Iranian Journal of Microbiology
Volume 17 Number 3 (June 2025) 404-410
DOI: http://doi.org/10.18502/ijm.v17i3.18823

ORIGINAL ARTICLE

Evaluation of the prevention, treatment and synergistic activity between *Helicobacter pylori* IgY antibodies and pantaprazole on AGS cell line and C57BL/6 Mice

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Received: September 2024**, Accepted:** December 2024

ABSTRACT

Background and Objectives: The growing resistance of *Helicobacter pylori* to antibiotics poses significant challenges in managing gastric ulcers. The application of IgY antibodies against *H. pylori* is a promising strategy. The current study evaluated the preventive and synergistic effects of IgY antibodies in conjunction with pantoprazole for treating *H. pylori* infection.

Materials and Methods: This investigation specifically focused on the inhibitory effects of IgY in the AGS cell line infected with *H. pylori*. In addition, the synergistic activity of IgY with pantoprazole and its preventive and therapeutic effects were assessed in male C57BL/6 mice.

Results: The findings indicated that IgY antibodies possess a substantial inhibitory effect on the adhesion of *H. pylori* to the AGS cell line. Furthermore, IgY antibodies resulted in a significant reduction ($P<0.05$) in the population of *H. pylori* and the severity of gastritis in infected C57BL/6 mice in both treatment and prevention groups. Notably, the optimal outcome was observed when IgY was administered alongside pantoprazole.

Conclusion: The use of IgY has the potential to repair damaged cells and prevent infection by decreasing bacterial adherence to gastric epithelial cells. Given its synergistic effect with pantoprazole, IgY can be recommended as a suitable complementary treatment in conjunction with pantoprazole.

Keywords: Immunoglobulin Y; Therapeutic; *Helicobacter pylori*; Cell line; C57BL Mice; Gastrointestinal diseases

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INTRODUCTION

Helicobacter pylori is a gram-negative bacterium that lives in the human stomach and is responsible for various conditions, including gastric and duodenal ulcers, as well as lymphoma (1).

Chronic *H. pylori* infection has been strongly associated with stomach cancer, leading to its classification as a class I carcinogen by the International Agency for Research on Cancer (IARC) in 1994 (2, 3). The prevalence of *H. pylori* infection is relatively high, ranging from 25% in developed countries to 80% in developing countries, depending on social and economic factors. However, in 2022 reports, its overall prevalence worldwide is decreasing (4-8).

Antibiotic therapy is widely employed for the treatment of *H. pylori* infections. This approach generally consists of a combination regimen including clarithromycin, amoxicillin, metronidazole, and a proton pump inhibitor (9-11). However, the effectiveness of this treatment regimen has decreased due to antibiotic resistance, particularly clarithromycin resistance, leading to treatment failure in approximately 20% of cases (9-12). Antibiotic treatments have associated microbiota disruption and high costs (13). Therefore, alternative therapeutic approaches are needed to enhance current regimens' effectiveness without disrupting the microbiota (14-16).

Passive immunization, specifically using egg yolk immunoglobulin Y (IgY), has gained attention as a potential therapeutic strategy (17, 18). IgY antibodies are inexpensive and can be obtained without causing harm or stress to animals, making them a practical source of antibodies (19, 20). Unlike mammalian serum immunoglobulins, IgY antibodies do not activate the mammalian complement system, reducing the risk of allergic reactions (21, 22). In light of the substantial prevalence of *H. pylori* and the increasing concerns regarding antibiotic resistance, as well as the significant impact of gastric cancer in Iran—ranked as the second most common cancer with the highest mortality rates, according to the World Health Organization's 2018 reports— The present study investigated the efficacy of the IgY antibody in preventing infection by examining its inhibitory effect on bacterial attachment and adhesion to the AGS cell line and mouse gastric tissue, as well as the treatment and reduction of gastric injury in a C57BL/6 mouse model. Furthermore, its synergistic effect with pantoprazole was also determined compared to the antibody and pantoprazole alone. These findings could provide valuable insights for the use of this product as a safe and cost-effective adjunct in the management of *H. pylori*.

