)	Proportion w/w (%)
Dangshen	*Codonopsis pilosula* (Franch.) Nannf.	Root	15	10.27
Baizhu	*Atractylis macrocephala* (Koidz.) Hand.-Mazz.	Rhizoma	10	6.85
Fuling	*Poria cocos* (Schw.) Wolf	Sclerotia	10	6.85
Gancao	*Glycyrrhiza uralensis* Fisch	Rhizoma	3	2.05
Chenpi	*Citrus reticulata* Blanco	Peel	6	4.11
Banxia	*Pinellia ternata* (Thunb.) Makino	Tuber	6	4.11
Muxiang	*Aucklandia Lappa* Decne.	Root	10	6.85
Sharen	*Amomum villosum* Lour.	Fruit	6	4.11
Ezhu	*Curcuma aeruginosa* Roxb.	Rhizome	10	6.85
Baihuasheshecao	*Oldenlandia diffusa* (Willd.) Roxb.	Whole herb	30	20.55
Yujin	*Curcuma wenyujin* Y. H. Chen & C. Ling	Root tuber	10	6.85
Yunmushi[a]	Mica	NA	30	20.55
Total	NA	NA	146	100.00

[a]Yunmushi is a type of ore and may be used as medicine in Traditional Chinese Medicine. NA, not applicable.

atractylenolide II were prepared to a solution of 1 mg/ml in methanol. The six compounds were mixed at equal proportions for HPLC analysis. Jianpiyiqi formula water extraction solution (3 g/ml crude drug) was diluted to 1 g/ml solution with methanol for HPLC analysis. All samples were separated using a C_{18} chromatographic column (5 μm; 250x4.6 mm) in a Waters 2695 HPLC system and detected in a Waters 2489 detector (Waters Corp.). Mobile phrases were water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B). The mobile phase elution procedure was as follows: Solvent A was varied from 95 to 85% from 0 to 30 min, solvent A was varied from 85 to 70% from 30 to 45 min, solvent A was varied from 70 to 30% from 45 to 55 min and solvent A remained at 30% from 55 to 60 min. The column temperature was 30˚C, the sample loading volume was 25 μl, the flow rate was 1 ml/min and the detection wavelength was 230 nm.

H&E staining. The gastric tissues of the rats were fixed in 4% formalin at 25˚C for 24 h and underwent routine ethanol dehydration and paraffin embedding. The embedded tissues were cut into 5-μm slices using a Leica Biosystems RM2245 wheel slicer (Leica Microsystems GmbH). The slices were baked in an oven at 60˚C for 2 h and were then dewaxed and rehydrated. Subsequently, the slices were stained with hematoxylin (Nanjing Jiancheng Bioengineering Institute) at 25˚C for 3 min and eosin (Nanjing Jiancheng Bioengineering Institute) at 25˚C for 30 sec. The stained slices were dehydrated with ethanol, cleared with xylene, mounted on glass slides with neutral balsam and covered with coverslips. The rat gastric tissues were imaged using the Olympus CKX-41 inverted light microscope (Olympus Corporation) at x100 magnification. The atrophy and inflammation of gastric glands were scored according to the visual analogue scale of the new Sydney system (16,17). The pathology scoring was carried out independently by two examiners, and the mean value was finally taken.

ELISA. The contents of GAS (cat. no. E-EL-R0472c; Elabscience, Inc.), PP (cat. no. E-EL-R0719c; Elabscience, Inc.), SS (cat. no. E-EL-R0914c; Elabscience, Inc.) and PGE_2 (cat. no. H099-1; Nanjing Jiancheng Bioengineering Institute) in rat serum were detected according to the manufacturer's protocols for the ELISA kits. The rat serum was added to the coated 96-well microplate. The antibodies and HRP-conjugated streptomycin were added to the plate. Subsequently, the 96-well microplate was sealed with parafilm and incubated at 37˚C for 1 h. The solution in the wells was then completely discarded. The microplate was washed five times with washing buffer and incubated with diaminobenzidine (DAB) chromogenic reagent at 37˚C for 10 min, and reaction termination solution was then immediately added. The optical density (OD) value of all wells was measured at the wavelength of 450 nm using a Tecan M200 pro automatic microplate reader (Tecan Group, Ltd.). According to the standard curve linear regression equation and the OD values of the samples in each group, the corresponding sample concentration was calculated.

Immunohistochemistry (IHC). The paraffin-embedded stomach tissues were cut into 3-μm slices. After the slices were baked, the slides were submersed in citrate unmasking solution for antigen retrieval and boiled at 100˚C for 5 min. The slices were incubated with 3% hydrogen peroxide for 10 min and blocked with 0.5 ml blocking solution for 1 h at room temperature. The slides were coated with 0.2 ml Wnt1 (1:300 dilution; cat. no. ab15251; Abcam), β-catenin (1:300 dilution; cat no. 7074; Cell Signaling Technology, Inc.), GSK-3β (1:300 dilution; cat. no. 7074; Cell Signaling Technology, Inc.) and cyclin D1 (1:300 dilution; cat no. 7074; Cell Signaling Technology, Inc.) primary antibodies and incubated overnight at 4˚C. The slices were then incubated with 0.2 ml HRP-conjugated secondary antibody (1:500 dilution; cat no. 7074; Cell Signaling Technology, Inc.) in a humidified chamber at 25˚C for 1 h. The slides were incubated with 0.5 ml DAB solution for 5 min and the sections

Table II. Sequences of primers used for quantitative PCR.

