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ORIGINAL ARTICLE

Basic Study

Mechanistic research: Selenium regulates virulence factors, reducing adhesion ability and inflammatory damage of *Helicobacter pylori*

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Abstract

BACKGROUND

The pathogenicity of *Helicobacter pylori* is dependent on factors including the environment and the host. Although selenium is closely related to pathogenicity as an environmental factor, the specific correlation between them remains unclear.

AIM

To investigate how selenium acts on virulence factors and reduces their toxicity.

METHODS
H. pylori strains were induced by sodium selenite. The expression of cytotoxin-associated protein A (*CagA*) and vacuolating cytotoxin gene A (*VacA*) was determined by quantitative PCR and Western blotting. Transcriptomics was used to analyze *CagA*, *CagM*, *CagE*, *Cag1*, *Cag3*, and *CagT*. C57BL/6A mice were infected with the attenuated strains subjected to sodium selenite induction, and *H. pylori* colonization, inflammatory reactions, and the cell adhesion ability of *H. pylori* were assessed.

RESULTS
CagA and *VacA* expression was upregulated at first and then downregulated in the *H. pylori* strains after sodium selenite treatment. Their expression was significantly and steadily downregulated after the 5th cycle (10 d). Transcriptome analysis revealed that sodium selenite altered the levels affect *H. pylori* virulence factors such as *CagA*, *CagM*, *CagE*, *Cag1*, *Cag3*, and *CagT*. Of these factors, *CagM* and *CagE* expression was continuously downregulated and further downregulated after 2 h of induction with sodium selenite. Moreover, *CagT* expression was upregulated before the 3rd cycle (6 d) and significantly downregulated after the 5th cycle. *Cag1* and *Cag3* expression was upregulated and downregulated, respectively, but no significant change was observed by the 5th cycle. C57BL/6A mice were infected with the attenuated strains subjected to sodium selenite induction. The extent of *H. pylori* colonization in the stomach increased; however, sodium selenite also induced a mild inflammatory reaction in the gastric mucosa of *H. pylori*-infected mice, and the cell adhesion ability of *H. pylori* was significantly weakened.

CONCLUSION
These results demonstrate that *H. pylori* displayed virulence attenuation after the 10th d of sodium selenite treatment. Sodium selenite is a low toxicity compound with strong stability that can reduce the cell adhesion ability of *H. pylori*, thus mitigating the inflammatory damage to the gastric mucosa.

Key Words: *Helicobacter pylori*; Sodium selenite; Virulence factors; Adherence; Inflammation

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Core Tip: The situation caused by *Helicobacter pylori* drug resistance is critical. Selenium is one of the trace elements in the human body, and its content in the stomach is related to the degree of *H. pylori* infection. Initial studies have shown that sodium selenite has inhibitory effects on *H. pylori*, so we further investigated the mechanism of action of sodium selenite on *H. pylori*. This study provides an experimental basis for use of the trace element selenium in the treatment of *H. pylori* infection.

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INTRODUCTION

Helicobacter pylori is a common gram-negative bacterium that colonizes the stomach. Half of the global population is infected with this bacterium, which causes gastritis, stomach ulcers, and other diseases. In 1994, the World Health Organization listed *H. pylori* as a Class I carcinogen. In 2022, the United States listed this bacterium on the list of microorganisms associated with cancer[1,2].

The control and treatment of *H. pylori* infections can reduce gastric cancer incidence. However, because of the extensive use of antibiotics against *H. pylori*, the drug resistance rate has increased, and the eradication rate has significantly decreased[3]. Therefore, new antibacterial drugs or prevention and control programs need to be developed to alleviate the public health threat posed by *H. pylori*. Imitating live vaccines that lower *H. pylori* toxicity and reduce pathogenicity serves as better control methods.

CagA and *VacA*, the main *H. pylori* virulence factors, are associated with inflammation, apoptosis, autophagy, and the epithelial–mesenchymal transformation[4]. *CagA*, a bacterial oncoprotein[5], and *VacA*, which promotes the survival of *H. pylori* in gastric epithelial cells, are associated with phenomena such as mitochondrial damage[4]. The *cag* pathogenicity island (CagPAI) contains secretory protein-encoding *CagA* genes and type IV secretion system (T4SS) genes including *CagE*, *CagM*, *Cag3*, *CagT*, *CagX*, and *CagY*. These genes are involved in the T4SS activity[6]. However, the mutual regulation of these genes is not well understood.

The incidence of *H. pylori* infection is 10%-15%. Most people infected with *H. pylori* do not develop disease; however, the reason for this remains unclear. The mechanism underlying the interaction between *H. pylori* and the environment and body needs to be further explored. Selenium (Se), a trace element in the human body[7,8], plays a major role in immunoregulation, antioxidation, and antitumor and antibacterial functions[9-12]. As an environmental factor in the stomach, Se is closely associated with *H. pylori* pathogenesis. Ustündağ *et al*[13] found that the stomach Se level is higher in the early stage of *H. pylori* infection but significantly decreases when *H. pylori* induces precancerous lesions. Burguera *et al*[14] found that the stomach Se content is significantly lower in patients with gastric ulcers and gastric cancer than in those with gastritis. Liu[15] found that sodium selenite can promote gastric ulcer healing[16,17], but it remains unknown why Se content in the patient's stomach decreases and how Se promotes ulcer healing.

Therefore, in this study, we explored how the Se-rich environment acts on *H. pylori* virulence factors by inducing *H. pylori* with sodium selenite and investigated the interaction between Se and *H. pylori* in light of the toxicity and inflammatory injury mediated by strains in the stomach. These findings provide an experimental basis for Se application for the prevention and treatment of *H. pylori* infection-related diseases.

MATERIALS AND METHODS

The cells and bacteria

The human gastric mucosal epithelial cell line GES-1 (No. CC4026; Guangzhou Cellcook Biotech Co., Ltd., Guangzhou, China) and human gastric cancer cell line BGC823 (Nanjing Kaijian Biotech Co., Ltd., Nanjing) were purchased. *H. pylori* strains (159, 26695, G27, and NSH57) were all provided by Professor Bi Hongkai, Laboratory of Pathogen Biology, Nanjing Medical University (Nanjing, China).

Induction and culture of H. pylori cells with sodium selenite

Hp G27 and NSH57 bacterial solutions were prepared at an initial concentration of 1×10^5 colony-forming units (CFU)/mL. The sodium selenite concentration for induction was determined according to the minimal inhibitory concentration (MIC) results, and the induction time was determined according to the growth curve. Sequential induction was performed using culture medium containing the same sodium selenite concentration for continuous passages.

Quantitative PCR targeting virulence factors in H. pylori strains to determine mRNA expression after sodium selenite induction

RNA was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (No. RC112-01; Vazyme Biotech Co., Ltd., Nanjing, China). cDNA was obtained through RNA reverse transcription using a dsDNase-containing reverse transcription premix (No. MR05101M; Monad Biotech Co., Ltd., Suzhou, China). The MonAmp SYBR Green qPCR Mix (No. MQ10301S; Monad Biotech) was used to configure the quantitative PCR (qPCR) system, and the Light Cycler 96 was used for amplification, with the procedures adjusted as follows: initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 30 s; all steps were performed for 40 cycles. The corresponding calculations were made according to the following equation: $2^{-\Delta CT\ (test)\ -\Delta CT\ (calibrator)}$ = relative expression. The mRNA expression of *CagA*, *VacA*, 16s RNA, interleukin 6 (IL-6), IL-8, tumor necrosis factor-alpha (TNF-α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *Cag1*, *Cag3*, *CagT*, *CagM*, and *CagE* was determined. Table 1 lists the design and synthesis of the primers.

Western blotting of H. pylori virulence factor proteins after sodium selenite induction

In total, 3-7 mL of *H. pylori* bacterial solution was centrifuged at 12000 rpm for 2 min. The supernatant was removed and dosed with approximately 130 mL RIPA lysate (No. P0013B; Shanghai Beyotime Biotech Co., Ltd., Shanghai, China). Then a protease inhibitor and 0.1% EDTA were added at a 100:1 ratio. Thereafter, the solution was mixed on ice and ultrasonicated for approximately 5 min until the solution became clear. The solution was centrifuged at 13000 rpm for 5 min (4 °C) to obtain the supernatant protein solution. The protein concentration of the obtained solution was determined and the sample was divided to ensure 100 mg per tube. Then the samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) loading buffer, denatured in boiling water at 100 °C for 5–10 min, and stored at -80 °C.

A PAGE Gel Fast Preparation Kit (10%, No. PG112; Shanghai Epizyme Biomedicine Technology Co., Ltd., Shanghai, China) was used for colloid preparation. Electrophoresis was performed using the loading protein and marker at 80 V for 30 min and 120 V for 1 h, respectively; the proteins were electrotransferred to a PVDF membrane at a constant current of 300 mA for 3 h. The protein gel was placed in a PVDF membrane for 1 h for blocking; transferred into the primary antibody dilution buffer containing *CagA* (A-10), *VacA*, and GAPDH antibodies at dilution ratios of 1:500, 1:500, and 1:6000, respectively; and incubated at 4°C overnight.

