Original Article

Effects of Neutrophil-Activating Protein of *Helicobacter pylori* on Th Cytokines and Airway Inflammation in Allergic Asthma

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Abstract

Background: The study conclusively demonstrated that *Helicobacter pylori* (*H. pylori*) neutrophil-activating protein (HP-NAP) has the ability to switch a pro-allergic Th2 response to a Th1 response. Furthermore, it investigated the effects of HP-NAP on TH1 and TH2 cytokines, as well as airway inflammation in a rat model of allergic asthma, and compared these effects with those of *H. pylori*.

Materials and Methods: *H. pylori* and HP-NAP were prepared based on a previous study. Sixty male Blab/c mice were used for histopathological and immunological studies, divided into six groups of 10. Five mice from each group were selected for histopathological studies, and five were selected for measuring changes in TH cytokine levels and eosinophil count. The study was conducted over 31 days.

Results: Histological staining was performed on six selected groups. After treatment with HP-NAP, the recipient treatment group demonstrated a significant decrease in interleukin (IL)-4, IL-5, and total immunoglobulin E (IgE) levels, along with a significant increase in IL-2 and interferon-gamma (IFN-γ) levels. Furthermore, the number of eosinophils in the bronchoalveolar lavage fluid (BALF) of mice treated with HP-NAP significantly decreased compared to the positive control samples.

Conclusion: Our research findings conclude that HP-NAP can significantly reduce serum levels of IgE, IL-5, and IL-4, while increasing levels of IL-2 and IFN-γ, compared to *H. pylori*. HP-NAP can be a useful therapeutic agent for preventing and treating allergic asthma.

Keywords: Allergic asthma, cytokine, *Helicobacter pylori*, neutrophil-activating protein

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Introduction

Allergic asthma is a chronic inflammatory condition that affects the bronchi of the lungs. It is characterized by an overproduction of mucus in response to allergens, leading to the thickening of the bronchial walls and the transformation of goblet cells. This common condition can occur during childhood or later in life, significantly reducing the quality of life. Viral and bacterial infections, rather than allergens, may cause exacerbations of asthma in elderly patients.

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The immune system plays a crucial role in the development and progression of asthma due to its complexity. Extensive research has been conducted on various receptors and cells, including Th1/Th2, Th17, regulatory T-cells (T-regs), Toll-like receptors (TLRs), and dendritic cells (DCs). These studies have demonstrated that these cells collectively form a complex intercellular network, providing a comprehensive framework for asthma immunological research.[1–3] Prioritizing asthma prevention, detection, diagnosis, management, and treatment

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is crucial for all populations. An asthma attack is a direct consequence of immune system dysfunction. Histological assessment indicating eosinophil accumulation and infiltration is indicative of bronchial inflammation. In allergic asthma, interleukin (IL)-4 plays a crucial role in the process of antibody class switching to immunoglobulin E (IgE).[4–7] IL-5 plays an essential role in the eosinophilic inflammatory process, whereas IL-13 stimulates mucus production.[8,9]

Microbial metabolites directly affect the cells of the innate immune system, such as natural killer (NK) cells and dendritic cells (DCs). They induce the release of interferon-gamma (IFN-γ), IL-12, and IFN-α and trigger the conversion of Th2 cells into Th1 cells. These processes require further investigation.[10,11] Research has established that T cells responding to foreign antigens can be classified into two distinct types: Th1 and Th2 cells. These cells exhibit different activities, and an imbalance in the Th1/Th2 ratio is a critical factor in the immune response associated with asthma.[12,13] Th1 stimulates macrophages, enhancing the production of IL-12, IFN-γ, and tumor necrosis factor-beta (TNF-β), thereby promoting cellular immunity. In contrast, Th2 stimulates B cells and primarily releases IL-4, IL-5, and IL-13, ultimately triggering the activation of humoral immunity.[14–16] Epidemiological studies and the health hypothesis suggest that infectious infections may affect the development of allergy disorders.[17] Infections impede Th2 allergic responses by shifting the immunological balance toward the prevalence of Th1 responses, leading to the production of IFN-γ and IL-12.[18,19] Providing bacteria or their components that trigger a Th1 response can prevent the onset of asthma.[20] Notably, studies have found a correlation between *H. pylori* infection and a reduced risk of developing allergic asthma.[21,22]