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Product length (bp)
Wnt1	AACAGTAGTGGCCGATGGTG	GGGTTCTGTCGGATCAGTCG	143
Wnt5a	CCAGTACCAGTTCCGGCATC	GCCTATTTGCATCACCCTGC	82
β-catenin	CAGATCCCATCCACGCAGTT	TCTGTGACGGTTCAGCCAAG	70
Glycogen synthase kinase-3β	TCCTTATCCCTCCTCACGCT	GATGCAGAAGCGGCGTTATT	118
Cyclin D1	CAAGTGTGACCCGGACTGC	GACCAGCTTCTTCCTCCACTT	137
MMP7	GATGGGCCAGGAAACACTCT	TCAAAGTGAGCATCTCCGCC	71
GAPDH	GTGAAGGTCGGTGTGAACGG	CCACTTTGTCACAAGAGAAGGC	76
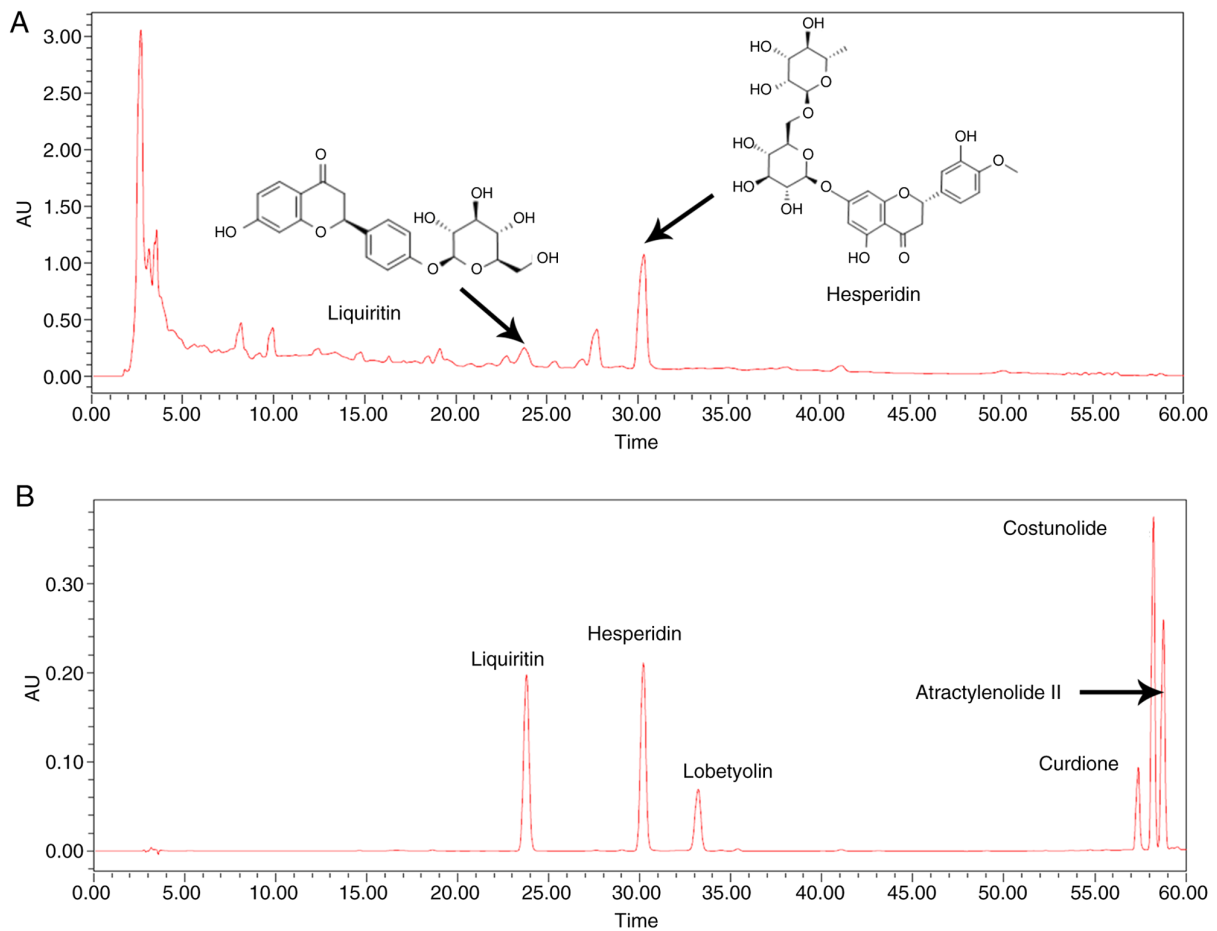
Figure 1. Generation of the HPLC chromatogram of Jianpiyiqi formula. (A) Water extract of Jianpiyiqi formula (contained 1 g/ml crude drug) was analyzed via HPLC. (B) The mixture of six reference compounds (liquiritin, hesperidin, lobetyolin, curdione, costunolide and atractylenolide II) was analyzed via HPLC. HPLC, high-performance liquid chromatography; AU, absorption units.