MATERIALS AND METHODS

Preparation of IgY antibody. This study used IgY against Inactivated *H. pylori*, which was produced in our previous study under Review.

The effect of IgY-Hp on the attachment of *H. pylori* on AGS cell line. The AGS cells were cultured in RPMI1640 medium (Gibco, Germany), supplemented with 10% fetal bovine serum (v/v), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. The culture conditions were maintained at 37°C in a 5% CO_2 environment for a duration of 24 hours.

To evaluate the inhibition of *H. pylori* attachment to the AGS cell line by IgY-Hp, AGS cells (10^5 cells/mL) were seeded on a chamber slide (SPL, South Korea) and incubated for 24 hrs. After 24 hrs, the chamber slides were washed with 1 mL of sterile PBS. AGS cells were incubated with 400 µL of IgY-Hp (13 mg/mL) and pre-incubated with 400 µL of *H. pylori* (10^8 CFU/mL) to investigate bacterial attachment and the level of *H. pylori* colonization.

Two chamber slides were used as controls, one containing AGS cells infected with *H. pylori* (10^8 CFU/mL) and the other containing only AGS cells (10^5 cells/mL). Following a four-hour incubation period at 37°C in a CO_2 incubator, the supernatant was carefully removed, and the chamber slides were subsequently washed with phosphate-buffered saline (PBS).

The cells were fixed using a 3% (v/v) glutaraldehyde solution for a duration of 24 hrs, followed by a wash with phosphate-buffered saline (PBS). Subsequently, the cells were dehydrated through a series of graded ethanol concentrations (50-95%) and subjected to two washes with absolute ethanol for 15 minutes each. The dehydrated cells in absolute ethanol were processed using a critical point dryer, subsequently coated with gold, and analyzed via scanning electron microscopy (SEM) (TESCAN, Czech Republic) to assess the attachment of *H. pylori* to AGS cells.

C57BL/6 mice challenges. A total of 30 male C57BL/6 mice, aged between 6 and 8 weeks on average, were sourced from the Animal Care Center

of Mashhad University of Medical Sciences. The mice were divided into five groups, each consisting of six individuals. All groups were administered a daily oral antibiotic cocktail of 200 µl, containing ciprofloxacin (500 mg), metronidazole (400 mg), and erythromycin (500 mg), for seven days before the intervention.

This measure aimed to reduce the normal flora of the gastrointestinal tract. Following the antibiotic regimen, the mice were given 200 µl of 0.1 M sodium bicarbonate (pH=7.4) to decrease stomach acidity. Afterward, 300 µl of *H. pylori* suspension of 1×10^9 CFU/mL was inoculated into the mice five times, with each inoculation occurring every other day.

To confirm *H. pylori* infection in the mice, some mice were randomly selected, and their blood samples were analyzed by Indirect ELISA with anti-mouse-IgG. Furthermore, these mice were sacrificed, and their stomachs were aseptically isolated. The antrum region of the stomach was rinsed with PBS and then preserved in 10% formaldehyde for tissue staining over 3 days.

Once the presence of *H. pylori* was confirmed in the tissue samples, along with resulting inflammation and damage to the mice's stomachs, they were divided into five separate cages.

The first group received only IgY antibody at a dosage of 500 mg/kg, the second group received pantoprazole at a dosage of 30 mg/kg, the third group received a combination of pantoprazole (30 mg/kg) and IgY (500 mg/kg), and the fourth or control group was administered only PBS at a dosage of 500 mg/kg once a day for a total of 18 days.

Additionally, one group was designated for prophylaxis. This group initially received IgY antibody at a dosage of 500 mg/kg daily for 18 days and then was infected with *H. pylori* (1×10^9 cfu/ml). Finally, all mice were sacrificed, and their stomach, kidney, liver, and heart tissues were isolated under sterile conditions (18).

