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ORIGINAL ARTICLE

Basic Study

BanXiaXieXin decoction treating gastritis mice with drug-resistant *Helicobacter pylori* and its mechanism

Xiao-Hua Li, Jia-Yin Xu, Xue Wang, Li-Juan Liao, Liang Huang, Yan-Qiang Huang, Zeng-Feng Zhang

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is the main pathogen that causes a variety of upper digestive diseases. The drug resistance rate of *H. pylori* is increasingly higher, and the eradication rate is increasingly lower. The antimicrobial resistance of *H. pylori* is an urgent global problem. It has been confirmed that Banxia Xiexin decoction (BXXXT) demonstrates the effects of treating gastrointestinal diseases, inhibiting *H. pylori* and protecting gastric mucosa. The purpose of the present study is to further explore the therapeutic effects of BXXXT on drug-resistant *H. pylori*.

AIM

To confirm that BXXXT demonstrates therapeutical effects *in vivo* and *in vitro* on gastritis mice with drug-resistant *H. pylori* and explain its mechanism to provide an experimental basis for promoting the application of BXXXT.

METHODS

The aqueous extract of BXXXT was gained by water decocting method. The inhibitory effect of the aqueous extract on *H. pylori* was detected by dilution *in vitro*; drug-resistant *H. pylori* cells were used to build an acute gastritis model *in*

vivo. Thereafter, the model mice were treated with the aqueous extract of BXXXT. The amount of *H. pylori* colonization, the repair of gastric mucosal damage, changes of inflammatory factors, apoptosis, *etc*., were assessed. In terms of mechanism exploration, the main medicinal compositions of BXXXT aqueous extract and the synergistic bacteriostatic effects they had demonstrated were analyzed using mass spectrometry; the immune function of peripheral blood cells such as $CD3^+$ T and $CD4^+$ T of mice with gastritis before and after treatment with BXXXT aqueous extract was detected using a flow cytometry; the *H. pylori* transcriptome and proteome after treatment with BXXXT aqueous extract were detected. Differently expressed genes were screened and verification was performed thereon with knockout expression.

RESULTS

The minimum inhibitory concentration of BXXXT aqueous extract against *H. pylori* was 256-512 μg/mL. A dose of 28 mg/kg BXXXT aqueous extract treatment produced better therapeutical effects than the standard triple therapy did; the BXXXT aqueous extract have at least 11 ingredients inhibiting *H. pylori*, including berberine, quercetin, baicalin, luteolin, gallic acid, rosmarinic acid, aloe emodin, *etc*., of which berberine, aloe emodin, luteolin and gallic acid have a synergistic effect; BXXXT aqueous extract was found to stimulate the expressions of $CD3^+$ T and $CD4^+$ T and increase the number of $CD4^+$ T/$CD8^+$ T in gastritis mice; the detection of transcriptome and proteome, quantitative polymerase chain reaction, Western blotting and knockout verification revealed that the main targets of BXXXT aqueous extract are *CFAs* related to urea enzymes, and *CagA*, *VacA*, *etc*.

CONCLUSION

BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro* and *in vivo* and its mechanism comes down to the synergistic or additional antibacterial effects of berberine, emodin and luteolin, the main components of the extract; the extract could activate the immune function and enhance bactericidal effects; BXXXT aqueous extract, with main targets of BXXXT aqueous extract related to urease, virulence factors, *etc*., could reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa.

Key Words: Banxia Xiexin decoction; *Helicobacter pylori*; Drug resistance; Therapeutic effects; Mechanism

Core Tip: The failure rate of treating *Helicobacter pylori* (*H. pylori*) infectious diseases is increasing, leading to an urgent need to study and develop anti-*H. pylori* drugs. Banxia Xiexin decoction (BXXX) has a good effect on Hp infection and Hp-infection-related diseases. However, its pharmacological mechanism remains unclear, and whether it has an effect on drug-resistant *H. pylori* infection has not been confirmed by animal experiments. Our study confirms that BXXX decoction (BXXXT) has good therapeutic effects on drug-resistant *H. pylori* infection through *in vivo* and *in vitro* experiments in mice, then the composition of BXXXT and effective components, the immunomodulatory effect, the main target were verified. We preliminarily explain why BXXXT has a good effects.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the main pathogen that causes a variety of upper digestive diseases[1], such as chronic gastritis, peptic ulcer, and gastric cancer[2]. At present, the treatment options for *H. pylori* infections include standard triple therapy, bismuth-containing quadruple therapy, and sequential therapy. The drug resistance rate of *H. pylori* is increasingly higher, and the eradication rate is increasingly lower. Clarithromycin-resistant *H. pylori* has been listed as a focus for the research and development of antibiotics by the World Health Organization in 2017. The antimicrobial resistance of *H. pylori* is an urgent global problem. Multiple antibiotic resistance existed in 16847 *H. pylori* strains

isolated in Wenzhou from 2013-2020. Separate statistics on the resistance rates of *H. pylori* to the six commonly used antibiotics revealed that the resistance rates to levofloxacin, clarithromycin and metronidazole were high in the region, respectively 32.81%, 26.02%, and 95.67%. Therefore, how to avoid, overcome and eradicate *H. pylori* and *H. pylori*' s drug resistance brings challenges to the current clinical work. In China, traditional Chinese medicine could be used to treat a variety of refractory diseases, reducing drug resistance and improving the eradication rate of *H. pylori*[3]. "BXXXT" comes from Zhang Zhongjing' s Treatise on Febrile Diseases, which consists of 15 g *Pinellia ternate*, 9 g *Radix scutellariae*, 9 g *Dried ginger*, 9 g *Ginseng*, 9 g *Roasted licorice*, 3 g *Coptis chinensis*, and 4 *Jujubes*. At present, experimental studies and clinical efficacy of this prescription have been reported for gastrointestinal dysfunction, peptic ulcer, chronic gastritis, atrophic gastritis and other digestive system diseases[4-6]. There are numerous studies at home and abroad on the efficacy of this prescription[7-9], and it has been confirmed that BXXXT demonstrates the effects of treating gastrointestinal diseases, inhibiting *H. pylori* and protecting gastric mucosa[10-12]. However, whether it has the same effect on the refractory gastritis caused by drug-resistant *H. pylori* remains unreported.

Therefore, the purpose of the present study is to further explore the therapeutic effects of BXXXT on drug-resistant *H. pylori* through establishing mouse models, and to study the molecular mechanism of BXXXT applied in the treatment of the refractory gastritis caused by drug-resistant *H. pylori* through ingredient analysis, immune regulation, identification of therapeutical targets, *etc*., so as to provide an experimental basis for improving the application efficiency and of this prescription worldwide.

MATERIALS AND METHODS

Strains and culture conditions

H. pylori strains (standard 26695, G27, NSH57, and multi-drug-resistant BHKS159 all provided by Professor Bi Hongkai of Nanjing Medical University) containing the preservation solution and stored at -80 °C, clinical strains HPBS001-HPBS016 that had been isolated by Huang Yanqiang Laboratory, Youjiang Medical College for Nationalities were used. The *H. pylori* strains were cultured in a Columbia (OXOID, United Kingdom) medium with 10% serum or a BHI (OXOID, United Kingdom) medium in a microaerobic environment (85% nitrogen, 5% oxygen, and 10% carbon dioxide) at 37 °C.

Experimental animals

SPF C57BL/6 mice aged six to eight weeks were purchased from Changsha Tianqin Biological Co., Ltd.; the number of SPF animal license: SYXK Gui 2017-0004; animal experiment ethics number: No. 2019112501.

Preparation of BXXXT aqueous extract

The prescription consists of 15 g *Pinellia ternate*, 9 g *Radix scutellariae*, 9 g *Dried ginger*, 9 g *Ginseng*, 9 g *Roasted licorice*, 3 g *Coptis chinensis*, and 4 g *Jujubes*. In the first round, the medical materials used to prepare BXXXT were crashed to crude powder that was placed in a beaker with sterile distilled water at a ratio of 1:10, soaked for 4 h, treated with diluted and boiled for 0.5 h with the liquid filtered out. In the second round, the filtered powder was treated with distilled water in a ratio of 1:5 and boiled for 20 min with the filtrate filtered out; in the third round, the filtered powder was treated following the same steps described in the second round but boiled for 5 min with the liquid filtered out. The liquids prepared during the three rounds were combined, concentrated to crude drug (1 g/mL), evaporated under atmospheric pressure, sterilized, and stored for later use.