were counterstained with hematoxylin at 25˚C for 30 sec. The slides were mounted with neutral balsam mounting medium and covered using coverslips. The rat gastric tissues were imaged using the Olympus CKX-41 inverted light microscope (Olympus Corporation) at x100 magnification. Staining of the IHC sections was determined using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Reverse transcription-quantitative (RT-q)PCR. The total RNA was extracted from rat gastric tissues using TRIzol reagent. The extracted RNA was reverse transcribed into the complementary DNA using the Maxima First Strand cDNA Synthesis Reverse transcription PCR kit according to the manufacturer's protocol. The reaction conditions were 25˚C for 10 min, 50˚C for 15 min and 85˚C for 5 min. The relative mRNA expression levels of Wnt1, Wnt5a, β-catenin, GSK-3β, cyclin D1 and MMP7 were detected using Applied Biosystems StepOnePlus fluorescence quantitative PCR equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the Maxima SYBR Green/ROX quantitative PCR kit according to the manufacturer's protocol. The thermal cycling conditions were: One cycle of 95˚C for 15 min, then 40 cycles of 95˚C
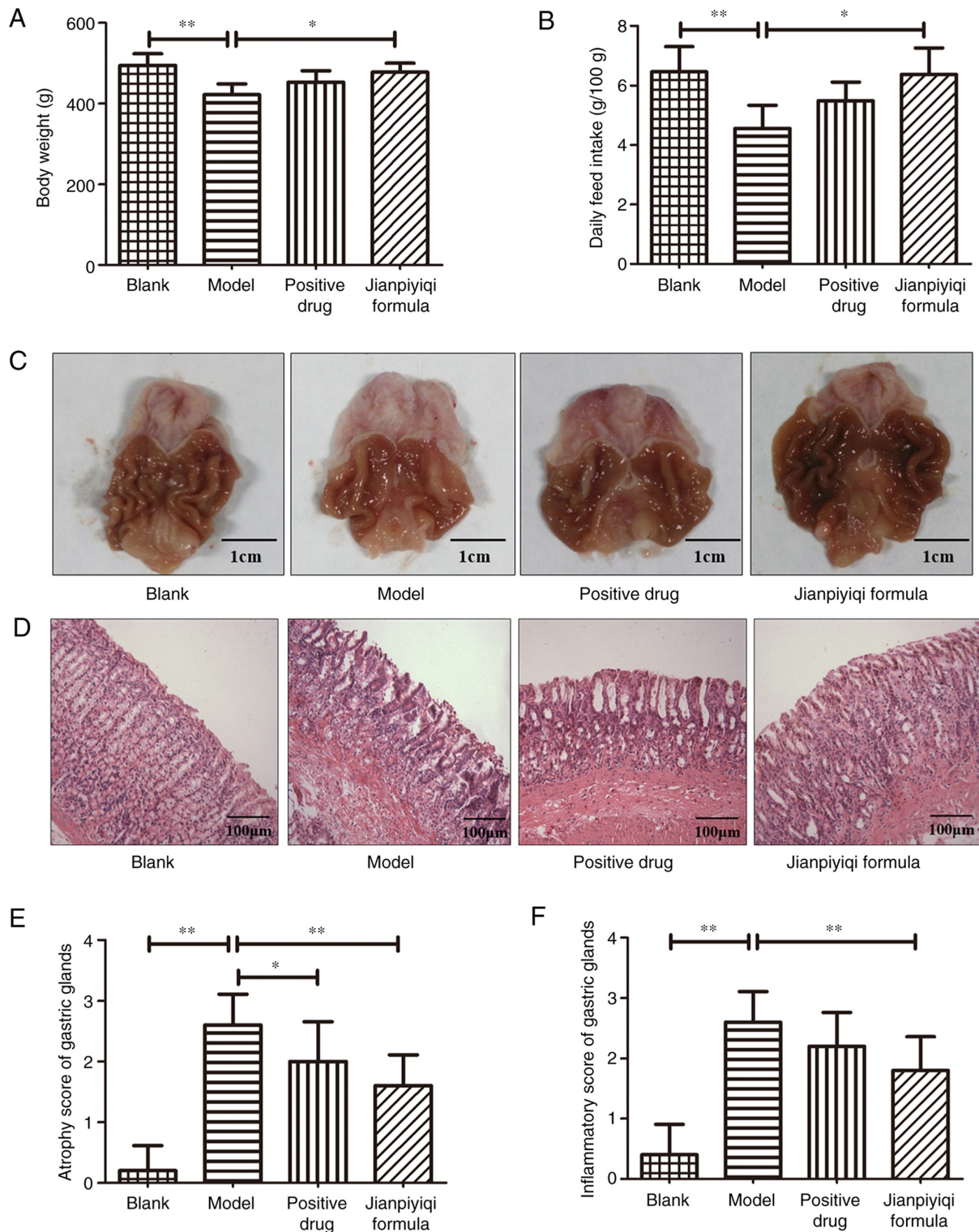
Figure 2. Effect of Jianpiyiqi formula on body weight, feed intake, gastric morphology and pathology of rats with CAG. (A) Body weight and (B) daily feed intake were measured using an electronic scale at the end of the animal experiment. (C) For a representative sample in each group, the macroscopic view of the gastric mucosa was captured with a camera (scale bar, 1 cm). (D) Rat gastric tissues were fixed and stained with H&E. The pathologically stained sections were imaged with a microscope (scale bar, 100 μm). (E) Atrophy score and (F) inflammatory score were measured according to the visual analogue scale of the new Sydney system. *P<0.05 and **P<0.01 vs. model group. CAG, chronic atrophic gastritis.

for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The relative mRNA expression was analyzed and calculated using the $2^{-\Delta\Delta Cq}$ method (18), with the housekeeping gene GAPDH used as a control gene. The sequences of all qPCR primers are listed in Table II.