Thereafter, the PVDF membrane was washed three times with 0.1% Tween 20 Detergent (1 ×) on a shaker (10 min each time). The secondary antibody dilution buffer containing the mouse IgGκ light chain binding protein horseradish peroxidase (HRP) antibody and the goat anti-rabbit IgG (H + L) HRP antibody (both diluted at 1:6000) was added. After incubation for 2 h, the aforementioned washing steps were repeated.

Using an enhanced chemiluminescence detection kit, the image was developed in the automatic chemiluminescence image analyzer. The gray values of the bands were quantified using ImageJ software. The expression of all proteins was compared with that of GAPDH. Table 2 presents the antibody information.

Table 1 Primer list

Name	Sequence	Company performing synthesis
CagA	F: ACCCCTAGTCGGTAATG	Shanghai Invitrogen Biotech Co., Ltd.
	R: GCTTTAGCTTCTGATACTGC	
VacAs1a	F: GTCAGCATCACACCGCAAC	Shanghai Generay Biotech Co., Ltd.
	R: CTGCTTGAATGCGCCAAAC	
16sRNA	F: CTGGAGAGACTAAGCCCTCC	Shanghai Invitrogen Biotech Co., Ltd.
	R: AGGATCAAGGTTTAAGGATT	
IL-6	F: GCAGAAAAAGGCAAAGAATC	Wuhan Genecreate Biotech Co., Ltd.
	R: CTACATTTGCCGAAGAGC	
IL-8	F: CACCGGAAGGAACCATCTCA	Wuhan Genecreate Biotech Co., Ltd.
	R: TGGCAAAACTGCACCTTCACA	
TNF-α	F: TCTTCTCGAACCCCGAGTGA	Wuhan Genecreate Biotech Co., Ltd.
	R: CCTCTGATGGCACCACCAG	
GAPDH	F: GGACCTGACCTGCCGTCTAG	Wuhan Genecreate Biotech Co., Ltd.
	R: GTAGCCCAGGATGCCCTTGA	
Cag1	F: GCTATGGGGATTGTTGGGATAA	Shanghai Sangon Biotech Co., Ltd.
	R: GCTTCAGTTGGTTCGTTGGTAA	
Cag3	F: GACACCTTGAATGTGAATGACAAA	Shanghai Sangon Biotech Co., Ltd.
	R: GTTGTAATACCCATTGACTTGCTCTAA	
CagT	F: TCTAAAAAGATTACGCTCATAGGCG	Shanghai Sangon Biotech Co., Ltd.
	R: CTTTGGCTTGCATGTTCAAGTTGCC	
CagE	F: GCGATTGTTATTGTGCTTGTAG	Shanghai Sangon Biotech Co., Ltd.
	R: GAAGTGGTTAAAAAATCAATGCCCC	
CagM	F: ACAAATACAAAAAAGAAAAAGAGGC	Shanghai Sangon Biotech Co., Ltd.
	R: ATTTTTCAACAAGTTAGAAAAAGCC	

16sRNA: 16S ribosomal RNA; CagA: Cytotoxin-associated protein A; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IL-6: Interleukin 6; TNF-a: Tumor necrosis factor-alpha; VacAs1a: Vacuolating cytotoxin gene As1a.

Table 2 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

CagA: Cytotoxin-associated protein A; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HPR: Horseradish peroxidase; H: Heavy chain; L: Light chain; m-IgGk BP-HRP: Mouse IgGκ light chain binding protein; VacA Vacuolating cytotoxin gene A.

Differentially expressed genes of H. pylori after sodium selenite induction were determined by transcriptome sequencing

To explore the effect of the half inhibitory concentration of sodium selenite on the Hp G27 strain, the bacterial solution was prepared (1 × 10^8 CFU/mL); treated with sodium selenite at concentrations of 1/8, 1/5, 1/4, 1/2, and 1 times the

MIC; and cultured for 0, 2, and 8 h. Thereafter, 1 mL solution was coated over the plate and the plate was cultured for 3 d. The colonies were counted to determine the concentration of the original bacterial solution. The concentration at which the colony count remained relatively stable for 0, 2, and 8 h after sodium selenite treatment was considered the half inhibitory concentration and used as the transcriptome induction concentration.

The transcriptome analysis samples were treated with sodium selenite at a concentration of 1/5 the MIC. The intervention experiment was repeated three times to obtain three sample groups (n = 9 samples). The markers S_0, S_2, and S_8 represent the treatment groups at 0, 2, and 8 h, respectively. Sequencing and analysis were performed by Nanjing Fengzi Biomedical Technology Co., Ltd. (Nanjing, China).

Detection of the cell adhesion ability of H. pylori strains after induction with sodium selenite

Phosphate-buffered saline (PBS), *H. pylori*, induction, and *H. pylori* + 4 mol/L sodium selenite groups were used for the Alma blue experiments. GES-1 cells were cultured in a 96-well plate for 18-24 h (1×10^4 cells/well) and infected with *H. pylori* for 1 h (multiplicity of infection [MOI] = 300:1). Then the cells that did not adhere to the GES-1 cells were slowly removed, and the adhering cells were treated with 50 mL of 0.4% saponin solution for 5 min. Thereafter, the cells with the spent solution were removed, added to the culture medium containing 100 mL *H. pylori*, treated with 10 mL Alma blue, inoculated in a three-gas incubator for 4-6 h, and analyzed using a multimode reader.

The fluorescence experiential groups were divided into the same categories as those in the Alma blue experiment, as previously described. After culturing BGC823 cells in a 24-well plate for 18-24 h (5×10^4 cells/well), the cells were fluorescently stained with SYTO9 reagent, infected with *H. pylori* strains (MOI = 100:1), and cultured for another 3 h. The cells adhering to *H. pylori* strains were observed under an inverted fluorescence microscope.

Determination of inflammatory factor levels in H. pylori-infected cells induced with sodium selenite

The GES-1, GES-1 + G27, GES-1 + G27 induced, GES-1 + G27 + Se, GES-1 + Se, and Se groups were included in this experiment, and all groups were treated with 4 mol/L sodium selenite. GES-1 cells were cultured in a 6-well plate for 18-24 h (3×10^5 cells/well). After 24 h of infection with *H. pylori* strains (MOI = 100:1), the levels of inflammatory factors, such as IL-6, were determined.

Determination of the pathogenicity of H. pylori after sodium selenite induction in mice

Thirty specific pathogen-free (SPF) C57BL/6J mice (age: 6-7 wk) were purchased from Changsha Tianqin Biological Co., Ltd. (No. 430726210100134118; SPF Animal Use License No. SYXK Gui 2017-0004; Animal Experiment Ethics No. 2019112501; Changsha, China). The mice were randomly divided into the following five groups: PBS, Se, NSH57, NSH57-induced, and NSH57 + Se groups. Mice in the Se group were intragastrically administered sodium selenite (4 mol/L), and those in the induction group were administered sodium selenite (4 mol/L) for six cycles. The corresponding bacterial solutions were centrifuged at 12000 rpm for 2 min, and the supernatant was removed to obtain the precipitate. Thereafter, the solutions were resuspended in fresh medium or medium containing four sodium selenite, with the concentration adjusted to 1×10^9 CFU/mL.

The mice were fasted and deprived of water for 12 h before intragastric administration. Thereafter, they were intragastrically administered the solutions (0.5 mL/mouse), once every alternate day five consecutive times, and the diets were resumed diet 4 h after intragastric administration. After all rounds of intragastric administration, the mice were kept for 3 wk and dissected following the aseptic operation procedure. The tissues collected from stomachs, livers, spleens, and kidneys were fixed in 10% formalin and stained with hematoxylin and eosin (H&E). The whole stomach was divided into two parts along its major and minor curves, with the stomach contents gently scraped away. One part was fixed in H&E staining, and a fluorescence immunoassay was conducted to measure the levels of the inflammatory factors IL-1β, IL-6, and TNF-α. The other part was placed in a sterile Eppendorf tube containing magnetic beads, and the medium was weighed and recorded before and after induction, and crushed well using a multisample tissue grinder at 50 Hz three times (3 min each time). Then 100 mL abrasive solution was diluted 10-, 100-, and 1000-fold. Thereafter, 100 mL of each of the three diluted concentrations was coated over a solid plate containing selective antibiotics and cultured for 3-4 d. Then the number of *H. pylori* CFU/g or the extent of *H. pylori* colonization in the stomach was calculated.