Evaluation of MIC of BXXXT against H. pylori in vitro

A medium of BXXXT aqueous extract that had been diluted two-fold was prepared as the treatment group: BXXXT aqueous extract was mixed with BHI and diluted two-fold with berberine set as the positive drug control group. *H. pylori* cells were adjusted at OD^{600} = 0.03 (equivalent to 1.0 × 10^7 CFU/mL). Bacterial solution (100 μL, equivalent to 1.0 × 10^6 CFU/mL) was inoculated into a 96-well plate that contained medicated BHI and incubated at 37 °C for 48 h to determine the results with the lowest concentration of drugs that could inhibit *H. pylori* growth being minimum inhibitory concentration (MIC).

Detection of therapeutic effects of BXXXT aqueous extract on mice with drug-resistant H. pylori gastritis in vivo

BXXXT aqueous extract, amoxicillin (Sigma-Aldrich, Germany), clarithromycin (Sigma-Aldrich, Germany) and omeprazole (Sigma-Aldrich, Germany) were all dissolved and diluted to 10 mg/mL. C57BL/6 model mice (BHKS159) were divided into four groups: The omeprazole + amoxicillin + clarithromycin group (the dose was 138.2 mg/kg of omeprazole, 28.5 mg/kg of amoxicillin, and 14.3 mg/kg of clarithromycin), the omeprazole + BXXXT aqueous extract (28 mg/kg), the omeprazole + BXXXT aqueous extract (7 mg/kg) and the phosphate-buffered saline (PBS) group, with six mice in each group.

Mice were given administration once a day for three consecutive times. Two days after drug withdrawal, the blood was collected from the eyeballs of the mice. The mice were then sacrificed through cervical dislocation with tissues taken from their stomach and broken to acquire *H. pylori* that was then isolated, cultured, and identified with the amount of colonization calculated. Part of the stomach tissues was made into paraffin sections with HE staining, TUNEL immunohistochemistry and fluorescence immunoassay performed thereon.

Main pharmacodynamic components of BXXXT aqueous extract

This analysis was performed by Beijing Bio-Tech Pack Technology Company Ltd using a TripleTOF5600 + and an AB SCIEX™ with the ion source being ESI. The chromatographic column was SHIMADZU InerSustain C18 (100.0 mm × 2.1 mm, 2 μm) with the column temperature being 35 °C and flow rate of 0.300 (mL/min). The mobile phase: (1) Equate = "acetonitrile"; and (2) equate = "0.1% CH3COOH-H2O". The chromatographic conditions were shown in Table 1. The scanning range of mass spectrometry conditions was m/z 100-1500. The scanning mode: DIA. Capillary voltage: 5000 V (positive) and 4500 V (negative). Capillary Temp: 500 °C, DP 60 V, CE 35 V, and CES 15 V.

Synergistic antimicrobial effects of main pharmacodynamic components of BXXXT aqueous extract

According to the protocols of the checkerboard method, drug A in the first row and drug B in the first column were diluted two-fold respectively. Then the transverse and longitudinal drugs were also cross-diluted two-fold and treated with 100 μL bacterial suspension, with the optimal combination effect selected to calculate the antimicrobial concentration index (FICI) after culture for 48 h. FICI= MIC of A drugs used in combination/MIC of A drugs used alone + MIC of B drugs used in combination/MIC of B drugs used alone. Criteria: synergistic effects (FICI ≤ 0.5); additive effect (0.5 < FICI ≤ 1.0); no effect (1.0 < FICI ≤ 2.0); antagonistic effects (FICI > 2.0).

Immunobactericidal effects of BXXXT aqueous extract

During the process of testing the efficacy of BXXXT aqueous extract described previously in section 1.5, the peripheral blood of mice before and after administration was collected and cells thereof were treated with anticoagulant and then with 50 μL antibody mixtures: CD3, CD4, and CD8, *etc*. After the membranes were fixed and broken, the cells were treated with 100 μL antibody mixtures: IFN and IL-4. After filtration, the expressions of immune cells and cytokines were detected using a flow cytometry.

Effect mechanism of BXXXT aqueous extract on H. pylori

BHKS159 bacteria were cultured on a Columbia plate overnight. Thereafter, the single colony was selected and diluted to 0.5 Mcfarland standard (MCF) with 2 μL taken and added to 5 mL BXXXT aqueous extract (1/2 MIC, prepared by being treated with BHI). The negative control group was induced with PBS, shaken at 37 °C and centrifuged to collect the bacteria solutions after it had been treated with the aqueous extract for 4 h and 8 h. The bacteria solutions were delivered to Nanjing Medical University where they were observed under a transmissive electron microscope. *H. pylori* cells were treated with BXXXT aqueous extract at the half inhibitory concentration that had been detected before for 8 h, after which the samples were collected and frozen in liquid nitrogen for 10 min, frozen with dry ice and delivered to Beijing Allwegene Technology Co., Ltd. for detection and analysis of transcriptome and proteome. The transcriptome analysis was performed using the Illumina PE150 sequencing strategy; the length of RNA fragments was detected using Agilent 2100; the alignment and transcript assembly analysis were performed using Boetie2 and the Rockhhoper software; quantitative protein analysis was performed using the ITRAQ labeling quantitative strategy, ITRAQ/TMT labeling performed using isoheavy isotope labeling, and the quantitative detection of target genes performed using a fluorescent polymerase chain reaction (PCR) instrument and Western blotting. Strains with low expressions of related target genes were used to verify MIC changes and mutant strains were constructed with reference to the protocols described in the previous studies[13]. The relevant mRNA amplification primers are shown in Table 2, and the relevant antibody information is displayed in Table 3.

Statistical analysis

Statistical analysis and mapping were performed using the Graphpad Prism software, version 8.0. Continuous data were expressed as mean ± SD. Differences between groups were analyzed using the one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The MIC of BXXXT aqueous extract on H. pylori detected in vitro

The MICs of BXXXT aqueous extract on three sensitive *H. pylori* strains and 11 drug-resistant *H. pylori* strains were detected by applying the solid plate method. BXXXT aqueous extract was found to have

Table 1 Chromatographic conditions

Time (min)	Parameter
0	A: 0%, B: 100%
10	A: 50%, B: 50%
13	A: 95%, B: 5%
14	A: 0%, B: 100%
15	A: 0%, B: 100%

Table 2 Primer information

No.	Primer	Sequence (5' to 3')	Company
1	UREA F	GCCAATGGTAAATTAGTT	Shanghai Invitrogen Biotech Co., Lt
2	UREA R	CTCCTTAATTGTTTTTAC	
3	UREB F	TCTATCCCTACCCCACAACC	
4	UREB R	CCATCCACGAACACATGGTA	
5	CagA F	ACCCCTAGTCGGTAATG	
6	CagA R	GCTTTAGCTTCTGATACTGC	
7	VacA F	GTCAGCATCACACCGCAAC	
8	VacA R	CTGCTTGAATGCGCCAAAC	
9	16sRNA F	CTGGAGAGACTAAGCCCTCC	
10	16sRNA R	AGGATCAAGGTTTAAGGATT	

Table 3 Antibody information

Name	Art. No.	Company
CagA (A-10)	sc-32746	Santa Cruz
VacA	sc-28368	Santa Cruz
m-IgGk BP-HRP	sc-516102	Santa Cruz
GAPDH Ab	AF7021	Affinity Biosciences
Goat anti-rabbit IgG (H+L) HPR	S0001	Affinity Biosciences

antibacterial effects on strains, both resistant and sensitive (the MIC was 256-512 μg/mL). The antibacterial effect of BXXXT aqueous extract was compared with that of berberine (the MIC was 512-2048 μg/mL), as shown in Table 4. The results suggested that there was a two-to-four-fold difference between them and that BXXXT aqueous extract produced better antibacterial effects than 98% pure berberine. The reason might be that although berberine accounts for a small portion of the prescription, there might be other antibacterial components, or there might be synergistic or additive effects among these components.