Statistics analysis. Values are expressed as the mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. The data were analyzed using SPSS 22.0 software
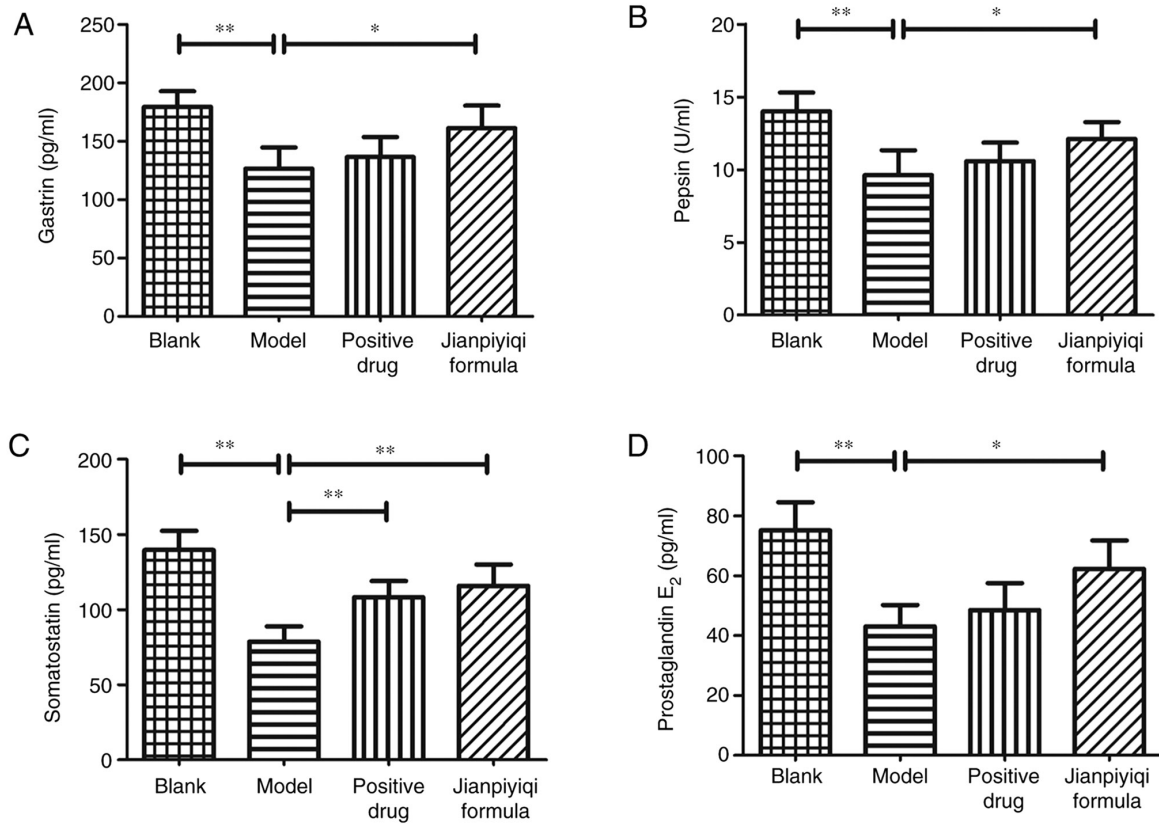
Figure 3. Effect of Jianpiyiqi formula on GAS, PP, SS and PGE$_2$ in serum of rats with CAG. At the end of the animal experiment, the rat serum was collected and used to detect the contents of (A) GAS, (B) PP, (C) SS and (D) PGE$_2$ according to the ELISA protocol. *P<0.05 and **P<0.01 vs. model group. CAG, chronic atrophic gastritis; GAS, gastrin; PP, pepsin; SS, somatostatin; PGE$_2$, prostaglandin E$_2$.

(IBM Corp.) and graphs were plotted using GraphPad Prism 5.0 software (GraphPad Software, Inc.).

Results

HPLC chromatogram of Jianpiyiqi formula. The TCM prescription Jianpiyiqi formula is an extract of 12 Chinese medicinal herbs/ingredients (Table I). The chemical composition of the formula is complex. In the present study, Jianpiyiqi formula was prepared and its HPLC chromatogram was examined (Fig. 1A). The HPLC analysis indicated that relatively high concentrations of liquiritin and hesperidin were present in the extract (Fig. 1A). HPLC results showed hesperidin was the major active component of Jianpiyiqi formula. The six compounds liquiritin, hesperidin, lobetyolin, curdione, costunolide and atractylenolide II were used as controls to identify the pharmaceutically relevant components of the formula (Fig. 1B).