Following a 72 hr fixation period, tissue dehydration was conducted using a series of ascending ethanol concentrations ranging from 50 to 100%. Subsequently, clearing was done with Gezilol (in concentrations ranging from 1 to 3) for 2 hrs. This was followed by a triple impregnation with paraffin. Following sample blocking, Giemsa and Hematoxylin and Eosin (H&E) staining procedures were under methodologies outlined in previous articles. Then *H. pylori* infection indicators were reported based on mild to severe severity 1, 2, 3, 4 (23).

Statistical analysis. Figures in the article represent data from three independent experiments and are expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using SPSS 16 and GraphPad Prism 8 software, employing the Student's t-test and ANOVA test. In this analysis, a P-value of less than 0.05 was deemed to indicate statistical significance.

RESULTS

Effect of IgY-Hp antibodies on the attachment of *H. pylori* on AGS cells. The scanning electron microscope image in Fig. 1 shows AGS cells, including AGS cells infected with *H. pylori* (10^8 CFU/mL), in the presence or absence of IgY-Hp (13 mg/mL). Numerous rod-shaped bacilli of *H. pylori* attached to the surface of AGS cells were observed when the cells were incubated with *H. pylori* (Fig. 1B). In contrast, the number of attached *H. pylori* organisms decreased when cells were treated with IgY-Hp (Fig. 1C)

Treatment of mice infected with *H. pylori* with IgY antibody. Our findings indicate that the presence of the IgY antibody led to improvements in various pathological injuries, including inflammation, hemorrhage, edema, and atrophy, as well as a reduction in the count of *H. pylori*. For scoring purposes, we used a scale of 1 to 4, with 1 indicating mild and four indicating severe. The pantoprazole, combined pantoprazole +IgY, and IgY treatment groups showed improvement rates of 64.7%, 68.9%, and 55.4%, respectively, compared to the control group (Table 1). The results of H&E and Giemsa staining suggest that combined treatment with pantoprazole and IgY is more effective than monotherapy with pantoprazole, as shown in Figs. 2 and 3. In the prophylaxis group with IgY, we observed a 59.7% improvement compared to the control group, which was statistically significant ($P<0.005$).

In addition, the degree of pathological damage in different treatment and prophylaxis groups showed a reduction in the combined treatment group, pantoprazole, prophylaxis, and IgY antibody compared to the control group, respectively, as shown in Fig. 4. Inflammation, hemorrhage, edema, atrophy, and *H.*

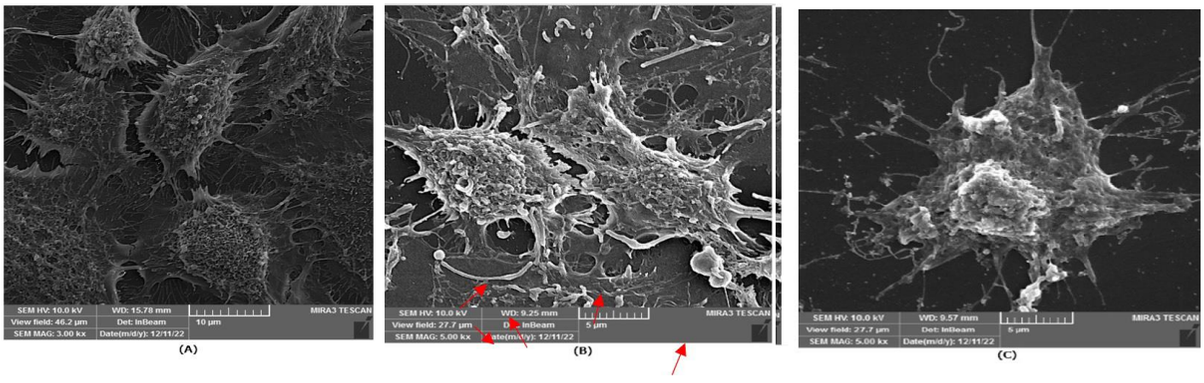

Fig. 1. Examination of scanning electron microscopy (SEM) for AGS cells infected with *H. pylori* in the presence or absence of IgY-Hp. (A) AGS cells; (B) AGS cells infected with *H. pylori* (10^8 CFU/ml), *Helicobacter pylori* bacilli are marked with red arrows; (C) AGS cells infected with *H. pylori* (10^8 CFU/ml) pretreated with IgY-Hp (13 mg/ml).