Statistical analysis

Statistical analysis and mapping were performed using GraphPad Prism 8.0. The continuous data are expressed as the mean ± standard deviation. Differences in means between the groups were analyzed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Induction with sodium selenite in vitro downregulated the expression of the H. pylori virulence factors CagA and VacA

According to the MIC and cytotoxic effects of sodium selenite on *H. pylori* observed in Supplementary Table 1 and Figure 1, 0, 4, 8, and 16 μmol/L sodium selenite was used to induce Hp G27 (48 h as a cycle), and sequential induction was performed after the medium was changed.

qPCR results revealed that after Hp G27 was sequentially induced with sodium selenite for 1-6 cycles, the mRNA expression of *CagA* was slightly downregulated in the 1st cycle, upregulated in the 2nd cycle, significantly upregulated in the 3rd cycle, downregulated again in the 4th cycle, and downregulated steadily and significantly throughout the 5th and 6th

Table 3 Identified differentially expressed genes (S_2 *vs* S_0)

Gene_id	Readcount_S_2	Readcount_S_0	Log2 fold change	*P* value	P_{adj}	Gene name	Description
Gene509	376.5506144	256.0954025	0.55616	0.0066152	0.035684	HPG27_RS02500	Cag pathogenicity island protein CagI
Gene521	1368.459246	801.0552768	0.77258	5.49E-05	0.00089411	HPG27_RS02560	Type IV secretion system apparatus protein CagT
Gene511	1617.425068	1154.535615	0.48639	0.005033	0.28476	HPG27_RS02510	Type IV secretion system apparatus protein Cag3
Gene540	181710.1299	131892.6179	0.46228	0.0039181	0.023777	HPG27_RS02655	Type IV secretion system oncogenic effector CagA
Gene536	1945.873607	3339.614831	-0.77926	0.00032024	0.0036791	HPG27_RS02635	VirB4 family type IV secretion/conjugal transfer ATPase

Table 4 Identified differentially expressed genes (S_8 *vs* S_0)

Gene_id	Readcount_S_8	Readcount_S_0	Log2 fold change	*P* value	P_{adj}	Gene name	Description
Gene509	494.7491661	231.643609	1.0948	1.57E-06	3.04E-05	HPG27_RS02500	Cag pathogenicity island protein CagI
Gene521	1519.085968	724.4396343	1.0683	2.78E-07	7.47E-06	HPG27_RS02560	Type IV secretion system apparatus protein CagT
Gene511	1562.119955	1044.031209	0.58134	0.0021884	0.010463	HPG27_RS02510	Type IV secretion system apparatus protein Cag3
Gene529	311.1908412	460.4755565	-0.56532	0.0049482	0.020333	HPG27_RS02595	Type IV secretion system apparatus protein CagM

cycles (Figure 1A).

VacA mRNA expression was slightly upregulated in the 1st cycle, significantly upregulated in the 2nd cycle, downregulated again throughout the 3rd and 4th cycles, and downregulated steadily and significantly throughout the 5th and 6th cycles (Figure 1B). Western blotting results revealed that *CagA* protein expression changed in a manner consistent with that of mRNA in the 1st cycle. In the 3rd cycle, the amount of mRNA at 4 and 8 mol/L was significantly increased, whereas the protein expression basically remained unchanged. In the 6th cycle, the protein expression was downregulated in a manner consistent with the decreased mRNA expression.

VacA mRNA expression increased in the 1st and 3rd cycles, whereas its protein expression remained unchanged. In the 6th cycle, the protein expression was downregulated in a manner consistent with the decreased mRNA expression (Figure 1C-E). The mRNA expression of *CagA* and *VacA* was upregulated, whereas their protein expression remained unchanged. These results indicated that sodium selenite was possibly acting on biological processes posttranscriptionally or posttranslationally. The same results were obtained after the sequential induction of NSH57 strains for six cycles, and the protein expression was downregulated in a manner consistent with the decreased mRNA expression (Figure 1F-H). However, the aforementioned results suggested that the doses of 4, 8, and 16 μmol/L could induce virus attenuation, with 16 μmol/L being the best concentration.

Changes in the H. pylori transcriptome after sodium selenite induction

H. pylori induced with sodium selenite at a concentration of 1/5 the MIC exhibited only slight growth. These results suggested that this concentration could be used to achieve a half-maximal inhibitory effect (Figure 2A). Therefore, this concentration was selected for induction. The RNA sequencing (RNA-seq) correlation test of the transcriptional group revealed that R^2 values in the repeated intervention group were all > 0.9, thereby exhibiting good repeatability among the samples. The differences in R^2 values were noted among the different intervention groups (Figure 2B). Principal component analysis revealed significant differences between the repeated and non-repeated samples (Figure 2C). The transcriptome analysis results are as follows: (1) 329 differentially expressed genes (DEGs) were detected between S_2 and S_0; (2) 466 DEGs were detected between S_8 and S_0; and (3) 85 DEGs were detected between S_8 and S_2. Overall, 247, 25, and 54 identical genes were detected between (1) and (2), (1) and (2), and (2) and (3), respectively, and 13 identical DEGs were detected between (1), (2), and (3) (Figure 2D and E). Among the top 20 pathways where the DEGs of (1), (2), and (3) were enriched, signal transduction pathways of epithelial cells associated with *H. pylori* infection were found to be associated with virulence factors (as shown in Figure 2F-H). The "*CagPAI-CagA*" pathway related to *CagA* virulence was further identified (Figure 2I). Thereafter, follow-up verification experiments were conducted on the genes with upregulated expression, *Cag1*, *Cag3* and *CagT,* and the genes with downregulated expression, *CagM* and *CagE,* that had been identified based on significant differences, repeatability of differential expression between (1) and (2), and whether they encoded unknown proteins, as shown in Tables 3 and 4. Therefore, the transcriptional analysis suggested that virulence factors of *H. pylori* after sodium selenite induction were mainly enriched in *H. pylori* infection-associated