Effect of Jianpiyiqi formula on body weight, food intake, gastric morphology and pathology of rats with CAG. After the establishment of the CAG rat model induced by MNNG and ranitidine, the therapeutic effect of Jianpiyiqi formula on CAG was evaluated. The experimental results demonstrated that compared with the blank group, the body weight and feed intake of the rats were decreased, and gastric mucosal atrophy and inflammation appeared in the model group. Jianpiyiqi formula significantly improved the body weight and the food intake of the rats (Fig. 2A and B). All rat stomachs were cut along the side of the

large curve and gastric morphology was imaged using a camera. Compared with that in the blank group, the stomach tissue in the model group appeared slightly paler and had fewer gastric folds. Furthermore, Jianpiyiqi formula and the positive control drugs improved gastric morphology (Fig. 2C). Subsequently, the rat gastric tissue was stained with H&E for pathological observation. Atrophy and inflammation of gastric tissues were scored according to the new Sydney system (16). Marked gastric atrophy and inflammation were observed in the CAG model group. After Jianpiyiqi formula treatment, the atrophy and inflammation of gastric glands was improved. Furthermore, the positive control drugs significantly improved gastric atrophy (Fig. 2D-F).

Effect of Jianpiyiqi formula on GAS, PP, SS and PGE$_2$ in rats with CAG. Next, the gastric mucosa-related factors were detected to evaluate the curative effect of Jianpiyiqi formula on CAG. The contents of GAS, PP, SS and PGE$_2$ in rat serum were measured via ELISAs. Compared with those in the blank group, the contents of GAS, PP, SS and PGE$_2$ were decreased in the CAG rat model group. It was indicated that the positive control drugs significantly increased the content of SS. In addition, Jianpiyiqi formula significantly improved the secretion of gastric mucosa-related factors (GAS, PP, SS and PGE$_2$) in the serum of CAG rats (Fig. 3).

Effect of Jianpiyiqi formula on the protein expression levels of Wnt1, β-catenin, GSK-3β and cyclin D1 in rats with CAG. The Wnt/β-catenin signaling pathway was previously reported
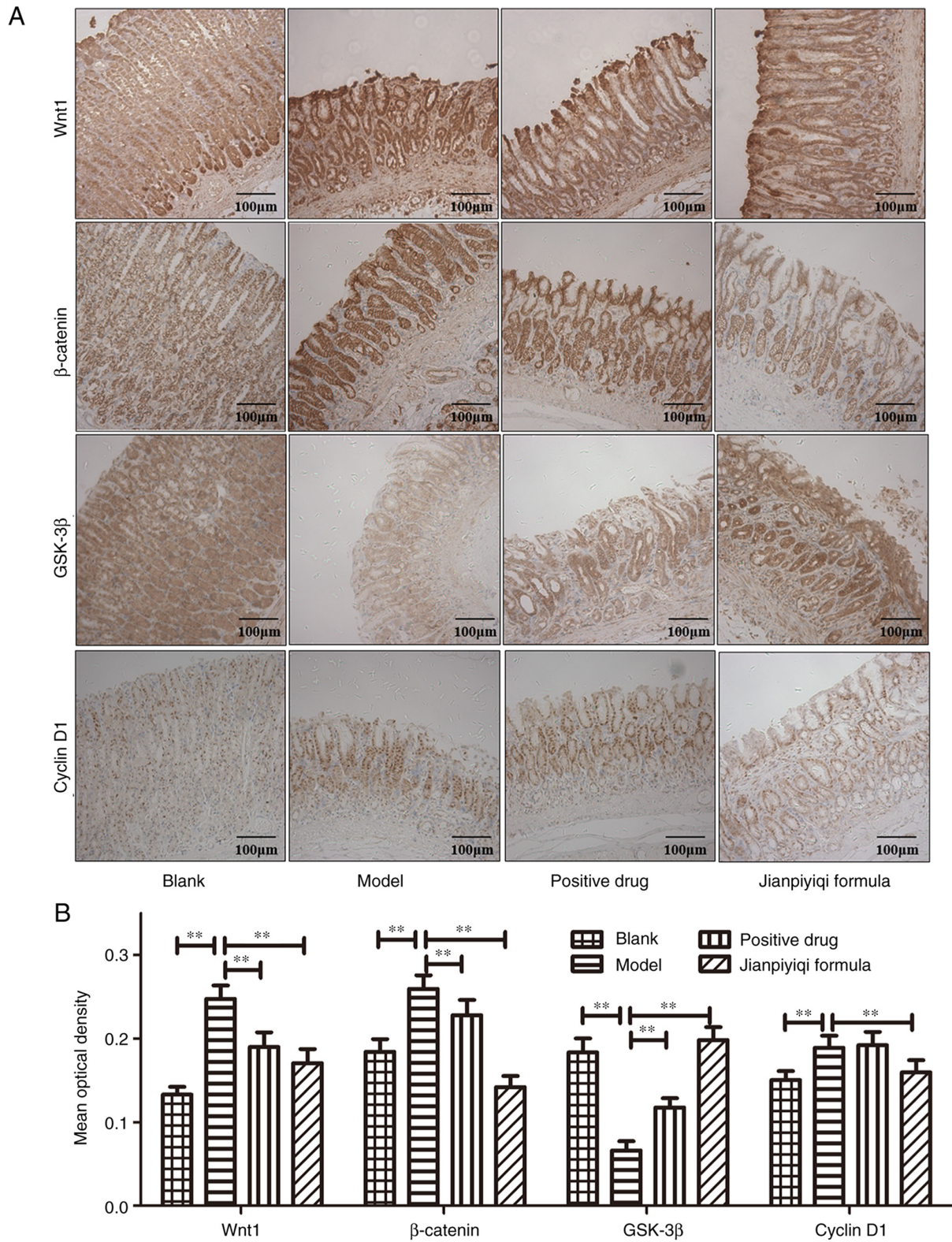
Figure 4. Effect of Jianpiyiqi formula on protein expression levels of the Wnt/β-catenin signaling pathway in the stomach tissue of rats with CAG. (A) Paraffin-embedded rat gastric tissues were used to detect the *in situ* protein expression levels of Wnt1, β-catenin, GSK-3β and cyclin D1 using IHC (scale bar, 100 μm). (B) Mean optical density of rat gastric tissue IHC sections was analyzed using Image-Pro Plus 6.0 software. [**]P<0.01 vs. model group. CAG, chronic atrophic gastritis; IHC, immunohistochemistry; GSK-3β, glycogen synthase kinase-3β.