Therapeutic effects of BXXXT aqueous extract were detected in vivo on mice with H. pylori-resistant acute gastritis

Model mice with acute gastritis caused by the drug-resistant strain BHKS159 were constructed and treated with PBS, omeprazole (OPZ) + amoxicillin clarithromycin (AC), OPZ + BXXXT (28 mg/kg) and OPZ + BXXXT (7 mg/kg), respectively. Although *H. pylori* colonization, inflammatory factors IL-1β, IL-6 and tumor necrosis factor-alpha (TNF-α), inflammatory damage, and apoptosis factors Bcl-2 and Bax were improved in OPZ + AC treatment group, there were still significant differences compared with OPZ + BXXXT (28 mg/kg) treatment group. After OPZ + BXXXT (28 mg/kg) treatment, the mice could basically recover to the normal level, implying therapeutical effects significantly better than that of the triple group (Figure 1). However, amoxicillin which does not develop drug resistance, produced therapeutical effects that contributed to the improvement in the OPZ + AC treatment group when

Table 4 Minimum inhibitory concentration of Banxia Xiexin decoction aqueous extract against *Helicobacter pylori* (μg/mL)

Strain	Drug-resistanct strain	BXXXT aqueous extract	Berberine
26695	Sensitive	512	1024
G27	Sensitive	512	1024
NSH57	Sensitive	256	512
BHKS159	Resistant to levofloxacin, clarithromycin and metronidazole	512	1024
HPBS001	Resistant to levofloxacin, clarithromycin and metronidazole	512	1024
HPBS002	Resistant to metronidazole	512	1024
HPBS003	Resistant to clarithromycin	512	1024
HPBS004	Resistant to levofloxacin	512	1024
HPBS005	Resistant to levofloxacin and metronidazole	256	1024
HPBS006	Resistant to clarithromycin and metronidazole	256	1024
HPBS007	Resistant to clarithromycin	512	1024
HPBS010	Resistant to metronidazole, clarithromycin and levofloxacin	512	2048
HPBS011	Resistant to metronidazole and clarithromycin	512	1024
HPBS013	Resistant to metronidazole, clarithromycin and levofloxacin	512	1024
HPBS014	Resistant to metronidazole, clarithromycin, amoxicillin and levofloxacin	512	1024

BXXXT: Banxia Xiexin decoction. The minimum inhibitory concentrations of the drugs for sensitive and drug-resistant strains are amoxicillin ≥ 0.5 μg/mL, clarithromycin ≥ 1.0 μg/mL, levofloxacin ≥ 2.0 μg/mL, and metronidazole ≥ 8.0 μg/mL.

combined with omeprazole. BXXXT, which did not demonstrate good effects *in vitro*, produced obvious therapeutical effects *in vivo*, which might be related to the synergistic and immunomodulatory effects of BXXXT aqueous extract.

Main pharmacodynamic components of BXXXT aqueous extract

MS-DIAL 3.70 (MS-DIAL: Data independent MS/MS deconvolution for comprehensive metabolome analysis) (Nature Methods, 12, 523-526, 2015).The original LC-MS data of BXXXT aqueous extract were imported into MS-DIAL, version 3.70 for preprocessing (MS-DIAL: Data independent MS/MS deconvolution for comprehensive metabolome analysis) (Nature Methods, 12, 523-526, 2015), including peak value extraction, noise-removal, deconvolution and peak alignment, and thereafter the three-dimensional data matrix in comma-separated values format was derived (original data matrix). The peak information extracted was compared with the database, with the full database search of MassBank, Respect and GNPS (14951 records in total). About 428 monomer components were identified, among which, as the related literature suggests, there were a total of 78 major components related to *H. pylori* resistance. Eleven species including berberine, emodin, baicalin, quercetin have been widely reported and demonstrate good antibacterial effects. Their ion additions and molecular structure are displayed in Table 5. It could be suggested that among the components of BXXXT aqueous extract, in addition to berberine, other components such as emodin also have inhibitory effects, which provides experimental basis for the better antibacterial effects *in vitro* BXXXT aqueous extract could produce compared with berberine.

Synergistic antimicrobial effects of main pharmacodynamic components of BXXXT aqueous extract

Six groups of berberine and emodin, berberine and luteolin, luteolin and gallic acid, luteolin and rosmarinic acid, catechuic acid and quercetin, catechuic acid and emodin were selected from 12 main anti-HP components of water extract of BXXXT aqueous extract for combined drug sensitivity detection. The results suggested that the six groups demonstrated additive or synergistic effects on *H. pylori* (Table 6), berberine and emodin, luteolin and gallic acid in particular producing better synergistic effects. Similar effects might also be found in other component combinations that had not been verified, which provides further experimental evidence that BXXXT aqueous extract could produce better antibacterial effects *in vitro* than berberine.

Immunobactericidal effects of BXXXT aqueous extract on mice

The *t*-test was used to analyze the proportion of $CD3^+$ T, $CD4^+$ T, and $CD8^+$ T cells in total lymphocytes

Table 5 Information of main pharmacodynamic components of Banxia Xiexin decoction aqueous extract

Name	Molecular structural formula
Berberine	COC1=C(OC)C2=C[N+]3=C(C=C2C=C1)C1=CC2=C(OCO2)C=C1CC3
Baicalin	C1=CC=C(C=C1)C2=CC(=O)C3=C(C(=C(C=C3O2)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)C(=O)O)O)O)O)O)O
Luteolin	OC1=CC(O)=C2C(=O)C=C(OC2=C1)C1=CC(O)=C(O)C=C1
Gallic acid	OC(=O)C1=CC(O)=C(O)C(O)=C1
Gingerol	CCCCCC(O)CC(=O)CCC1=CC(OC)=C(O)C=C1
Wogonoside	COC1=C(O)C=C(O)C2=C1OC(=CC2=O)C1=CC=CC=C1
Rosmarinic acid	OC(=O)[C@H](CC1=CC(O)=C(O)C=C1)OC(=O)\C=C\C1=CC(O)=C(O)C=C1
Aloe-emodin	OCC1=CC2=C(C(O)=C1)C(=O)C1=C(C=CC=C1O)C2=O
Catechin	CCCCCC(CC(=O)CCC1=CC(=C(C=C1)O)OC)O
Naringenin	OC1=CC=C(C=C1)[C@@H]1CC(=O)C2=C(O)C=C(O)C=C2O1
Quercetin	OC1=CC(O)=C2C(OC(=C(O)C2=O)C2=CC(O)=C(O)C=C2)=C1

before and after administration. After administration, the proportion of $CD3^+$ T and $CD4^+$ T in total lymphocytes increased ($P1 = 0.0009$, $P2 = 0.0115$), as shown in Figure 2A-F; no significant difference was found between $CD8^+$ T cells and TH1 cells ($P3 = 0.1937$, $P4 = 0.8061$), Figure 2A, C, D, and G, and the ratio of $CD4^+$ T/$CD8^+$ T cells was increased ($P5 = 0.0280$) as displayed in Figure 2H. These results indicated that BXXXT aqueous extract could improve the ratio of lymphocytes $CD3^+$ T and $CD4^+$ T to the total number of lymphocytes and the ratio of $CD4^+$ T/$CD8^+$ T cells. However, BXXXT aqueous extract could enhance immune functions, thus helping improve the immunity and antibacterial ability of the body, which might explain why BXXXT could produce better treatment effects *in vivo*.

Mechanism of action of BXXXT aqueous extract on H. pylori

No significant changes were found at × 10000 magnification on the morphological structures of *H. pylori* after 4 h and 8 h of BXXXT treatment (Figure 3A), indicating that BXXXT did not produce antibacterial effects by directly destroying morphological structures. In the samples treated with BXXXT for 8 h, a total of 357 differentially expressed genes were detected after transcriptome analysis, among which 133 genes were up-regulated and 224 genes down-regulated (Figure 3B), mainly concentrating in five metabolic pathways including metabolic pathways, the epithelial cell signaling in *H. pylori* infection and the microbial metabolism in diverse environments (Figure 3C). The epithelial cell signaling in *H. pylori* infection pathway suggested that it is closely related to urease genes and virulence genes, as shown in Figure 3D. The proteome detection found 86 differentially expressed genes, among which 44 were up-regulated and 42 down-regulated (Figure 3E), mainly concentrating in oxidoreductases and transferases pathways (Figure 3F). Among the related genes and proteins found in transcriptome and proteome, respectively, and concentrating in the main pathways, five possible proteins of BXXXT were screened, among which four were urease-related and one was related to the virulence gene *CagA* (Table 7). The quantitative PCR (Q-PCR) and Western blot detection were performed to confirm the correlation between BXXXT action and virulence genes and urease genes. The results suggested that the mRNAs and protein expressions of *CagA* and *VacA* after BXXXT treatment were significantly decreased (Figure 4A-D), providing additional evidence for the obvious effects of BXXXT *in vivo* from the point that BXXXT could reduce virulence of *H. pylori*. The gene *CFAs* related to environmental regulation, urease and drug resistance was mutated (Figure 4E), after which the MIC of the mutant strains increased 2-4 times (Figure 4F), further proving that the urease-related gene *CFAs* might be one of the main targets of BXXXT. However, the decrease of urease could affect the adaptive regulation of stomach acid for which *H. pylori*' s ability to colonize would be significantly reduced.