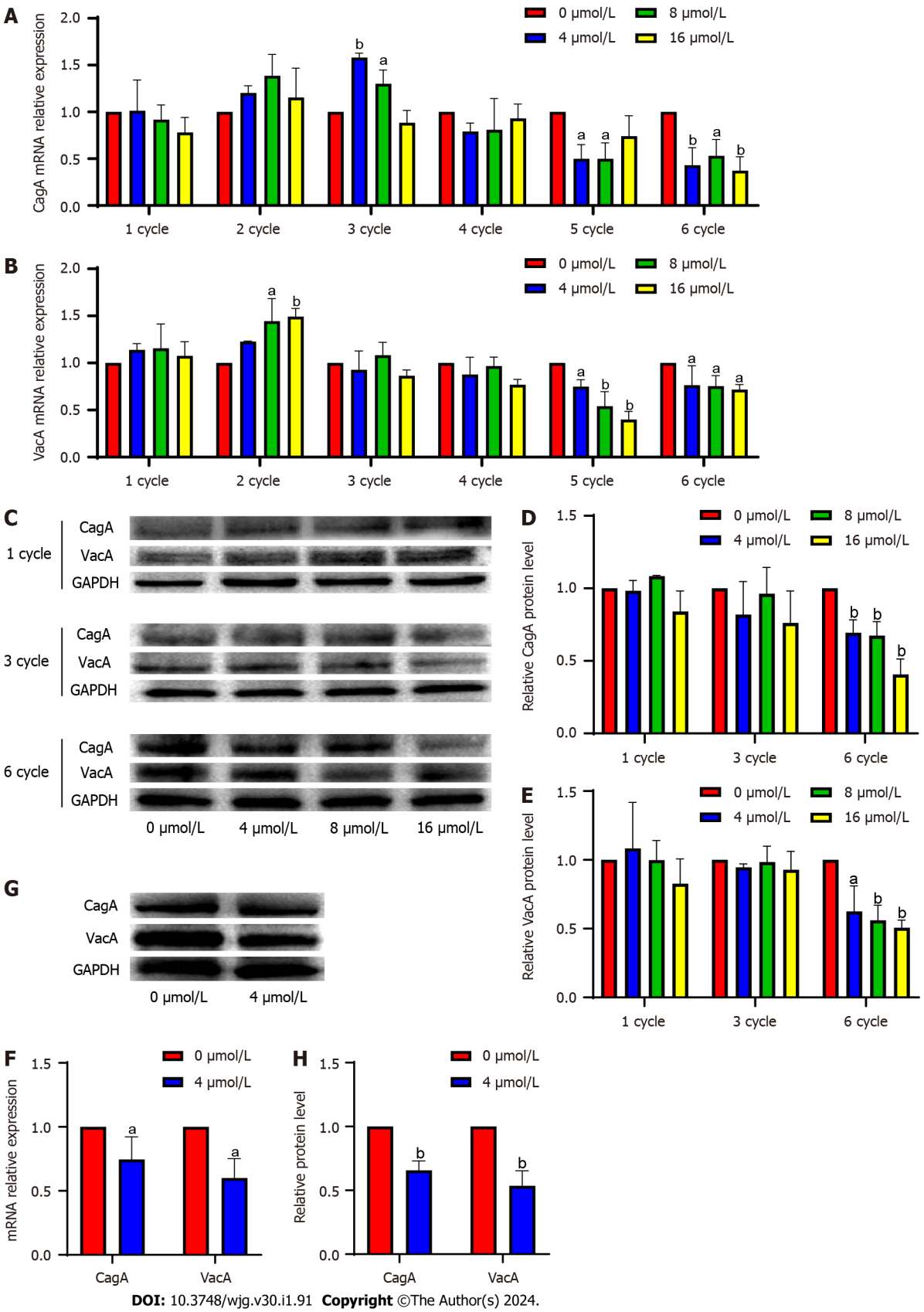

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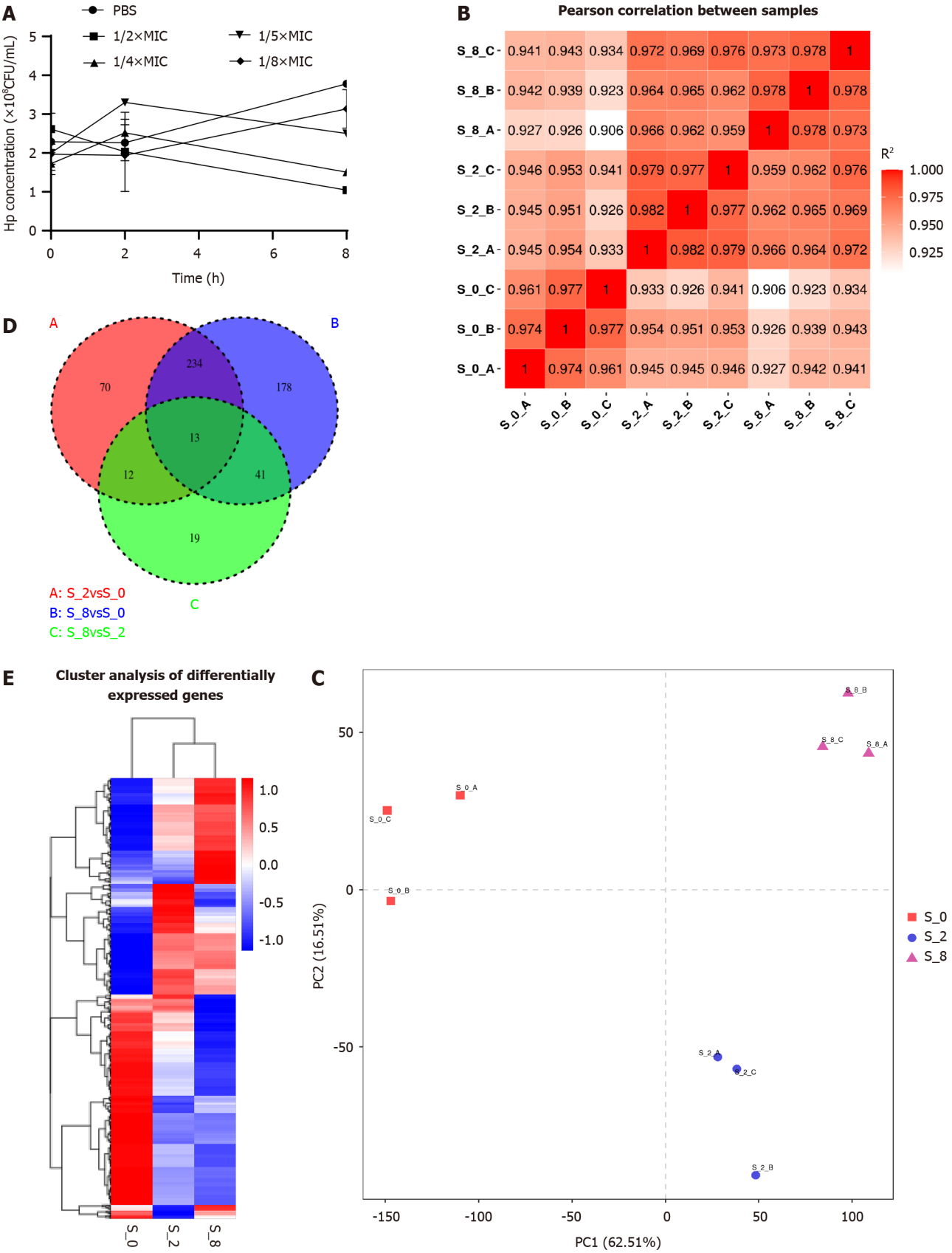
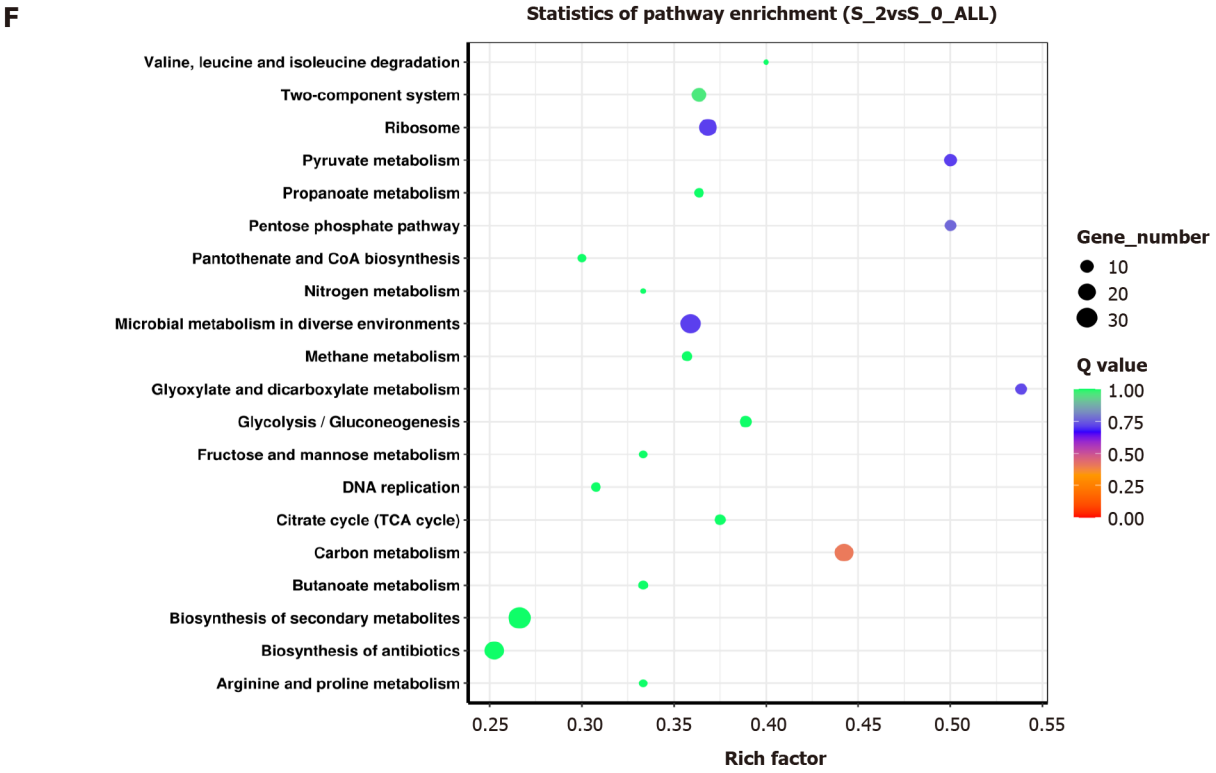
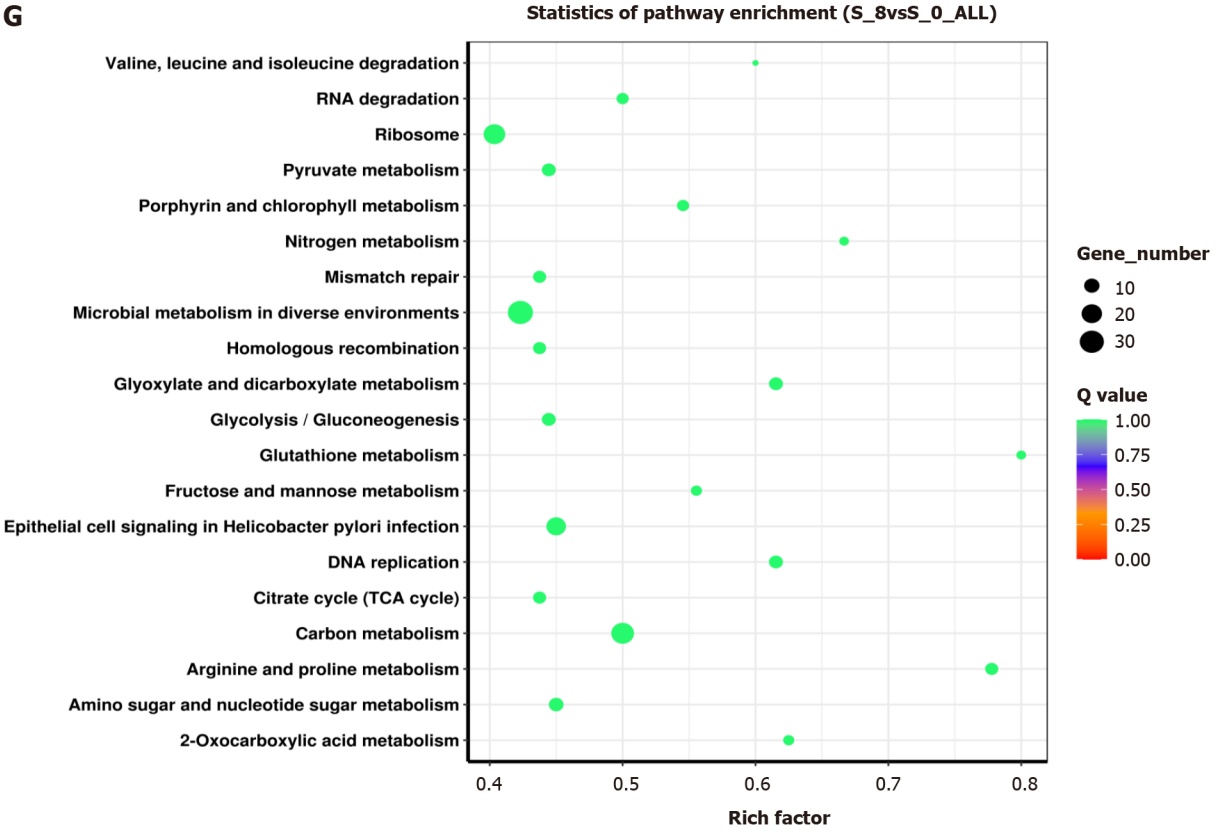
Figure 1 Induction of *Helicobacter pylori* with sodium selenite downregulates cytotoxin-associated protein A and *vacuolating cytotoxin gene A* expression. A: Effect of induction of the Hp G27 strain with sodium selenite on cytotoxin-associated protein A (*CagA*) for 1-6 cycles; B: Effect of induction of the Hp G27 strain with sodium selenite on vacuolating cytotoxin gene A (*VacA*) for 1-6 cycles; C-E: Effect of induction of the Hp G27 strain with sodium selenite on the *CagA* and *VacA* proteins for 1, 3, and 6 cycles; F: Effect of induction of the Hp G27 strain with 4 mol/L sodium selenite on *CagA* and *VacA* of the NSH57 strain for 6 cycles; G and H: Effect of induction of the NSH57 strain with 4 μmol/L sodium selenite on *CagA* and *VacA* protein. $^{a}P < 0.05$; $^{b}P < 0.01$.