to be closely associated with the efficacy of Jianpiyiqi formula in rats with CAG (13). The *in situ* protein expression levels of Wnt1, β-catenin, GSK-3β and cyclin D1 in gastric tissues were detected via IHC. The results indicated that compared with the blank group, the protein expression levels of Wnt1, β-catenin and cyclin D1 were increased and the protein expression of GSK-3β was decreased in the model group. Furthermore, compared with that in the model group, the protein expression
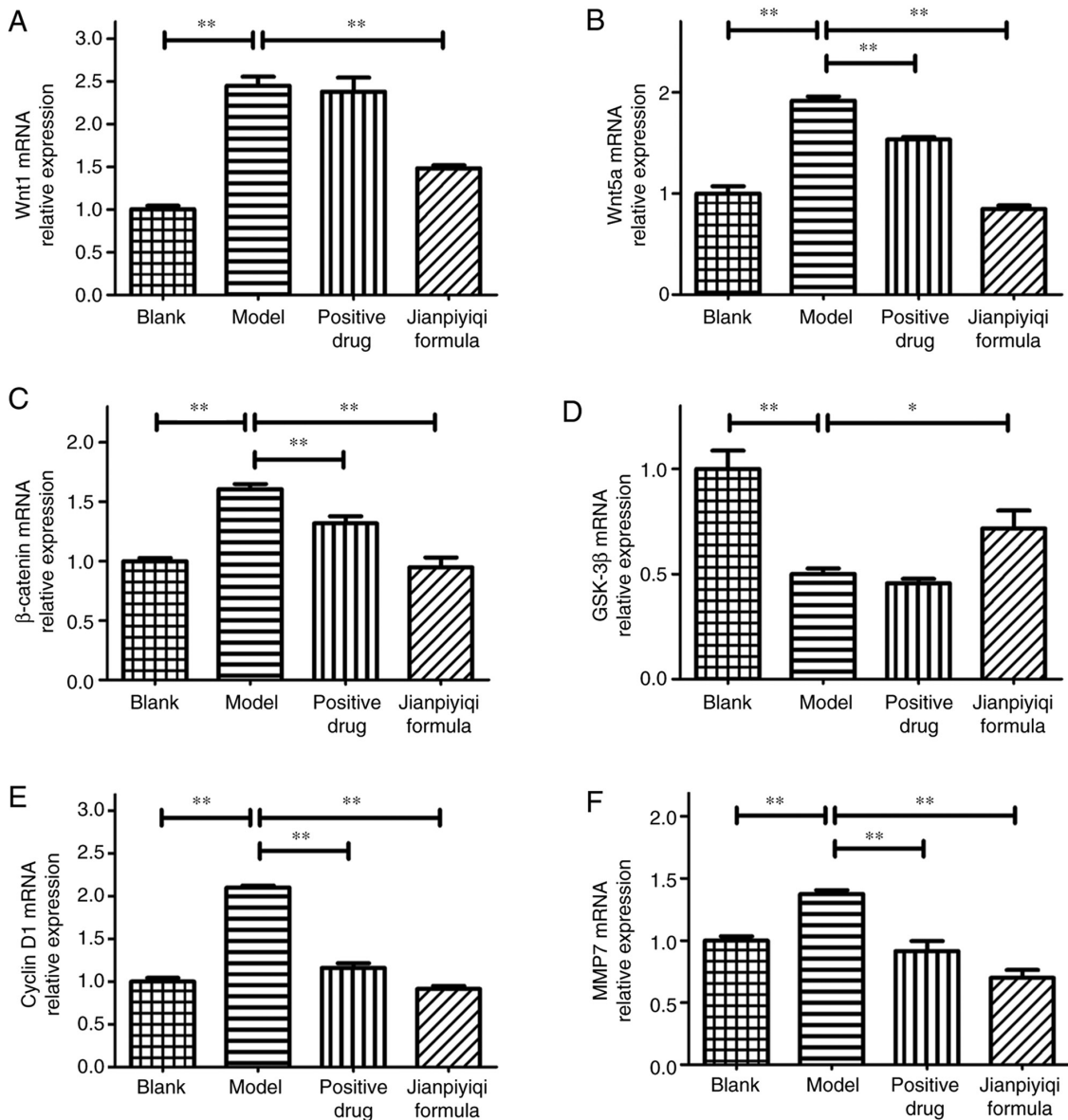
Figure 5. Effect of Jianpiyiqi formula on gene expression levels of the Wnt/β-catenin signaling pathway in stomach tissues of rats with CAG. The total RNA of rat gastric tissue was extracted and specific mRNAs were determined via reverse transcription-quantitative PCR. Relative mRNA expression levels of (A) Wnt1, (B) Wnt5a, (C) β-catenin, (D) GSK-3β, (E) cyclin D1 and (F) MMP7. $^*P<0.05$ and $^{**}P<0.01$ vs. model group. CAG, chronic atrophic gastritis; GSK-3β, glycogen synthase kinase-3β.

of GSK-3β was significantly increased and the protein expression levels of Wnt1 and β-catenin were significantly decreased in the positive control drug group. It was also observed that Jianpiyiqi formula significantly decreased the protein expression levels of Wnt1, β-catenin and cyclin D1 and increased the expression of GSK-3β in rats with CAG (Fig. 4).