DISCUSSION

There are about 4.4 billion people with *H. pylori* infections worldwide, with an average infection rate of 62.8%. Southeast Asia could be considered as high-incidence areas, mainly China, Japan, and South Korea[14,15]. The eradication of *H. pylori* has proven to prevent gastric cancer. However, the overuse of antibiotics leads to serious drug resistance. The drug resistance rate varies in different countries and regions and will change over time, indicated by those of clarithromycin, metronidazole, and levloxacin, all increasing over time. For example, the drug resistance rate of clarithromycin rose to 21% between 2012 and 2016[16-18]. Therefore, the failure rate of treating *H. pylori* infectious diseases is increasing,

Table 6 Minimum inhibitory concentrations of main antibacterial components in Banxia Xiexin decoction aqueous extract (μg/mL)

Strain	MIC of drugs used alone		MIC of drugs used in combinations		FIC	Effect
	Berberine	Emodin	Berberine	Emodin		
G27	1024	512	256	256	0.75	Addictive
26695	1024	512	256	128	0.50	Synergistic
BHKS159	1024	512	256	128	0.50	Synergistic
	Berberine	Luteolin	Berberine	Berberine		
G27	1024	1024	512	512	1.00	Addictive
26695	1024	1024	512	256	0.75	Addictive
BHKS159	1024	1024	512	512	1.00	Addictive
	Luteolin	Gallic acid	Luteolin	Gallic acid		
G27	1024	1024	256	256	0.50	Synergistic
26695	1024	1024	256	256	0.50	Synergistic
BHKS159	1024	1024	256	256	0.50	Synergistic
	Luteolin	Rosmarinic acid	Luteolin	Rosmarinic acid		
G27	1024	1024	512	256	0.75	Addictive
26695	1024	1024	512	512	1.00	Addictive
BHKS159	1024	1024	512	256	0.75	Addictive
	Catechuic acid	Quercetin	Catechuic acid	Quercetin		
G27	1024	1024	512	256	0.75	Addictive
26695	1024	1024	512	256	0.75	Addictive
BHKS159	1024	1024	512	256	0.75	Addictive
	Catechuic acid	Emodin	Catechuic acid	Emodin		
G27	1024	512	512	256	1.00	Addictive
26695	1024	512	512	256	1.00	Addictive
BHKS159	1024	512	256	256	0.75	Addictive

MIC: Minimum inhibitory concentration.

leading to an urgent need to study and develop anti-*H. pylori* drugs. In 2018, Hu *et al*[19] from Peking University proposed that non-antibiotic drugs such as traditional Chinese medicine, mucosal protective agents and probiotics could be used to treat *H. pylori* infection. Traditional Chinese medicine, including BXXXT, has a good effect on *H. pylori* infection and *H. pylori*-infection-related diseases[20,21]. However, its pharmacological mechanism remains unclear, and whether it has an effect on drug-resistant *H. pylori* infection or not has not been confirmed by animal experiments.

This study confirms that BXXXT has good therapeutic effects on drug-resistant *H. pylori* infection through *in vivo* and *in vitro* experiments in mice, which provides an experimental basis for elaborating that BXXXT could treat refractory gastritis caused by drug-resistant bacteria. While the efficacy of BXXXT is well established, explaining its mechanism is difficult. Traditional Chinese medicine, especially compound prescriptions, has complex components and a very complex mechanism of action in the body, which might be affected by multiple factors, especially those in stomach. Besides, it produces effects that are multi-target. As the content of the main components of the prescription is not high, the effects on the target may not always appear[22-24], which makes it difficult to elaborate on the mechanism of action. In the present study, the composition of BXXXT was analyzed, the effective anti-HP components were screened out with reference to the related literature and reports, the material basis of the efficacy was identified, and the synergistic effects among some of the effective components was verified. It was found that most of the components had additive or synergistic effects, such as berberine and emodin, luteolin and gallic acid. This indicated that though only accounting for a small portion, the active components of the Chinese medicine prescription, which could produce synergistic or additive effects demonstrated better antibacterial effects. The MIC of BXXXT against *H. pylori* is 256-512 μg/mL, much worse than that of clinical antibiotics but producing better therapeutical effects *in vivo*, especially

Table 7 Information of related proteins identified by transcriptome analysis after Banxia Xiexin decoction treatment

Uniprot Accession Number	Protein name	Protein amino acid sequence	*P* value	Reliability
E6NPU2	Urease subunit alpha OS = *Helicobacter pylori* (strain F57), OX = 866346, GN = urea, PE = 3, SV = 1	MKLTPKELDKLMLHYAGELARKRKEKGIKLNYVEAVALISAHIMEEARAGKKSAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIESNGKLVPGELFLK NEDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFGKRLDIASGT AVRFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIALHRAKERGFHGAKSDD NYVKTIKE	0.001211988	High
A0A2A6SFH9	Urease (fragment) OS = *Helicobacter pylori*, OX = 210, GN = BB479_08100, PE = 4, SV = 1	MKLTPKELDKLMLHYAGELARKRKEKGIKLNYVEAVALIXAHIMEEARAGKKTAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTXIEANGKLVPGELFLKN EDITINEGKKAVSVKVKNVGDRPVQIGSH	0.033815784	High
A0A0L0QH58	Urease subunit alpha OS = *Helicobacter pylori*, OX = 210, GN = urea, PE = 3, SV = 1	MKLTPKELDKLMLHYAGELAKKRKEKGIKLNYVEAVALISAHIMEEARAGKKSAAEL MQEGRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIEANGKLVPGELFLKN EDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFGKRLDIASGTAV RFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIALHRAKERGFHGAKSDDNY VKTIKE	0.033744196	High
N4TND2	Urease accessory protein UreG OS = *Helicobacter pylori* Hp A-11, OX = 992035, GN = ureG, PE = 3, SV = 1	MVKIGVCGPVGSGKTALIEALTRHMSKDYDMAVITNDIYTKEDAEFMCKNSVMPRER IIGVETGGCPHTAIREDASMNLEAVEEMHGRFPNLELLLIESGGDNLSATFNPELADFTIF VIDVAEGDKIPRKGGPGITRSDLLVINKIDLAPYVGADLKVMERDSKKMRGEKPFIFTNIR AKEGLNDVIAWIKRNALLED	0.016839824	High
Q8RRP6	Cytotoxin associated protein CagA (Fragment), OS = *Helicobacter pylori*, OX = 210, GN = cagA, PE = 4, SV = 1	ALADLKNFSKEQLAQQAQKNESFNAGKKFEFSQSVRNGVNGTLVGNGFSQAEATTL SKNFSDIKKELNAKLGNFNNNNINGLKNSTEPIYAKVNKKETGQAASPEEPIYTQVAKKVN AKIDRLNQIASGLGVVGQAAGFPLKRHDKVDDLSKVGRSVSPEPIYATIDDLGGPFPLKRH DKVDNLSKVGRSVSPEPIYATIDDLGGPFPLKRHDKVDNLSKVGLSRNQELTQKIDNLSQA VSEAKAGFFGNLEQTIDKLKDSTKHNVVNLWAESAKKVPASLSAKLDNYA	0.03652953	High

for drug-resistant *H. pylori*, why?

H. pylori can adhere to the gastrointestinal mucosa, produce virulence factors, damage gastric epithelial cells, and induce, control and regulate inflammatory responses. CagA encodes variety of proteins, including CagA and VacA, *ect*[25]. Karbalaei *et al*[26] found that *CagA* and *VacA* genes were potentially associated with resistance to clarithromycin, metronidazole, amoxicillin, tetracycline and levofloxacin. The VacA can not only destroy mitochondria[27,28], but also reduce the proliferation of T cells, B cells, and the other immune cells, and affect the immune response[29-31]. Among the components of BXXXT, Gingerol, a crude extract containing gingerol, is able to can inhibit the growth of *H. pylori* strains (MIC range is 0.78 μg/mL to 12.5 μg/mL) and has significant activity against $CagA^+$ strains[32]. Kaempferol is able to reduce the transcription of subunit protein A by the type IV secretory system, reduce the expression of pro-inflammatory cytokines (TNF-α, IL-1β) and IL-8 production in cells [33]. Urease can increase the pH value of the stomach and provide a suitable environment for *H. pylori* colonization. Palmatine in Coptis coptitis can act on sulfhydryl at the active site of urease and inhibit the conformational change of the urease molecules, and reduce urease activity[34]. Hesperidin can reduce the expression of UreA and UreB[35].