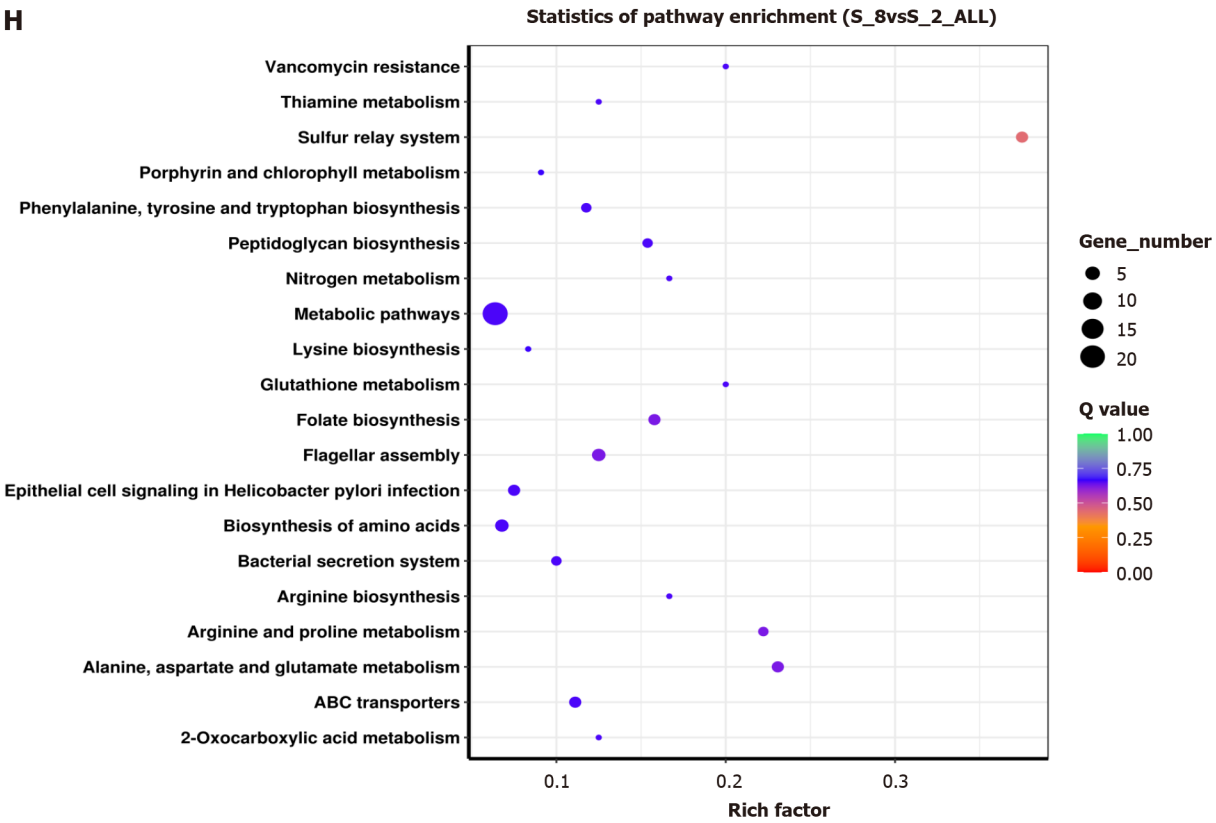

A
PBS
1/2×MIC
1/4×MIC
1/5×MIC
1/8×MIC
Hp concentration (×10⁸CFU/mL)
Time (h)
B
Pearson correlation between samples
S_8_C 0.941 0.943 0.934 0.972 0.969 0.976 0.973 0.978 1
S_8_B 0.942 0.939 0.923 0.964 0.965 0.962 0.978 1 0.978
S_8_A 0.927 0.926 0.906 0.966 0.962 0.959 1 0.978 0.973
S_2_C 0.946 0.953 0.941 0.979 0.977 1 0.959 0.962 0.976
S_2_B 0.945 0.951 0.926 0.982 1 0.977 0.962 0.965 0.969
S_2_A 0.945 0.954 0.933 1 0.982 0.979 0.966 0.964 0.972
S_0_C 0.961 0.977 1 0.933 0.926 0.941 0.906 0.923 0.934
S_0_B 0.974 1 0.977 0.954 0.951 0.953 0.926 0.939 0.943
S_0_A 1 0.974 0.961 0.945 0.945 0.946 0.927 0.942 0.941
S_0_A S_0_B S_0_C S_2_A S_2_B S_2_C S_8_A S_8_B S_8_C
R²
1.000
0.975
0.950
0.925
D
A
B
C
70
234
178
13
12
41
19
A: S_2vsS_0
B: S_8vsS_0
C: S_8vsS_2
E
Cluster analysis of differentially expressed genes
1.0
0.5
0.0
-0.5
-1.0
S_0
S_2
S_8
C
PC2 (16.51%)
PC1 (62.51%)
S_0
S_2
S_8

F


Statistics of pathway enrichment (S_2vsS_0_ALL)
Valine, leucine and isoleucine degradation
Two-component system
Ribosome
Pyruvate metabolism
Propanoate metabolism
Pentose phosphate pathway
Pantothenate and CoA biosynthesis
Nitrogen metabolism
Microbial metabolism in diverse environments
Methane metabolism
Glyoxylate and dicarboxylate metabolism
Glycolysis / Gluconeogenesis
Fructose and mannose metabolism
DNA replication
Citrate cycle (TCA cycle)
Carbon metabolism
Butanoate metabolism
Biosynthesis of secondary metabolites
Biosynthesis of antibiotics
Arginine and proline metabolism
0.25
0.30
0.35
0.40
0.45
0.50
0.55
Rich factor
Gene_number
10
20
30
Q value
1.00
0.75
0.50
0.25
0.00

G


Statistics of pathway enrichment (S_8vsS_0_ALL)
Valine, leucine and isoleucine degradation
RNA degradation
Ribosome
Pyruvate metabolism
Porphyrin and chlorophyll metabolism
Nitrogen metabolism
Mismatch repair
Microbial metabolism in diverse environments
Homologous recombination
Glyoxylate and dicarboxylate metabolism
Glycolysis / Gluconeogenesis
Glutathione metabolism
Fructose and mannose metabolism
Epithelial cell signaling in Helicobacter pylori infection
DNA replication
Citrate cycle (TCA cycle)
Carbon metabolism
Arginine and proline metabolism
Amino sugar and nucleotide sugar metabolism
2-Oxocarboxylic acid metabolism
0.4
0.5
0.6
0.7
0.8
Rich factor
Gene_number
10
20
30
Q value
1.00
0.75
0.50
0.25
0.00