Effect of Jianpiyiqi formula on gene expression levels of Wnt1, Wnt5a, β-catenin, GSK-3β, cyclin D1 and MMP7 in rats with CAG. The relative mRNA expression levels of Wnt1, Wnt5a, β-catenin, GSK-3β, cyclin D1 and MMP7 in rat gastric tissues were detected via RT-qPCR. The results demonstrated that the gene expression levels of Wnt1, Wnt5a, β-catenin, cyclin D1 and MMP7 were significantly increased and the gene expression levels of GSK-3β were significantly decreased in the CAG model group as compared with those in the blank group. In the positive

control drug group, no evident change in the mRNA expression of GSK-3β was observed, while the mRNA expression levels of Wnt5a, β-catenin, MMP7 and cyclin D1 were decreased compared with those in the model group. Furthermore, Jianpiyiqi formula significantly decreased the gene expression of Wnt1, Wnt5a, β-catenin, cyclin D1 and MMP7 and significantly increased the gene expression of GSK-3β compared with that in the model group (Fig. 5). Therefore, based on the results of the IHC and RT-qPCR experiments, it was suggested that Jianpiyiqi formula significantly inhibited the Wnt/β-catenin signaling pathway in rats with CAG.

Discussion

CAG is frequently accompanied by intestinal metaplasia and intraepithelial neoplasia, which are considered precancerous
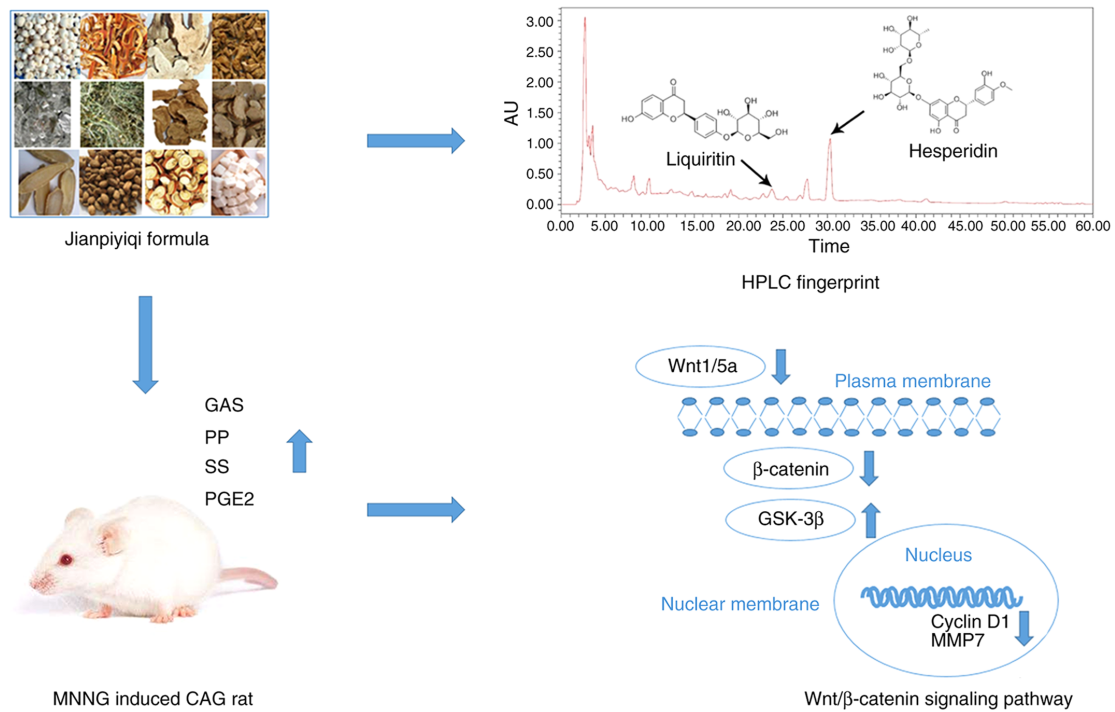
Figure 6. Proposed pharmacological mechanisms of Jianpiyiqi formula in the treatment of CAG via the Wnt/β-catenin signaling pathway. The HPLC chromatogram of Jianpiyiqi formula water extract was established. Jianpiyiqi formula relieved gastric atrophy and inflammation, as well as significantly increased GAS, PP, SS and PGE$_2$. Jianpiyiqi formula exerted a therapeutic effect on rats with CAG and inhibited the Wnt/β-catenin signaling pathway. CAG, chronic atrophic gastritis; AU, absorption units; GAS, gastrin; PP, pepsin; SS, somatostatin; PGE$_2$, prostaglandin E$_2$.