H. pylori is a gram-negative, spiral-shaped bacterium that causes a range of disorders, including chronic inflammation, stomach ulcers, and gastric cancer. Its flagella and helical morphology enable it to traverse the stomach mucosa and adhere to its epithelium with ease. *H. pylori* is a persistent bacterium that commonly colonizes the human stomach during childhood and can persist for many decades. *H. pylori* produces the urease enzyme to neutralize stomach acid in the periplasm.[10,12,13,23] *H. pylori* functions as an antigen and stimulates the Th1 immune response in the mucosal lining of the digestive tract, resulting in persistent inflammation of the stomach.[14,15]

H. pylori neutrophil-activating protein (HP-NAP) is a potent activator of TLR2, strongly stimulating neutrophils, monocytes, and DCs, leading to the robust activation and response of Th1 cells.[16,24,25] *H. pylori* primarily activates Th1 cells, resulting in the secretion of key cytokines such as IFN-γ, IL-12, and tumor necrosis factor-alpha (TNF-α) in the stomach.[26–29] HP-NAP enhances IFN-γ production and reduces IL-4, shifting the Th2 immune response toward a Th1 response. Research demonstrates that HP-NAP effectively decreases the symptoms of allergic asthma.[30] HP-NAP is an immunomodulatory substance that can be delivered intraperitoneally or intranasally. It makes it an effective therapeutic agent for both preventing and treating allergic asthma. Research indicates that the systemic administration of HP-NAP effectively reduces the levels of IgE, IL-5, and IL-4 in the bloodstream while simultaneously increasing the levels of IL-12 and IFN-γ.[31,32] Additionally, it inhibits the buildup of eosinophils in the respiratory passages.[33]

This study aims to investigate the effect of HP-NAP on Th1 and Th2 cytokines, as well as airway inflammation, in a mouse model of allergic asthma. This study aims to compare the effects of this protein with those of *H. pylori*. If the protein proves to be more effective in managing and treating allergic asthma in mice, it could lead to further clinical investigations for developing a suitable pharmaceutical or immunization as a novel intervention for preventing and treating allergic asthma.

Materials and Methods

Groups and animal

The groups studied in this experiment were the experiment involved six groups ($n = 10$) of mice: 1) mice sensitized with ovalbumin (OVA) (positive control), 2) healthy mice that received only phosphate-buffered saline (PBS) and were not sensitized with OVA (negative control), 3) mice sensitized with OVA and treated with *H. pylori* group (helico group), 4) mice sensitized with OVA were effectively treated with HP-NAP, 5) budesonide treatment was administered to mice sensitized with OVA (drug 1 control), and 6) healthy mice that did not receive OVA but were given PBS and HP-NAP (drug 2 control).

Asthma model producing

The standard strain of *H. pylori* and HP-NAP peptide were confidently prepared and confirmed, as described in a previous study (currently under publication). Sixty adult male BALB/c mice, aged 6–8 weeks, were confidently used to model and develop allergic asthma for histopathological studies and cytokine changes. The animals were divided into six groups and sensitized and challenged with OVA via intraperitoneal (IP) and intratracheal (IT) routes to induce asthma symptoms. On days 1 and 14, OVA with alum was injected intraperitoneally. On days 24, 26, 28, and 30, the mice were challenged with a 1% OVA solution using a nebulizer. The experiment was conducted with precision and accuracy. Additionally, on days 25, 27, and 29, the mice were treated with *H. pylori* and HP-NAP using a nebulizer, according to their respective groups. All groups were kept in an allergen- and pathogen-free environment. On day 31, all groups were euthanized and sampled [Figure 1].[4,5]

Histopathological studies

Lung tissue samples were extracted from five mice in each group and analyzed using toluidine blue and Masson's trichrome dyes for histopathological studies.[4,5]