In the present paper, the immunomodulatory effect of BXXXT was analyzed, and it was found that it could up-regulate the expressions of immune factors such as $CD4^+$ T and enhance immunity and the ability of sterilization; the main target of BXXXT was urease-related gene *CFAs*, which was related to the virulence factors *CagA* and *VacA*. When urease was destroyed, *H. pylori* cells could not survive in the gastric acid environment, and its colonization ability would be significantly weakened. With low expressions of *H. pylori* virulence factors, the inflammatory damage caused by *H. pylori* to gastric mucosa would be reduced. The effects of these three aspects could preliminarily explain why BXXXT has good effects *in vivo*. However, clarifying the action mechanism of BXXXT is very complicated and difficult, for there are many components of BXXXT, among which many are anti-*H. pylori*, the

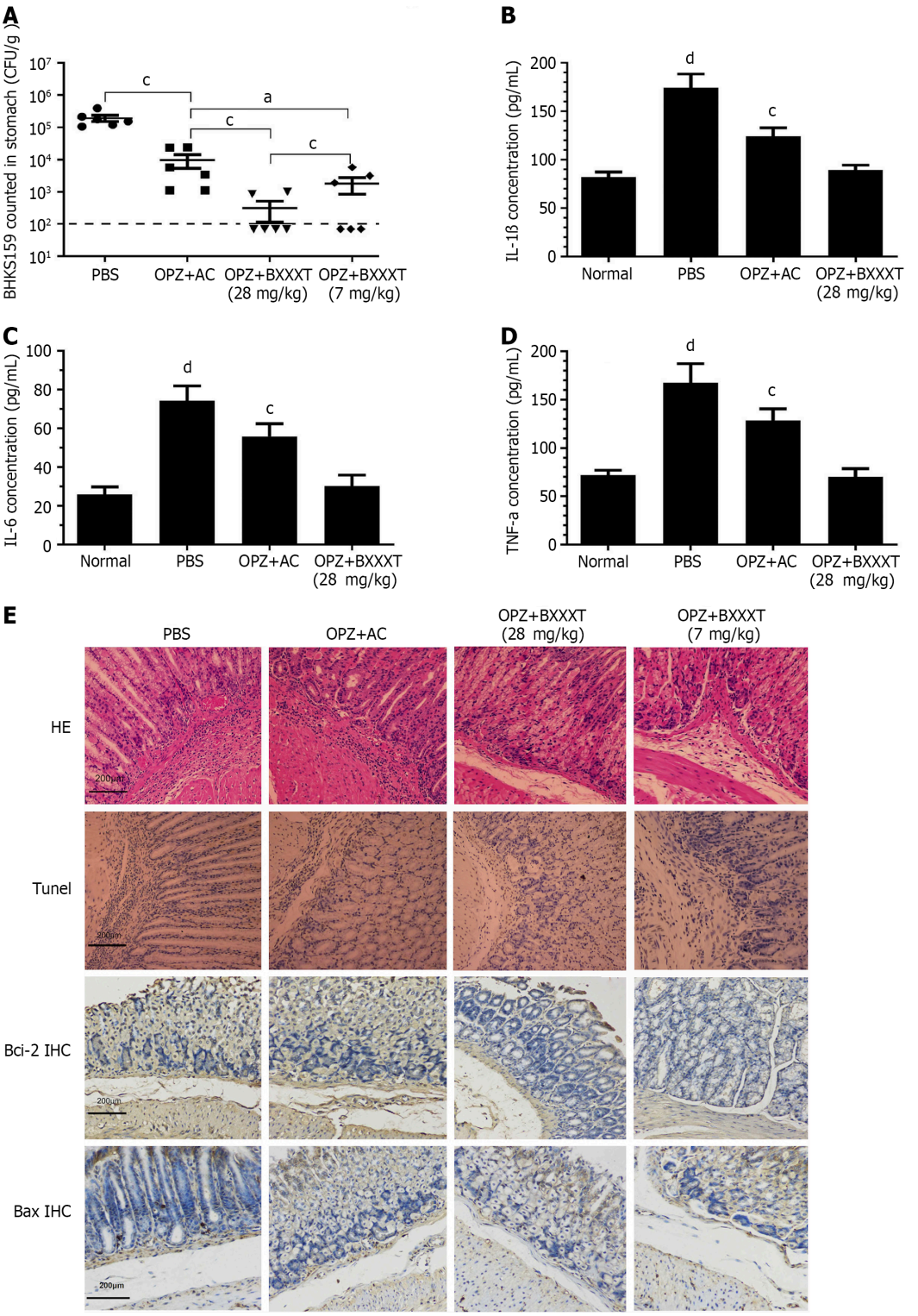

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Figure 1 Therapeutic effects of BanXiaXieXin decoction aqueous extract on mice with *Helicobacter pylori*-resistant acute gastritis. A: The amount of *Helicobacter pylori* colonization in model mice infected with drug-resistant strains; B: The expression of inflammatory factor IL-1β in model mice; C: The expression of inflammatory factor IL-6 in model mice; D: The expression of inflammatory factor tumor necrosis factor-alpha in model mice; E: The gastric mucosa injury and the expressions of apoptotic genes Bcl-2 and Bax, × 200. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; OPZ: Omeprazole; AC: Amoxicillin clarithromycin; PBS: Phosphate-buffered saline.

transcriptome and proteome analyses in this study also suggested that many membrane transporter genes were involved, such as ABC transporter, *etc*. Besides, we also found that berberine and other components of BXXXT could inhibit *HefA* gene to reverse drug resistance[25] and *CFAs* in this study might also be related to drug resistance[36,37]. Therefore, the action mechanism of BXXXT will be further studied.