lesions of GC (PLGC) (19). Therefore, it is urgent to identify novel and effective treatments for CAG. In recent years, significant progress has been made in the treatment of patients with CAG using TCM (20,21). Previous *in vivo* studies have indicated that TCM formulations are able to improve gastric mucosal atrophy, including Modified Sijunzi Decoction (22), Weiqi Decoction (23), Banxia Xiexin Decoction (24) and Huangqi Jianzhong Tang (25). Jianpiyiqi formula is a TCM prescription that may be used to effectively treat CAG in the clinic (7,8). However, the mechanisms of action of Jianpiyiqi formula in the treatment of CAG have remained to be elucidated. The mixture of pharmacologically active components in the water extract of the formula is complex. To the best of our knowledge, no previous study reported on the analysis of Jianpiyiqi formula by HPLC. Thus, the present study first constructed the HPLC chromatogram of Jianpiyiqi formula for drug quality control and subsequent investigation of its pharmacological mechanisms. Liquiritin and hesperidin were identified and major components of the formula. Although previous studies suggested that liquiritin and hesperidin have anti-inflammatory and tumor preventive properties (26,27), the two compounds do not represent all components of the formula. The effects of liquiritin and hesperidin on CAG need to be verified by further experiments in the future.

It has been reported that the Wnt signaling pathway serves an important role in the proliferation of gastric mucosal epithelium (12). Furthermore, the proliferation of gastric mucosal epithelial cells in patients with CAG is inhibited or delayed (1). Numerous studies have indicated that the Wnt/β-catenin signaling pathway is related to GC (11,14). CAG is a gastric precancerous disease, which is different from GC. However, limited studies have investigated the relationship between CAG and the Wnt/β-catenin signaling pathway (13,15). Therefore, it is worthwhile investigating whether the mechanisms of action of Jianpiyiqi formula in the treatment of CAG involve the Wnt/β-catenin signaling pathway.

In the present study, the MNNG-induced CAG rat model was established (28) and was used to evaluate the effect of Jianpiyiqi formula on CAG. It was indicated that the formula significantly improved gastric atrophy and reduced stomach inflammation. Furthermore, GAS, PP, SS and PGE$_2$ are closely associated with the development of CAG (1,4,29-31). GAS was able to promote the proliferation of gastric mucosal cells and increase gastric acid and pepsinogen secretion. Furthermore, PP promoted food digestion, while SS had a strong inhibitory effect on gastric acid secretion. It has been reported that PGE$_2$ is able to regulate the relaxation and contraction of blood vessels and exert a protective effect on gastric mucosal cells (29-31). The present study demonstrated that Jianpiyiqi formula significantly increased the contents of GAS, PP, SS and PGE$_2$ in rats with CAG.

The canonical Wnt/β-catenin signaling pathway serves an important role in regulating gastric cell proliferation (32,33). Wnt ligands specifically bind to the Frizzled receptors and then activate the disheveled family proteins. The activation of disheveled proteins leads to the inactivation of GSK-3β. As a result of the accumulation and migration of β-catenin from the cytoplasm to the nucleus, β-catenin is able to upregulate the expression of related genes (12). Furthermore, Wnt signal transduction may be closely associated with the pathogenesis of CAG. The present study reported that the Wnt/β-catenin signaling pathway was upregulated in the CAG rat model.

It was also identified that Jianpiyiqi formula inhibited the activation of the Wnt/β-catenin signaling pathway in CAG. Due to the consistency of rat modelling, the error of rat gene expression in the group was small. There is currently no gold standard for the treatment of CAG in the clinic. According to the specific symptoms of patients with CAG, corresponding treatment drugs are given. Folate, as a vitamin, is able to improve gastric mucosal damage and teprenone is a gastric mucosal protective agent (34,35). In the present study, the positive control drugs selected for the rat model of CAG were folate and teprenone, as these have a certain effect to improve gastric atrophy according to previous experimental results (23,25). Furthermore, the positive control drugs inhibited the expression levels of Wnt signal-related genes. Collectively, it was indicated that the therapeutic effect of Jianpiyiqi formula on CAG was more potent compared with that of the positive control drugs.

In conclusion, the present study demonstrated that Jianpiyiqi formula significantly ameliorated gastric gland atrophy and gastric mucosal inflammation in rats with CAG. Furthermore, Jianpiyiqi formula was demonstrated to inhibit the Wnt/β-catenin signaling pathway; the experimental evidence and proposed pathway are summarized in Fig. 6. According to the present results, the Wnt signaling pathway may serve an important role in the transition process from CAG to PLGC. However, these results should be further confirmed by additional experiments in the future. Overall, it was suggested that due to its promising efficacy, Jianpiyiqi formula may be more widely used in the clinical treatment of CAG.

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Not applicable.

Availability of data and materials

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

Author's contributions

The animal experiments were performed by ZY and TX. HPLC analysis was conducted by ZA. Immunohistochemistry and RT-qPCR were performed by WC and YX. ZY and FZ were the major contributors to the design of the study and writing of the manuscript. All authors confirm the authenticity of all the raw data and all authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiment of the present study was approved by the Ethics Committee of Jiangsu Province Integrated Chinese and Western Medicine Hospital (Nanjing, China; approval no. AEWC-20160810-12).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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