ELISA method

The study confidently analyzed the changes in cytokines IL-4, IL-5, IL-2, IFN-γ, and total IgE in the remaining five mice. To

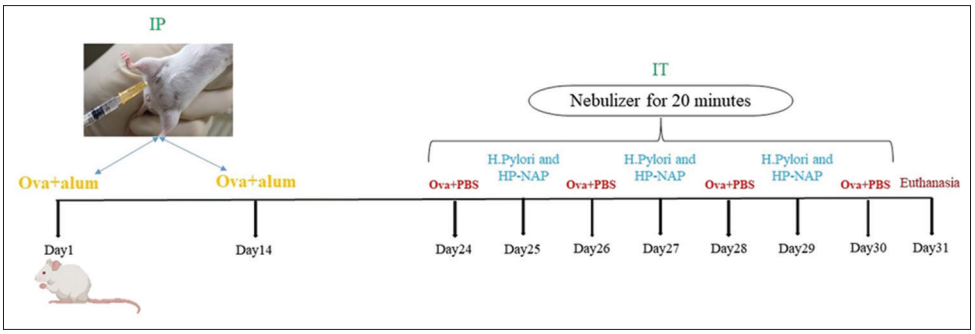

Figure 1: Allergic asthma model. Blab/c mice were sensitized by OVA and alum adjuvant on day 1 and repeated on day 14 via IP, and challenged on days 24, 26, 28, and 30 via IT with OVA aerosol to produce an asthma model. Treatment was done on days 25, 27, and 29. All groups were euthanized on day 31 and sampling was done

confidently extract the bronchoalveolar lavage fluid (BALF), a milliliter of PBS was confidently introduced through the trachea of the animal, and the lung was confidently massaged before confidently extracting the BALF fluid. The cytokines in the supernatant of BALF were confidently measured using the enzyme-linked immunosorbent assay (ELISA) method. The ELISA method was used to perform measurements of IL-4, IL-5, IL-2, and IFN-γ as instructed by the kits. Additionally, the total IgE in the serum of mice was measured using the ELISA method.

Eosinophil counting

To study and count eosinophils, BALF was extracted from the mice selected for this purpose. The fluid was centrifuged with the cytospin machine to evenly place the cells on a glass slide. The slide was stained with Giemsa dye, and the number of eosinophils was counted.

Statistical analysis

The results were statistically analyzed using SPSS software and presented as mean ± standard deviation (SD). Graphs were created using GraphPad Prism. A significance level of 0.05 was used.

Results

Histopathology

Peribronchial inflammation, perivascular inflammation, goblet cell hyperplasia, and mucus hypersecretion were increased in the significant ($P < 0.05$) asthma group (3.6 ± 0.3, 3.7 ± 0.2, 3.7 ± 0.3, and 3.9 ± 0.1) compared to the PBS group (0.5 ± 0.2, 0.5 ± 0.1, 0.5 ± 0.2, and 0.5 ± 0.1). Peribronchial inflammation and perivascular inflammation, goblet cell hyperplasia, and mucus hypersecretion were increased in significant ($P < 0.05$) in helico group (2.2 ± 0.1, 2.1 ± 0.1, 3.3 ± 0.3, and 3.2 ± 0.4) compared to HP-NAP group (1.5 ± 0.3, 1.7 ± 0.1, 2.2 ± 0.4, and 1.8 ± 0.2). Peribronchial inflammation, perivascular inflammation, goblet cell hyperplasia, and mucus hypersecretion were increased in significant ($P < 0.05$) HP-NAP group (1.5 ± 0.3, 1.7 ± 0.1, 2.2 ± 0.4, and 1.8 ± 0.2) compared to the PBS/HP-NAP group (0.5 ± 0.1, 0.5 ± 0.3, 1.0 ± 0.1, and 0.5 ± 0.2) [Figures 2 and 3].