Table 1. Inflammation evaluation indicators including: bleeding, edema, atrophy and the number of *H. pylori* in different groups (PBS, pantoprazole, IgY, combined and prevention) and their percentage of damage and improvement. To determine the Pathological injury degree, the grade of pathological damage (X) was divided by the theoretical total score (100 points) and multiplied by 100%.

Group (Samples n=5)	Evaluation indicator (Score)					Pathological injury degree (X/100 × 100%)	Pathological injury improvement degree
	Inflammation	Hemorrhage	Edema	Atrophy	*H. pylori*		
PBS	15	15	15	10	20	74.9%	25.1%
Pantoprazole	10	10	5	5	5	35.3%	64.7%
IgY-Hp	15	10	5	5	10	44.6%	55.4%
Pantoprazole + IgY-Hp	5	10	5	5	5	31.1%	68.9%
Prophylaxis	10	10	10	5	5	40.3%	59.7%

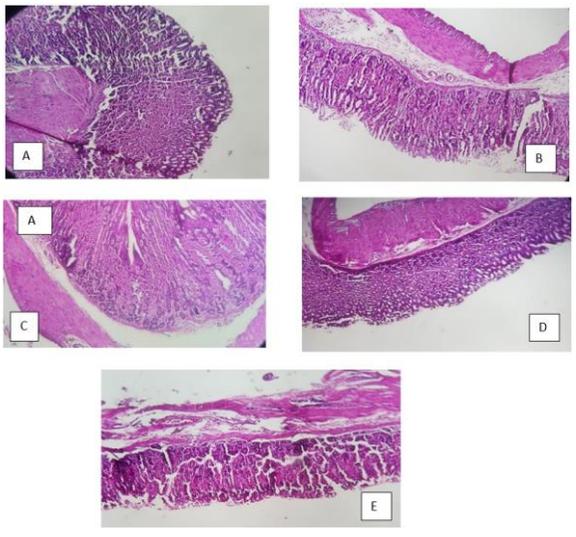

Fig. 2. Gastric tissue pathology after IgY treatment and prophylaxis of the Hp-infected mice. (HE staining, 40 × 10). A: PBS group; B: Pantoprazole group; C: IgY-Hp group; D: Pantoprazole + IgY-Hp group; E: Prophylaxis group. Mice gastric antrum samples were examined for the presence of curved bacilli and the Gastric pits and inflammation.

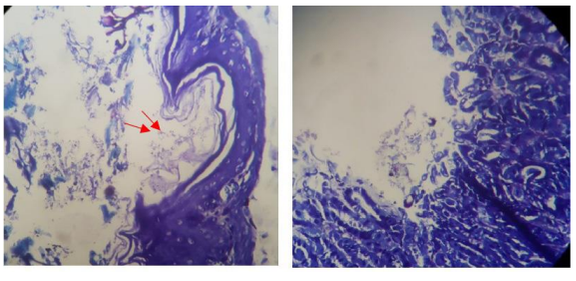

Fig. 3. Gastric tissue pathology in the Hp-infected mice. (Giemsa staining, 40 × 10). A: PBS group or negative control (The arrow indicates the *Helicobacter pylori*); B: IgY-Hp treatment group (There is no presence of *Helicobacter pylori*). Note: Red arrows indicate *Helicobacter pylori*.

pylori were given 1, 2, 3, and 4 points based on their severity from mild to severe, respectively. According to the statistical results, there was a significant difference between the treatment and prophylaxis groups (IgY-HP) compared to the control (PBS) ($P < 0.005$) (Table 1).