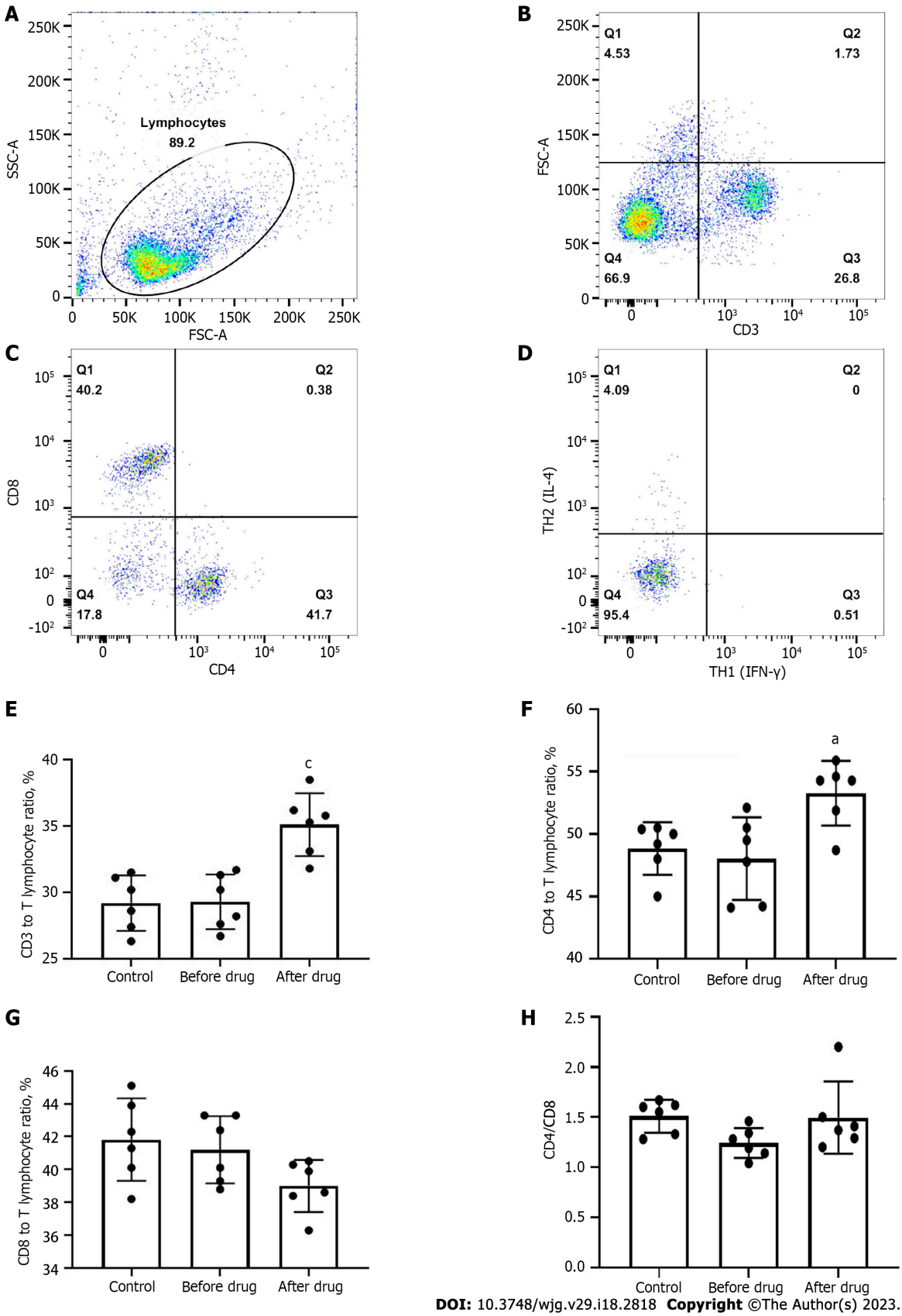

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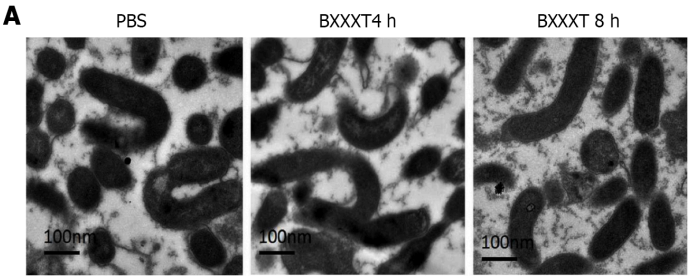
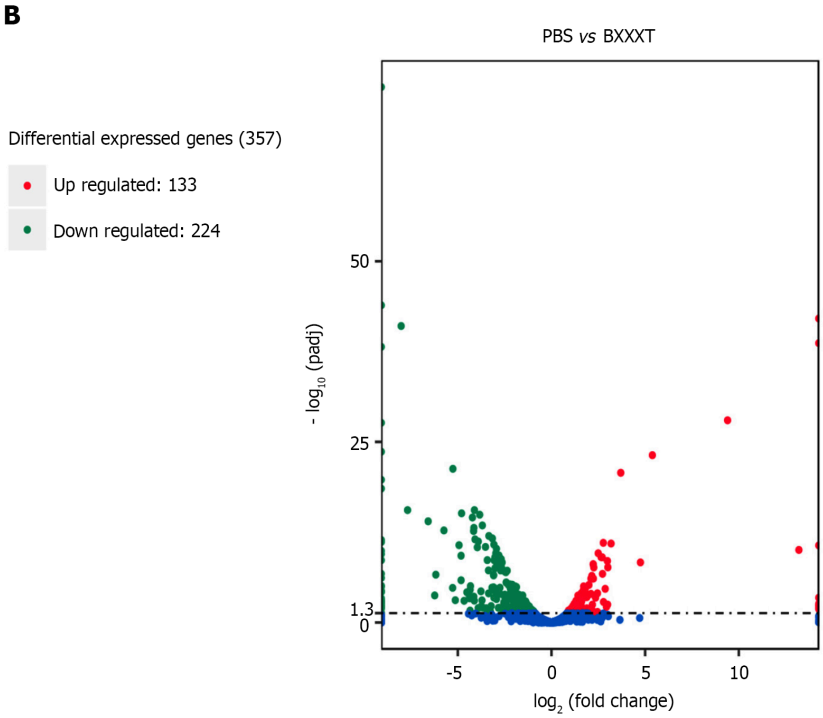
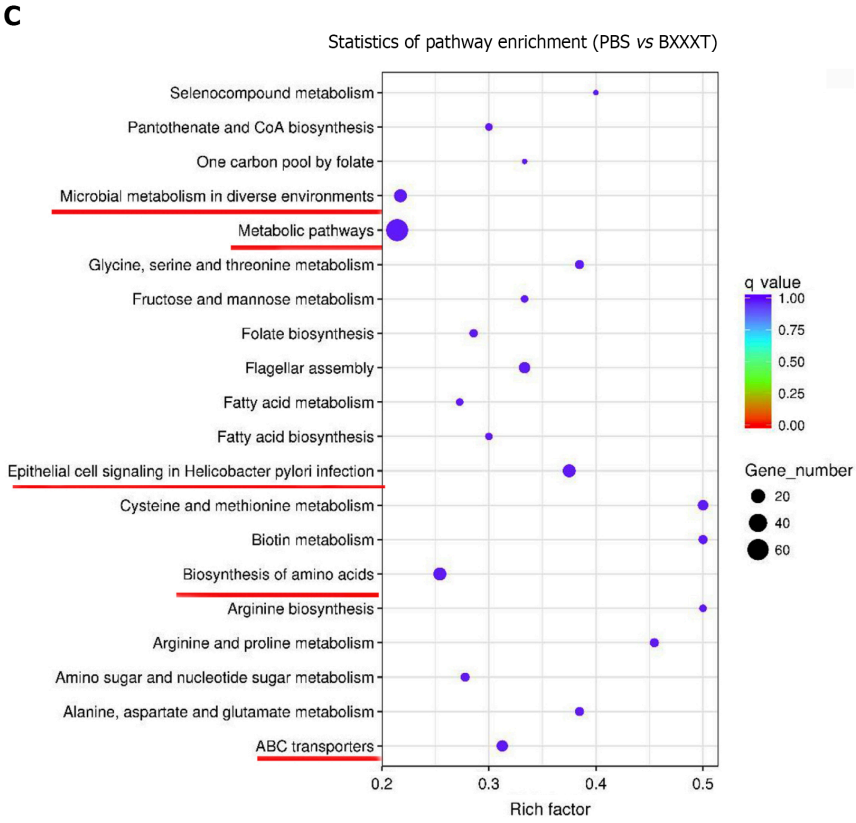
Figure 2 Immunobactericidal effects of BanXiaXieXin decoction aqueous extract on mice. A: Lymphocyte expression; B: CD3T cell expression; C: CD4T and CD8T cell expression; D: TH1 and TH2 expression; E: CD3T to lymphocyte ratio; F: CD4T to lymphocyte ratio; G: CD8T to lymphocyte ratio; H: CD4T /CD8T ratio. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

CONCLUSION

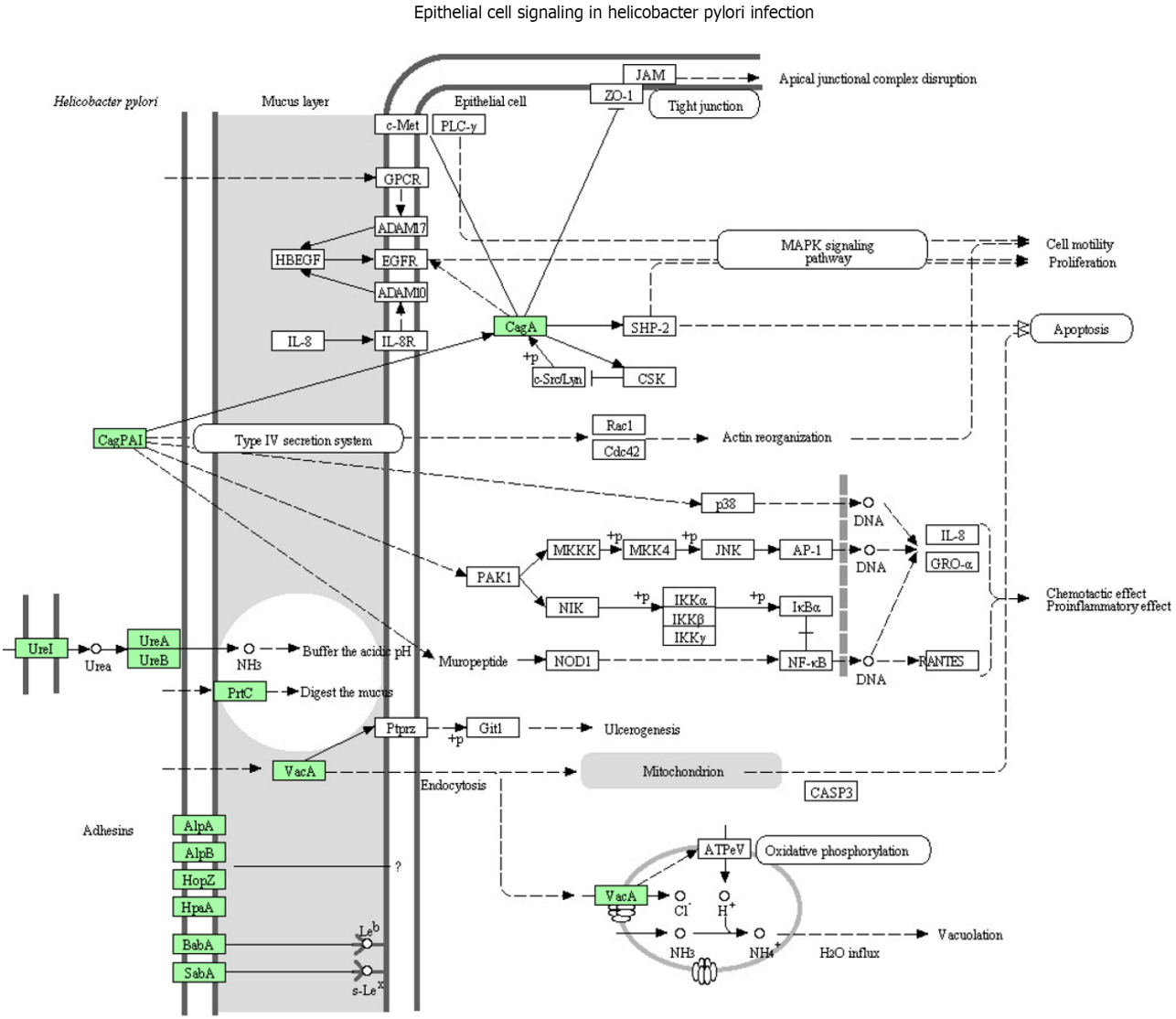
BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro*