Cytokines

The level of IL-4 and five cytokines in the asthma group (95 ± 7 pg/mL) (101 ± 9 pg/mL) increased significantly ($P < 0.05$) compared to the PBS group (41 ± 3 pg/mL) (53 ± 5 pg/mL). The level of cytokines IL-2 in the asthma group (29 ± 3 pg/mL) decreased not significantly ($P < 0.05$) compared to the PBS group (36 ± 4 pg/mL). The level of cytokines IFN-γ in the asthma group (22 ± 3 pg/mL) decreased significantly ($P < 0.05$) compared to the PBS group (73 ± 4 pg/mL). The level of IL-4, 5 cytokines in the helico group (65 ± 4 pg/mL) (64 ± 7 pg/mL) increased significantly ($P < 0.05$) compared to the HP-NAP group (52 ± 2 pg/mL), (55 ± 3 pg/mL). The level of cytokines IL-2 in the helico group (31 ± 3 pg/mL) and decreased not significantly ($P < 0.05$) compared to the HP-NAP group (30 ± 2 pg/mL). The level of cytokines IFN-γ in the helico group (62 ± 4 pg/mL) increased not significantly ($P < 0.05$) compared to the HP-NAP group (65 ± 4 pg/mL).

Total IgE

The level of total IgE in the asthma group (2612 ± 236 ng/mL) increased significantly ($P < 0.05$) compared to the PBS group (152 ± 17 ng/mL). The level of total IgE in the helico group (1578 ± 285 ng/mL) increased significantly ($P < 0.05$) compared to the HP-NAP group (1204 ± 209 ng/mL) [Figure 4].

Eosinophil counting

The level of eosinophil counting in the asthma group (74 ± 5) percent increased significantly ($P < 0.05$) compared to the PBS group (4 ± 1) percent. The level of eosinophil counting in the helico group (31 ± 5) percentage increased significantly ($P < 0.05$) compared to the HP-NAP group (19 ± 3) percentage [Figure 5].

Discussion

T-cell activity can be associated with the severity of asthma, the degree of airway narrowing, and the eosinophilia response. In atopic individuals with asthma, the immune response is mainly Th2, whereas in non-atopic individuals, the immune response is primarily Th1. Patients with asthma exhibit an abundant Th2 immune response, which is characterized by an increase in IL-4, 5, and 13. In contrast, a Th1 immune response is