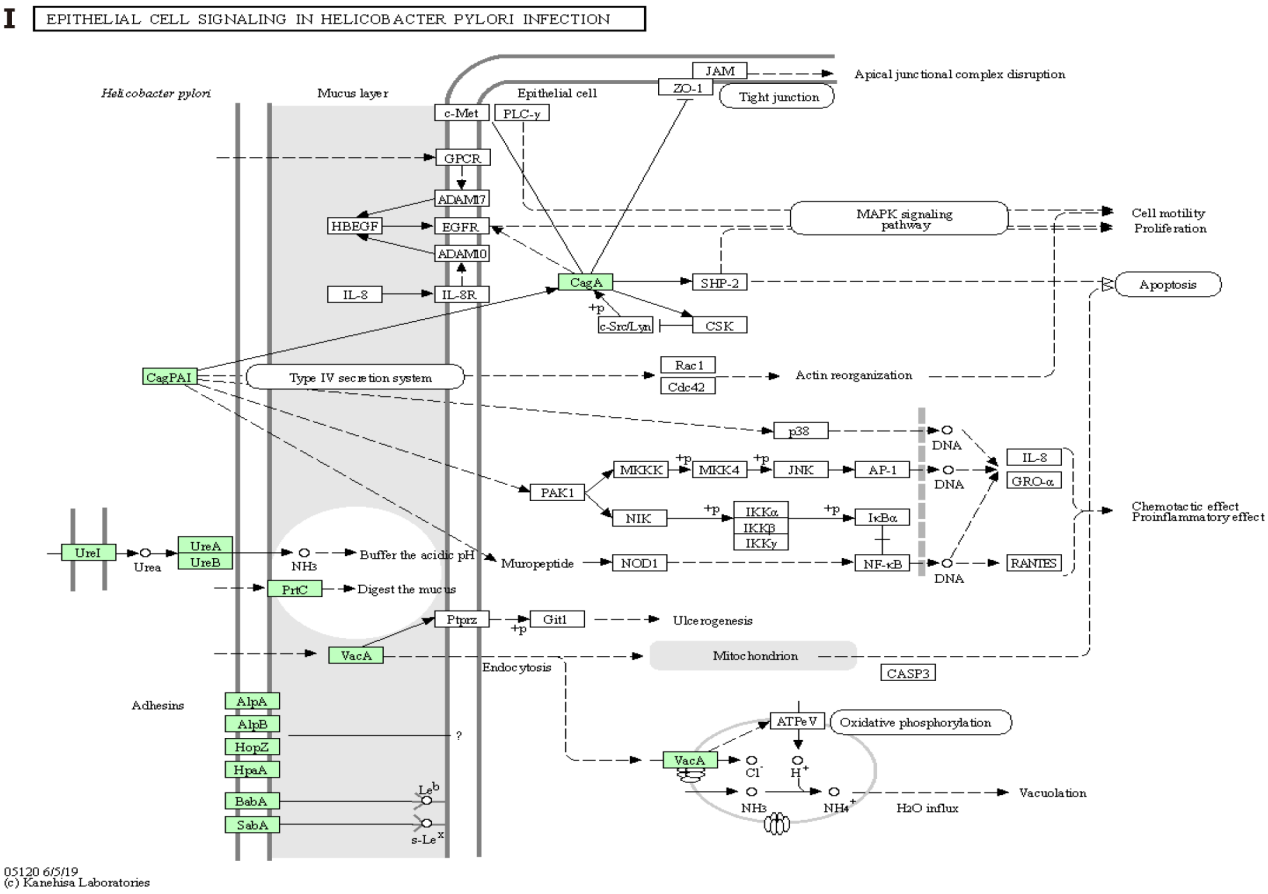


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Figure 2 Effect of sodium selenite on the *Helicobacter pylori* transcriptome. A: Half inhibitory concentration of sodium selenite given to *Helicobacter pylori*; B: RNA sequencing correlation examination; C: Principal component analysis of the transcriptome; D: The Venn diagram of differentially expressed genes (DEGs); E: Cluster analysis of DEGs; F-H: Gene set enrichment analysis of DEGs between treatment groups; I: Epithelial cell signal transduction pathways associated with *H. pylori* infection.

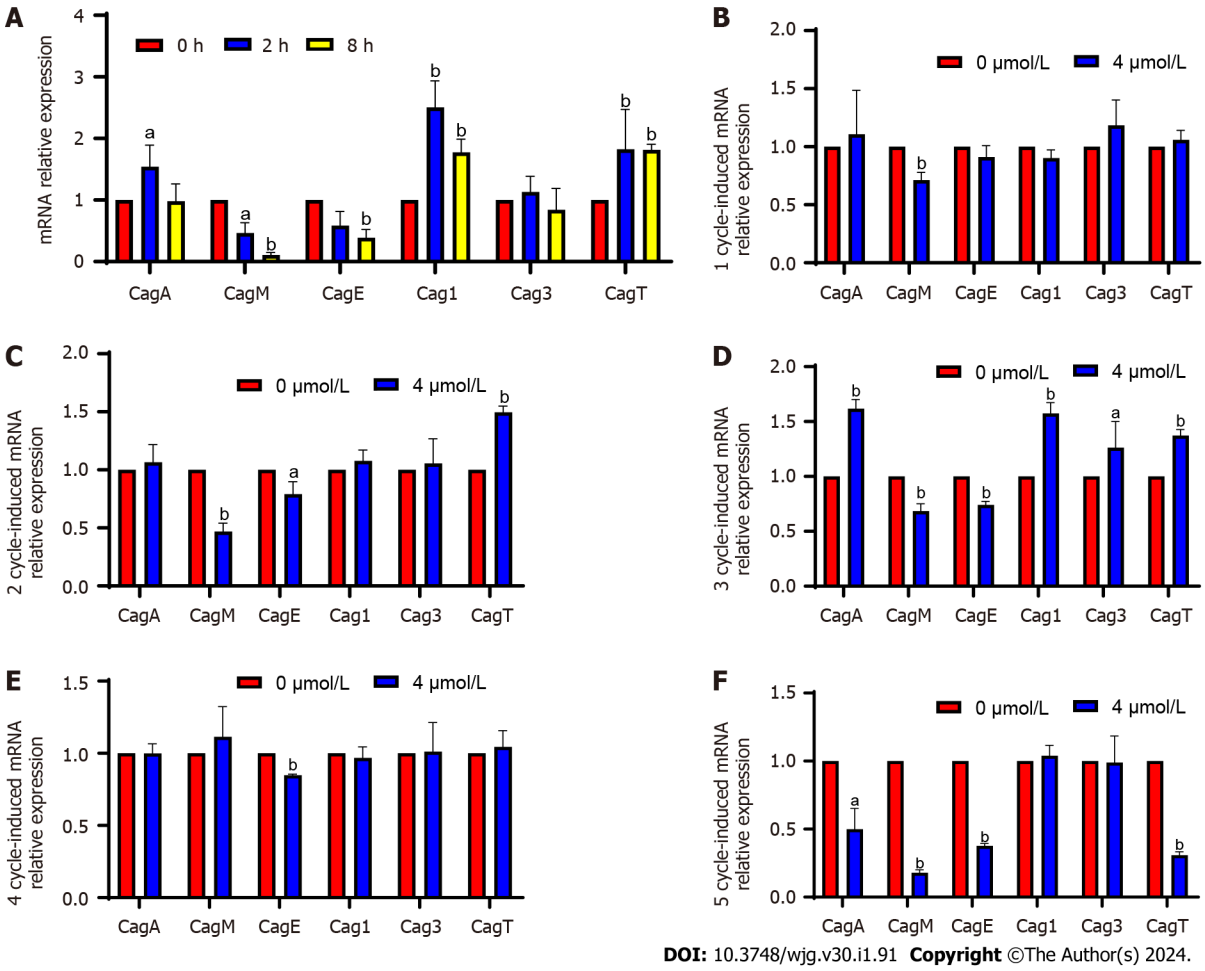

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Figure 3 Verification of differentially expressed genes and changes in screened differentially expressed genes following induction. A: Quantitative PCR verification of differentially expressed genes (DEGs); B-F: Changes in DEGs after induction with 4 μmol/L sodium selenite for 1-5 cycles. $^{a}P < 0.05$; $^{b}P < 0.01$.

epithelial cell signal transduction pathways. The main virulence factors of *CagPAI* included *CagA*, *CagM*, *CagE*, *Cag1*, *Cag3*, and *CagT*, with the urease gene and *VacA* also being key genes.

Verification of and changes in DEGs following induction

The main virulence genes of *CagPAI* were verified through qPCR (Figure 3). After the induction, *CagM* expression was significantly downregulated at 2 and 8 h and from the 1st to the 3rd cycles, upregulated again in the 4th cycle, and significantly downregulated in the 5th cycle (4-10 d). After the induction, *CagE* expression was significantly downregulated at 2 and 8 h with no significant change noted in the 1st cycle (2 d) and significantly downregulation from the 2nd to 5th cycles (4-10 d). The expression of these two genes was upregulated at some stages, but was significantly and continuously downregulated overall. *CagM* expression was upregulated at some stages but significantly and continuously downregulated overall; *CagM* and *CagE* expression was continuously and significantly downregulated at the fastest rate. *Cag1* expression was significantly upregulated at 2 and 8 h and in the 3rd cycle after induction, but no significant change was observed at the other time points. Although *Cag3* expression was significantly upregulated in the 3rd cycle after induction, no significant change was observed at other time points. Because no uniform trend of change was observed in the two genes, they might not be considered key genes in attenuation. *CagT* expression was significantly upregulated at 2 and 8 h and in the 2nd and 3rd cycles after induction, downregulated in the 4th cycle, and significantly downregulated in the 5th cycle. However, the expression at most of the detection sites was consistent with the result of the transcriptome analysis; the expression was upregulated, but whether the expression would continue to be downregulated after the 5th wk remains to be determined.