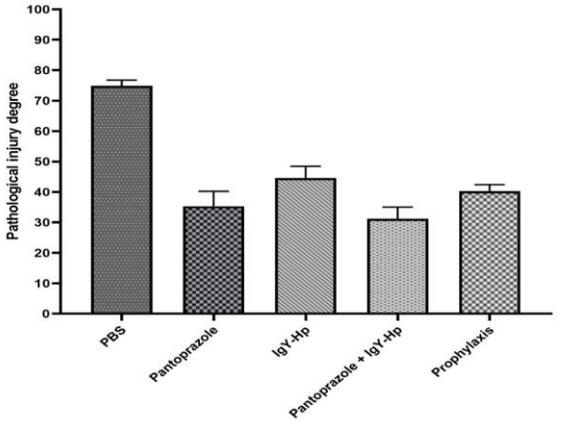

Fig. 4. Pathological injury degree in different treatment groups. Data are mean ± S.D. The P-value of <0.05 was considered statistically significant. 1) Pathological injury degree of PBS: 74.9 ± 1.85, 2) Pathological injury degree of Pantoprazole: 35.3 ± 4.93, 3) Pathological injury degree of IgY-Hp: 44.6 ± 3.78, 4) Pathological injury degree of Pantoprazole + IgY-Hp: 31.2 ± 3.86, 5) Pathological injury degree of Prophylaxis: 40.3 ± 2.08.

DISCUSSION

The global prevalence of *H. pylori* infection, coupled with the emergence of multi-drug resistant (MDR) strains, poses a significant challenge to the treatment of *H. pylori* infections and remains a public health threat in many regions (24). Approximately 20% of cases remain unresponsive to antibiotic treatment (25). Antibodies, specifically those that target specific antigens of *H. pylori*, offer a practical alternative that bypasses bacterial drug resistance (25). Given the need to replace antibiotic treatments for *H. pylori* infections and the ease of mass-producing egg yolk antibodies, IgY antibodies have been proposed as a suitable alternative (26, 27).

Our results showed that IgY-HP could significantly reduce (P<0.05) the number of *H. pylori* and the consequent degree of gastritis in C57/BL6 infected mice in both treatment and prevention groups. We demonstrated that oral administration of IgY antibodies had an impressive preventive effect against *H. pylori* colonization and infection in mice not yet exposed. One of the reasons for this is that the IgY antibody obtained in this study was produced against the whole-cell *H. pylori* that possesses the m1 allele of the *VacA* gene and the *CagA* gene. These genes are associated with increased virulence and a higher pathological impact, which could make the antibody more effective. As a result, it has a more comprehensive and practical effect on infected mice.

Using the Mongolian gerbil model, it has been demonstrated that IgY-HP reduces damage to gastric mucosal surfaces caused by *H. pylori* infection (26). The specificity of these antibodies in inhibiting *H. pylori* attachment to AGS cells provides further compelling evidence. These results indicate that IgY-HP can inhibit *H. pylori*'s ability to adhere to gastric epithelium surfaces (14, 17).

Our results confirm that IgY-Hp effectively inhibits the attachment of *H. pylori* to AGS cells, leading to a significant decrease in bacterial binding to these cells. Thus, the proposed mechanism is validated.

Using a novel approach, our study revealed a significant reduction in *H. pylori* colonization and gastric pathology in male C57BL/6 mice treated with IgY antibody. This finding, consistent with Suzuki et al.'s findings, was further enhanced in our study, indicating a more significant reduction in *H. pylori* counts and gastritis severity. These differences may be attributed to variations in antibody concentrations and specific strains of *H. pylori* used. Moreover, our study introduced a higher dose of IgY and a prophylactic group, demonstrating the potential of IgY antibodies to prevent *H. pylori* infection. These novel findings underscore the critical role of antibody dose in maximizing the therapeutic effects of IgY antibodies against *H. pylori.*

Another remarkable finding in our study is the preventive potential of this antibody in reducing bacterial count, likely attributed to the inhibition of bacterial attachment, as supported by our results on AGS cells. Although a reduction in histopathological parameters was evident, a statistically significant correlation was observed between the decrease in *H. pylori* count and the use of IgY-HP as a prophylactic measure compared to the control group. These findings contradict the study conducted by Sachiko Nomura, which focused on Mongolian gerbils. In their study, IgY against *H. pylori* urease, despite mitigating mucosal inflammation, did not demonstrate any impact on reducing bacterial colonization (12).