A
PBS
BXXXT4 h
BXXXT 8 h
100nm
100nm
100nm

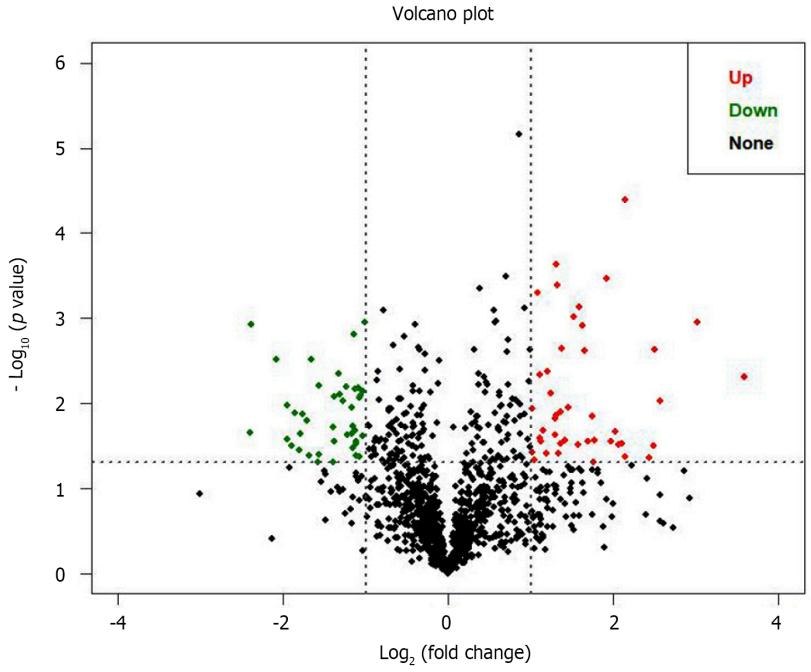

B
PBS *vs* BXXXT
Differential expressed genes (357)
Up regulated: 133
Down regulated: 224
50
25
1.3
0
- log10 (padj)
-5
0
5
10
log2 (fold change)


C
Statistics of pathway enrichment (PBS *vs* BXXXT)
Selenocompound metabolism
Pantothenate and CoA biosynthesis
One carbon pool by folate
Microbial metabolism in diverse environments
Metabolic pathways
Glycine, serine and threonine metabolism
Fructose and mannose metabolism
Folate biosynthesis
Flagellar assembly
Fatty acid metabolism
Fatty acid biosynthesis
Epithelial cell signaling in Helicobacter pylori infection
Cysteine and methionine metabolism
Biotin metabolism
Biosynthesis of amino acids
Arginine biosynthesis
Arginine and proline metabolism
Amino sugar and nucleotide sugar metabolism
Alanine, aspartate and glutamate metabolism
ABC transporters
0.2
0.3
0.4
0.5
Rich factor
q value
1.00
0.75
0.50
0.25
0.00
Gene_number
20
40
60

D


Epithelial cell signaling in helicobacter pylori infection
Helicobacter pylori
Mucus layer
Epithelial cell
JAM
ZO-1
Tight junction
Apical junctional complex disruption
c-Met
PLC-γ
GPCR
ADAM17
HBEGF
EGFR
ADAM10
MAPK signaling pathway
Cell motility
Proliferation
IL-8
IL-8R
CagA
SHP-2
Apoptosis
+p
c-Src/Lyn
CSK
CagPAI
Type IV secretion system
Rac1
Cdc42
Actin reorganization
p38
DNA
MKKK
MKK4
JNK
AP-1
IL-8
GRO-α
PAK1
NIK
IKKα
IKKβ
IKKγ
IκBα
Chemotactic effect
Proinflammatory effect
UreI
Urea
UreA
UreB
NH3
Buffer the acidic pH
Muropeptide
NOD1
NF-κB
RANTES
PrtC
Digest the mucus
Ptprz
Git1
Ulcerogenesis
VacA
Endocytosis
Mitochondrion
CASP3
Adhesins
AlpA
AlpB
HopZ
HpaA
BabA
SabA
?
Le^b
s-Le^x
ATPeV
Oxidative phosphorylation
Cl^-
H^+
NH3
NH4^+
H2O influx
Vacuolation

E


Volcano plot
Up
Down
None
- Log10 (p value)
Log2 (fold change)
6
5
4
3
2
1
0
-4
-2
0
2
4