Induction with sodium selenite in vitro reduced the cell adhesion ability of H. pylori

The cell adhesion ability of *H. pylori*, an important prerequisite for bacterial colonization and pathogenicity, was evaluated by inducing *H. pylori* with 4 μmol/L sodium selenite for 6 cycles. The Alamar Blue assay revealed that the number of Hp G27 cells adhering to GES-1 cells after induction was significantly decreased ($P < 0.05$) and that of Hp G27 cells adhering to cells after induction with 4 mol/L sodium selenite exhibited a decreasing trend ($P > 0.05$) (Figure 4A). The fluorescence experiment demonstrated that the number of Hp G27 cells that adhered to cells in the induction group was significantly reduced (the red fluorescence represents BGC823 cells, and the green fluorescence represents *H. pylori*

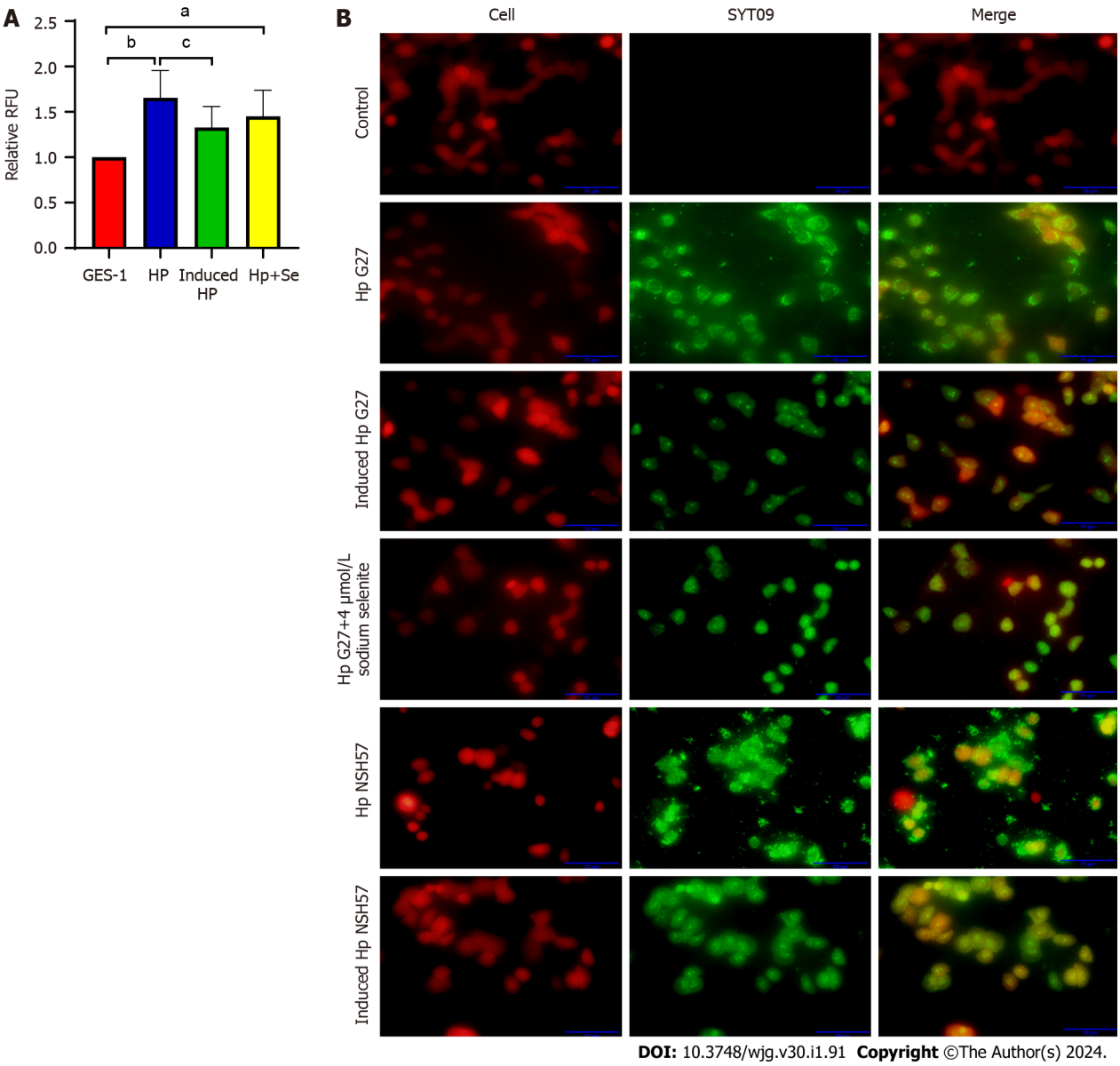

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Figure 4 Induction with sodium selenite decreased the adhesion ability of *Helicobacter pylori* strains. A: Number of cells adhering to *Helicobacter pylori* strains was determined using a fluorescence microscope; B: Number of cells adhering to *H. pylori* strains was determined with the SYTO 9 stain. [a] $P < 0.05$; [b]$P < 0.01$; [c]$P < 0.05$.

strains). The cell adhesion ability of NSH57 strains induced under the same conditions was the same as that of Hp G27 strains (Figure 4B). In conclusion, six induction cycles of different *H. pylori* strains with sodium selenite significantly reduced the cell adhesion ability of these strains.

Induction with sodium selenite reduced the inflammatory response triggered by H. pylori

After sodium selenite induction, GES-1 cells and mice were infected with *H. pylori* strains. The results of the cell infection experiment suggested that the relative mRNA expression of the inflammatory factors IL-6, IL-8, and TNF-α was significantly downregulated (Figure 5A-C). In the experiment including infected mice, the extent of *H. pylori* colonization in the *H. pylori*-induced groups was significantly increased ($P < 0.0001$), and inflammation was significantly reduced. However, inflammation was reduced in the mice in the noninduced *H. pylori* groups that had received sodium selenite intragastrically compared with that of those that had not in the same group. Furthermore, the degree of inflammation reduction in the noninduced group was not as good as that in the induced group. The gastric mucosal expression of IL-1β, IL-6, and TNF-α was reduced, similar to the degree of inflammation (Figure 5D and E).

The aforementioned results indicated that the inflammatory effect and virulence of *H. pylori* on cells and mice were significantly weakened after sodium selenite induction. However, the increased colonization might be a result of the weakened immune reaction in mice due to the reduced virulence of *H. pylori*.

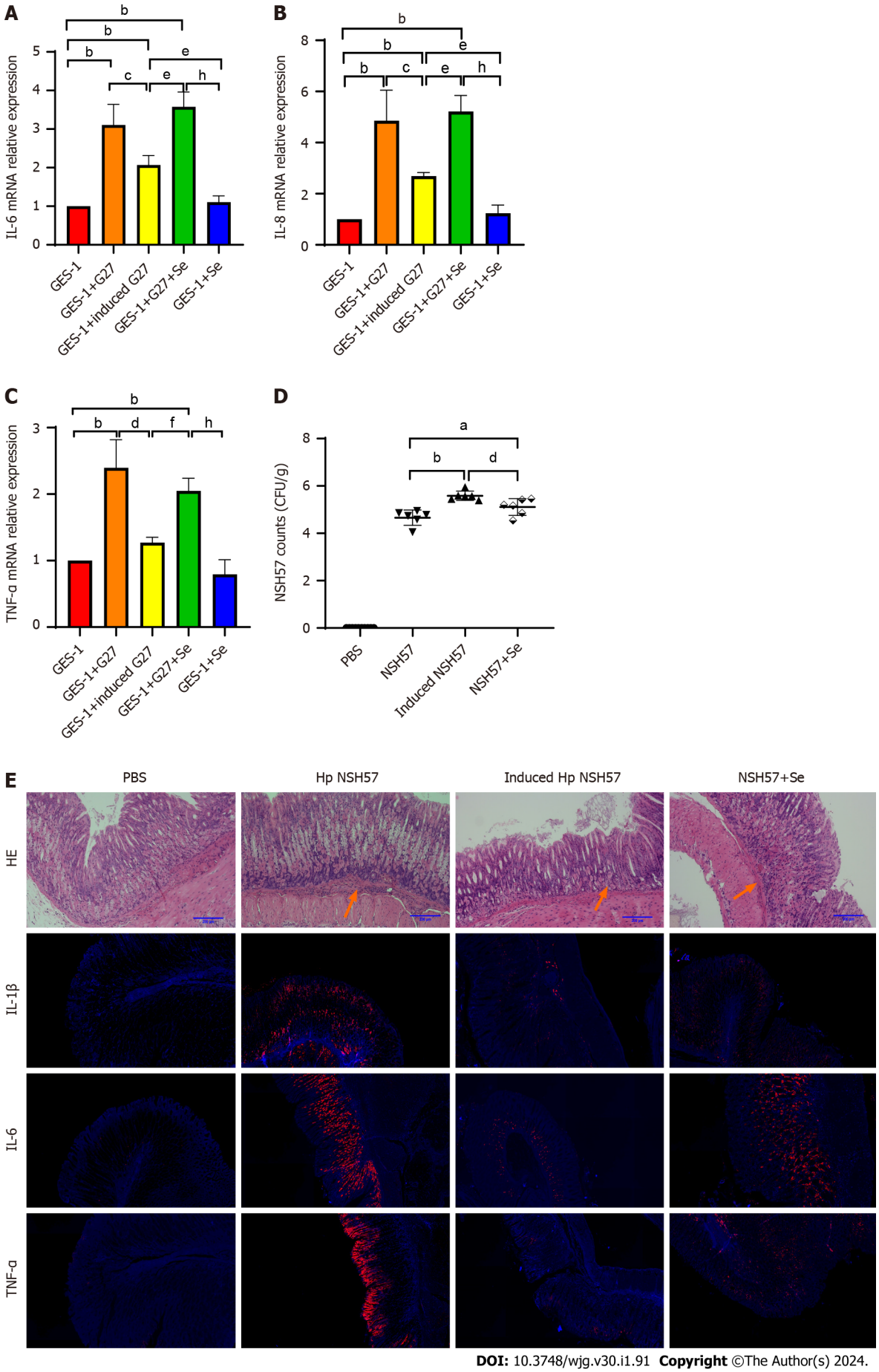

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Figure 5 Induction with sodium selenite decreased *Helicobacter pylori*-induced inflammation. A-C: Expression of IL-1β (A), IL-6 (B), and TNF-α