Our study demonstrated that administering IgY-HP over 18 days in the treatment group significantly reduced *H. pylori* levels. Additionally, histopathological symptoms like edema in mice were notably alleviated. These findings align with previous research carried out in Iran by Ziba Malek Shahi, focusing on anti-UreC-IgY, further reinforcing the potential ther-

apeutic efficacy of IgY-HP against *H. pylori* infections. However, the IgY against *H. pylori* whole cell lysate antigen at a lower dose compared to anti-urease C-IgY was able to produce a relative improvement. This efficacy could be attributed to the extensive antigenic diversity in the bacterial whole cell. In another similar study, it was observed that the use of IgY against *VacA* can cause a significant reduction in eosinophilic infiltration in mice's stomachs (28).

A 2019 study by Mony et al. investigated the use of IgY anti-urease to treat *H. pylori* infection in the C57BL/6 mice models. They used different concentrations of IgY antibodies to treat infected mice and reported a significant reduction in bacterial colonization and gastric inflammation at concentrations of 250 and 500 mg/kg. Our findings support the notion that IgY antibodies can serve as a significant intervention against *H. pylori*. We observed a marked reduction in both the count of *H. pylori* and the severity of gastritis in the group receiving IgY antibody monotherapy, as well as in the group undergoing pantoprazole combination therapy.

This study explores the combined use of IgY antibodies with pantoprazole, which enhances therapeutic effects and potentially reduces antibiotic dependence. Additionally, it evaluates IgY's dual role in treating and preventing *H. pylori* colonization, employing whole-cell lysate antigens to target a broader range of bacterial epitopes. Through a detailed assessment of gastric pathology and using the C57BL/6 mouse model, this work provides a comprehensive view of IgY's therapeutic potential, supporting its applicability as a safe, effective option for *H. pylori* management.

Limitations of our study, including the small sample size in the mouse challenge, may have affected the statistical results. On the other hand, the long-term effects or different doses of IgY in the treatment and prevention of *H. pylori* infections have not been investigated. In addition, we did not investigate the potential effect of this antibody on the immune response and changes in cytokine levels, so we cannot speak with complete confidence about the safety of this antibody in the long term. Prospective studies can focus on formulations and delivery systems to increase the stability and bioavailability of IgY and investigate its combination treatment with other drugs so that by identifying the appropriate antibody delivery system, the need for higher doses is reduced. On the other hand, in this study, only the synergistic effect of IgY with pentaprazole was investigated, and the results of using this antibody with other routine treatments, such as amoxicillin and clarithromycin, are questionable. The results of this study can be used as a preventive strategy and combined treatment of IgY and pantoprazole against *H. pylori* infection.

CONCLUSION

The findings of the present study indicate that oral administration of Hp-IgY has a preventive effect against the adhesion and colonization of *H. pylori* to gastric cells. On the other hand, its combined use with pantoprazole remarkably improved gastric tissue damage. Due to the absence of detectable side effects, Hp-IgY can be considered a safe complementary strategy in treating and especially preventing *H. pylori* infection.

However, future research can focus on determining the effects of long-term administration of Hp-IgY, examining cytokine levels in response to the antibody, and using its various formulations and doses to treat and prevent infection. The findings from this study are expected to provide valuable insights that will help future clinical trials and support the large-scale production of this therapeutic product.

ACKNOWLEDGEMENTS

We would like to express our sincere gratitude to the Parsian Protein Pishgam Shargh Company (Yadegar Production Group) (Mashhad, Iran) for their technical support for this research. This project was approved and ethically licensed by Mashhad University of Medical Science (IR.MUMS.AEC.1401.085) (Grant no. 4000697).

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