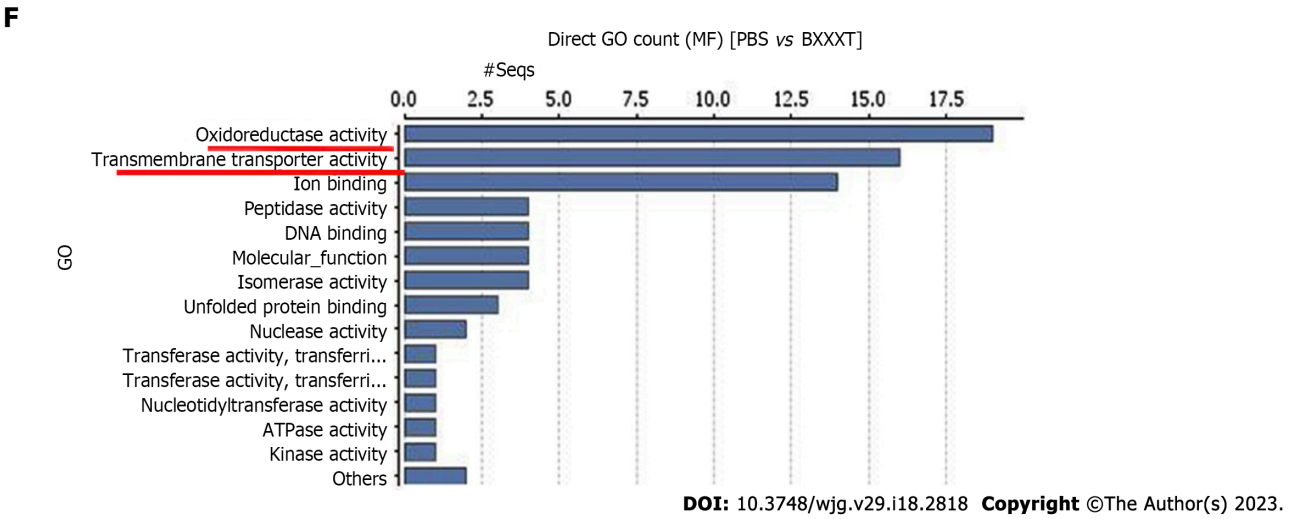


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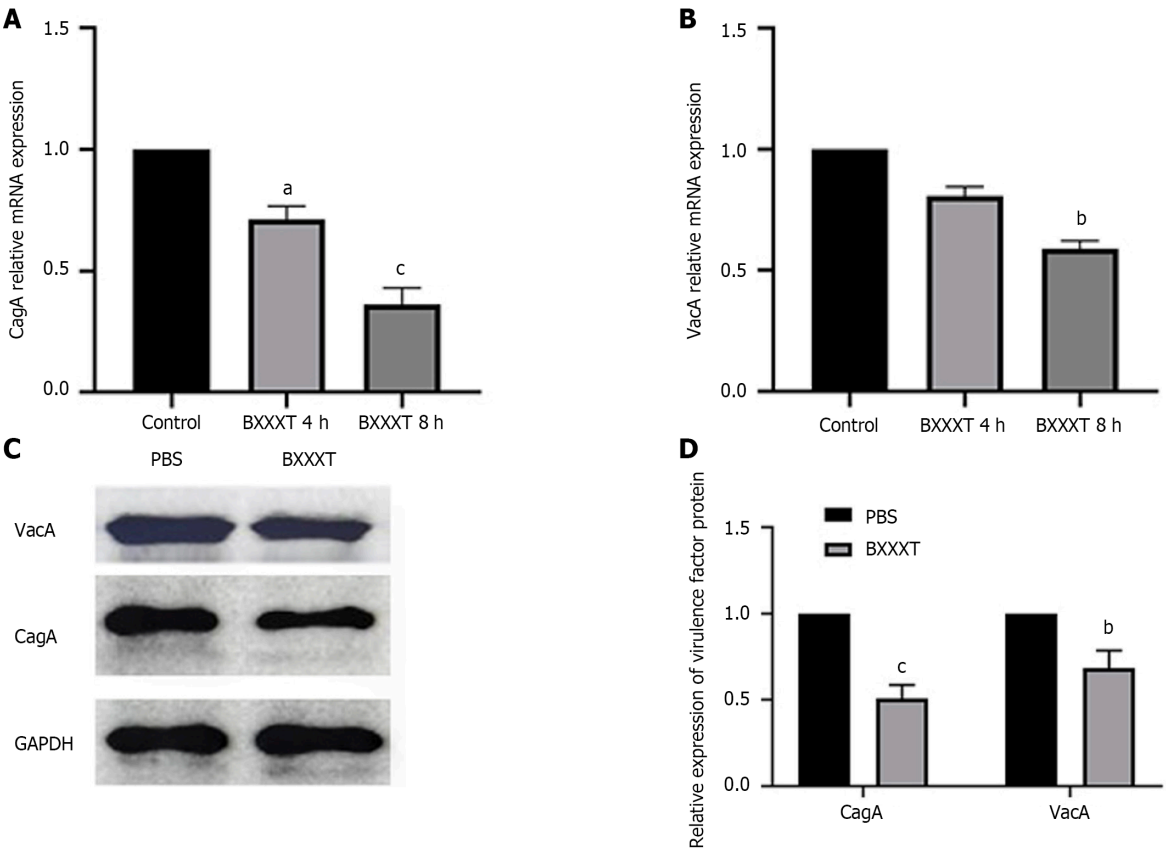
Figure 3 Detection of changes of *Helicobacter pylori* after 8 h treatment with BanXiaXieXin decoction using an electron microscopy, transcriptome, and proteome analyses. A: Observation of changes of *Helicobacter pylori* (*H. pylori*) using an electron microscope; B: The number of significantly differential genes in the transcriptome; C: Significantly differential genes concentrating in the GO enrichment pathway in the transcriptome; D: The epithelial cell signaling in *H. pylori* infection; E: The number of significantly differential genes in the proteome; F: Significantly differential genes concentrating in the GO enrichment pathway in the proteome. BXXXT: BanXiaXieXin decoction; PBS: Phosphate-buffered saline.

and *in vivo* and its mechanism comes down to the synergistic or additional antibacterial effects of berberine, emodin and luteolin, the main components of the extract; the extract could activate the immune function and enhance bactericidal effects; BXXXT aqueous extract, with main targets of BXXXT aqueous extract related to urease, virulence factors, *etc*., could reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa.



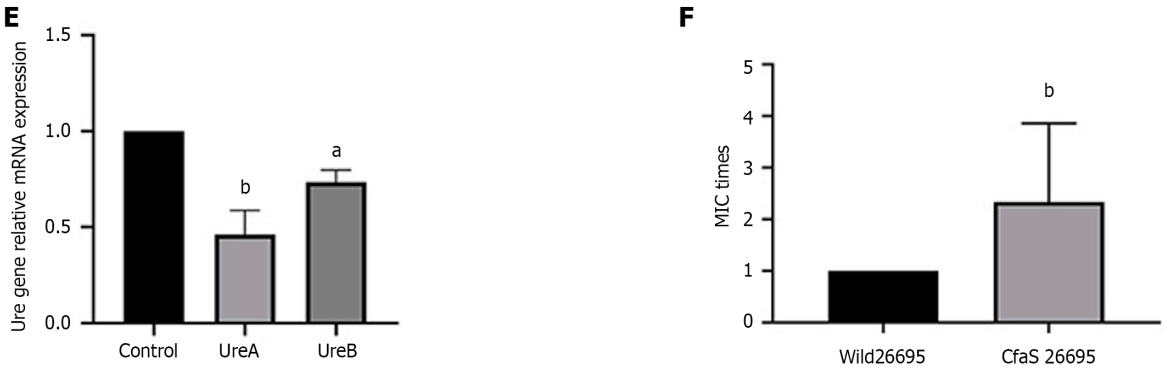


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Figure 4 Main targets of BanXiaXieXin decoction aqueous extract action. A: mRNA expression of *cagA*; B: mRNA expression of *VacA*; C: Protein expression of *CagA* and *VacA*; D: Quantitative expressions of *CagA* and *VacA* proteins; E: Mutant strain *CFAs* with a low urease expression; F: Changes of minimum inhibitory concentration of BXXXT against mutant strains with a low expression of urease. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; NS: Not significant. BXXXT: BanXiaXieXin decoction; PBS: Phosphate-buffered saline.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*)' s drug resistance brings challenges to the current clinical work. In China, traditional Chinese medicine could be used to treat a variety of refractory diseases, reducing drug-resistance, and improving the eradication rate of *H. pylori*. The study is to explore the therapeutic effects of Banxia Xiexin Decoction (BXXXT) on drug-resistant *H. pylori*.

Research motivation

H. pylori' s drug resistance brings challenges to the current clinical work. The study is to explore the therapeutic effects of BXXXT on drug-resistant *H. pylori*, avoid, overcome *H. pylori*' s drug resistance.

Research objectives

To confirm that BXXXT demonstrates therapeutical effects *in vivo* and *in vitro* on gastritis mice with drug-resistant *H. pylori* and explain its mechanism.

Research methods

The aqueous extract of BXXXT was gained by water decocting method. The inhibitory effect of the aqueous extract on *H. pylori* was detected by dilution in vitro. In terms of mechanism exploration, the main medicinal compositions of BXXXT aqueous extract and the synergistic bacteriostatic effects they had demonstrated were analyzed using mass spectrometry; the immune function of peripheral blood cells such as $CD3^+$ T and $CD4^+$ T of mice were detected using a flow cytometry; the *H. pylori* transcriptome and proteome were detected. Differently expressed genes were screened and verification was performed thereon with knockout expression.

Research results

BXXXT aqueous extract against *H. pylori* was better therapeutical effects *in vivo* and *in vitro*; BXXXT aqueous extract was found to stimulate the expressions of $CD3^+$ T and $CD4^+$ T and increase the number of $CD4^+$ T/$CD8^+$ T in gastritis mice; the detection of transcriptome and proteome, quantitative polymerase chain reaction, Western blot and knockout verification revealed that the main targets of BXXXT aqueous extract are *CFAs* related to urea enzymes, and *CagA*, *VacA*, *etc*.

Research conclusions

BXXXT aqueous extract could demonstrate good therapeutic effects on drug-resistance *H. pylori in vitro* and *in vivo* and its mechanism is related to reduce the urease and virulence of *H. pylori*, weaken its colonization, and reduce its inflammatory damage to the gastric mucosa, *etc*.

Research perspectives

BXXXT aqueous extract has good therapeutic effects on drug-resistance *H. pylori*, can overcome *H. pylori* 's drug resistance.

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FOOTNOTES

Author contributions: Li XH and Xu JY consulted literature, performed experiments, collected and analyzed data and wrote the first draft, making equal contributions to this work; Wang X and Liao LJ corrected it; Huang L, Huang YQ, and Zhang ZF designed, checked, modified, and completed the manuscript, making equal contributions to this work as co-corresponding authors, Huang L (youyihuangl@163.com) is the first corresponding author; all authors approved the final version of the article.

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