(C) in *Helicobacter pylori*-infected mice after induction; D: Gastric mucosal inflammation in mice with acute gastritis; E: *H. pylori* colonization in the stomach of mice with acute gastritis. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.05$; $^{d}P < 0.01$; $^{e}P < 0.05$; $^{f}P < 0.01$; $^{h}P < 0.01$.

DISCUSSION

Whether *H. pylori* infection causes disease depends on the bacteria, the environment, and the infected organism. Sodium selenite, a common inorganic Se, is also a common form of Se used for supplementation. Se has antibacterial effects[18,19]. The MIC of Se against *H. pylori* is 185 μmol/L, which is not strong enough to inhibit growth and makes it difficult to achieve a therapeutic effect, and high doses of Se can damage gastric mucosal cells. In addition, some infected people with high Se content in the body do not show any symptoms, although they carried *H. pylori* for a long time. This also reveals that the antibacterial effect of Se in the body is not the main factor and there are other factors affecting the development of *H. pylori* infection. In this study, after creating a Se-enriched environment and inducing *H. pylori* cells with sodium selenite, the expression of the *H. pylori* virulence factors *CagA* and *VacA* was significantly downregulated, and the downregulation of VacA expression was basically in line with the reports of Duan *et al*[20] and Shao *et al*[21]. However, as toxicity was not rapidly reduced after induction with sodium selenite, the reason and potential mechanism for the reduction in *H. pylori* virulence by sodium selenite is not clear.

In *H. pylori*, the *CagPAI*-encoded T4SS has 15-16 components. These components are mainly involved in *CagA* protein transport and play a certain role in immunogenicity[22]. T4SS can be divided into the core complex, inner membrane component protein, and outer membrane component protein. The core complex includes *CagT*, *CagX,* and *CagY*, the inner membrane component proteins include *CagE*, *CagW,* and *CagV*, and the outer membrane component proteins encompass *CagM* and *Cag3*[23]. Regarding the T4SS of *H. pylori*, studies have been conducted to determine changes in *CagPAI* expression to understand the relationship between this gene and diseases and the integrity of virulence genes to study the size of virulence factors[24,25]. *CagPAI* deletion caused changes in the T4SS, thereby weakening *H. pylori* toxicity; however, the bacterium was not completely detoxified[26,27]. Kumari *et al*[28] found for the first time that *CagW* could interact with *CagA* and play a crucial role in secretion while affecting the expression of flagellar components such as *CagL*. Similarly, *Cag1* deletion could lead to a decrease in *CagA* heterotopia function[29].

The DEGs of epithelial cell pathways were all screened from *CagPAI*. Meanwhile, *VacA,* which is outside *CagPAI,* was analyzed. *CagA* and *VacA* expression first increased and then decreased after sodium selenite induction and significantly decreased after 10 d. After sodium selenite induction for 2 h, *CagM* and *CagE* expression was continuously downregulated, whereas *CagT* expression was upregulated before the 6th d and downregulated significantly after the 10th d; while *Cag1* and *Cag3* expression was upregulated and downregulated, with no significant change noted on the 10th d. The above results suggest that these five genes may be key genes in the reduction of *H. pylori* virulence by sodium selenite in a Se-enriched environment and that the time needed to induce a reduction in *H. pylori* virulence is at least 10 d.

After induction of *H. pylori* by sodium selenite, its adhesion ability was tested *in vitro*, and its colonization and inflammatory damage to the gastric mucosa of mice was tested *in vivo*, both results demonstrated that sodium selenite could significantly reduce the adhesion ability and toxicity of inflammatory damage, and *H. pylori* stability was better in a Se-enriched environment. The application of sodium selenite to reduce the toxicity of *H. pylori* is an ideal method for the development of attenuated vaccines[30].

CONCLUSION

In this study, we investigated how a sodium-rich environment acts on *H. pylori* virulence factors, verified that sodium selenite could reduce *H. pylori* toxicity, and confirmed that a Se-rich environment could significantly reduce *H. pylori* pathogenicity. We also indicated that further studies are needed to elucidate the underlying molecular mechanisms. This study offers an experimental basis for the use of Se, a trace element, in *H. pylori* infection treatment in the future and a reference for ensuring that humans coexist with bacteria without developing diseases. Sodium selenite treatment is a potential method that will allow attenuated vaccines to alleviate severe drug resistance in *H. pylori*.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori is a common gram-negative bacterium that colonizes the stomach and is currently recognized as a class I carcinogen. The *H. pylori* infection rate is as high as approximately 60%, and the *H. pylori* eradication rate was markedly decreased with increasing rate of drug resistance. Finding new antimicrobial drugs or control regimens to mitigate the threat of *H. pylori* to human health and mimicking live vaccines is crucial. The preparation of live vaccines with reduced virulence and reduced pathogenicity is a promising control method. Selenium (Se) is one of the essential trace elements in the human body. Se has been proven to have an attenuating effect, but whether it has the same effect on *H. pylori* is unknown. The interaction among *H. pylori*, Se and its mechanism in the stomach has rarely been studied *H. pylori* has coexisted with humans for 100000 years, and eliminating this coexisting relationship is extremely difficult, this makes studying the effect of Se on *H. pylori* difficult. The influence of Se on the interaction between *H.pylori* and the organism

may provide new ideas and an experimental basis for the prevention and treatment of *H.pylori*.

Research motivation

Currently, *H. pylori* is a persistent threat to humans, and the increase in drug resistance makes it increasingly difficult to eliminate this threat. Studying the mechanism of the attenuating effect of the trace element Se on *H. pylori* and its interaction with the organism after attenuation will help to better apply Se to the development of *H. pylori* attenuated vaccines and alleviate the problem of *H. pylori* drug resistance.

Research objectives

The aim of this study was to investigate the effect and mechanism of action of the trace element Se on the virulence factors of *H. pylori* and to provide an experimental basis for the use of the trace element Se in the prevention and treatment of *H. pylori*.

Research methods

A Se-enriched environment was created with sodium selenite to induce *H. pylori*, and the effect of the Se-enriched environment on the virulence of *H. pylori* and its potential mechanisms were evaluated by real-time fluorescence quantitative polymerase chain reaction, protein immunoblotting, transcriptome gene sequencing, Alma blue assay, and cell adhesion assay. A mouse gastritis model was established to understand the attenuation of *H. pylori* in terms of the changes in virulence *in vitro* and *in vivo*.

Research results

Se-enriched environments may lead to a reduction in the virulence factors CagA and VacA and significantly attenuate the pathogenicity of *H. pylori* by affecting the CagPAI-encoded type IV secretion systems of *H. pylori*.

Research conclusions

The mechanism of action of sodium selenite leading to the reduction of *H. pylori* virulence was shown to be through the downregulation of virulence factor expression, which led to a significant reduction in adhesion capacity, as well as inflammatory damage, thus inhibiting the growth of *H. pylori* and presenting better stability under a Se-rich environment.

Research perspectives

This study demonstrated the antibacterial mechanism of sodium selenite against *H. pylori* in a Se-enriched environment through *in vitro* and *in vivo* experiments. These results provide theoretical support for further research and development of sodium selenite in the preparation of attenuated vaccines and contributes to the alleviation of drug resistance in *H. pylori*.

FOOTNOTES

Co-corresponding authors: Yan-Qiang Huang and Zan-song Huang.

Author contributions: Qin C and Huang GR were responsible for the experimental research, reviewed the literature, and drafted the manuscript; Qin C and Huang GR contributed equally to this study; Guan AX, Zhou WT, Chen H, Luo PP, and Luo XK were responsible for writing, reviewing, and editing the study; All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript; Huang YQ and Huang ZS contributed equally to this work as co-corresponding authors. The reasons for designating Huang YQ and Huang ZS as co-corresponding authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Huang YQ and Huang ZS contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Huang YQ and Huang ZS as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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