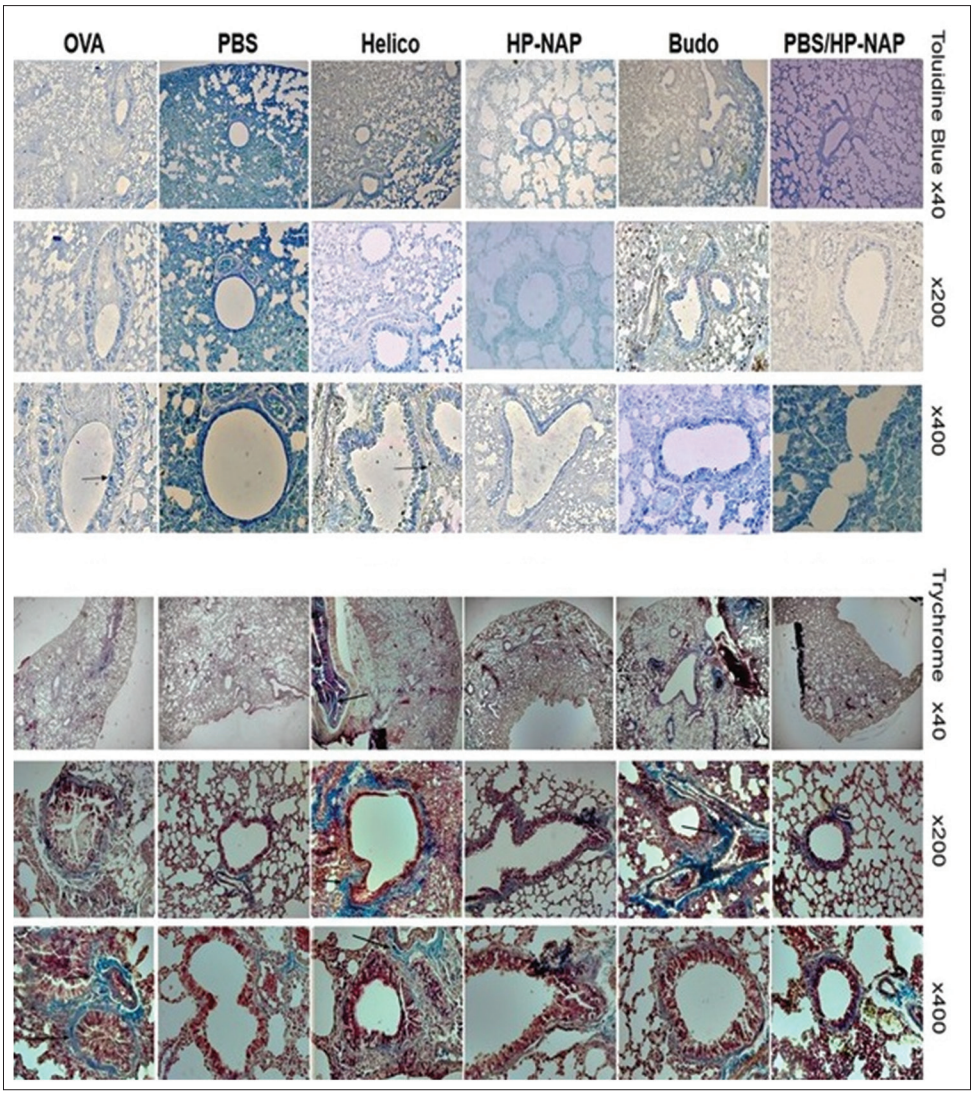

Figure 2: Toluidine blue staining of lung histopathology. The amount of per bronchial and perivascular inflammation, goblet cell hyperplasia, and the amount of mucus secretion in all groups were studied. Masson is trichrome staining of lung histopathology. The amount of per bronchial and perivascular inflammation, goblet cell hyperplasia, and the amount of mucus secretion in all groups were studied

characterized by an abundance of IL-2 and IFN-γ. Imbalances between Th1 and Th2 play a crucial role in the development of atopic diseases. The differentiation of Th0 to Th1 and Th2 is precisely regulated by transcription factors such as T-bet and GATA-3. GATA-3 is responsible for regulating Th2 expression, whereas T-bet controls Th1 differentiation. The expression of these factors regulates the balance between Th1 and Th2 cells, thereby impacting the allergic response.[34,35]

Allergic asthma is commonly linked to atypical Th-2 cell reactions. However, it has been observed that individuals with severe asthma exhibit a combination of Th-2 and Th-17 cell reactions in their air passages.[36] Th-17 cells and T-regs play a crucial role in asthma development.[37–39] Various asthma models have detected several cellular variables, including Th-2, Th-17, and eosinophil/neutrophil infiltration.[17–19] Eosinophilic asthma being exclusively a Th-2 condition and neutrophilic asthma being solely a Th-17 disorder is unlikely.[20] Reciprocal regulation between Th-2 and Th-17 inflammatory factors in asthma has been established.[21] A complex network comprising of A, Th-1/Th-2, and Th-17/T-regs, along with their respective cytokines, interacts in a highly intricate manner.[30,31]

H. pylori causes persistent immune-mediated pathogenic changes in the stomach, leading to dysbiosis. Additionally, it enhances immune activity regulation in the lungs and inhibits the onset of asthma through the modulation of DCs, T-regs, and other mechanisms. This supports the concept of the gut–lung axis.[40–42] Infection with *H. pylori* leads to a significant increase in T-reg activity in the gastric mucosa, with T-regs releasing more IL-10 in the peripheral blood than *H. pylori*-specific Th-1 cells. The formation of robust T-regs results in a decrease in the concentration of both total IgE and allergen-specific IgE. In animal models, inhibiting IL-10 can substantially increase IgE levels. The co-transmission of IL-10 and T-regs prevents allergies in the presence of *H. pylori*.[43]

Kyburz *et al*.[44] conducted experimental models on C57BL/6 mice to induce airway inflammation using various methods such as house dust, OVA, influenza A virus, *Citrobacter rodentium* infection, and exposure to *H. pylori* extract and its immune vacuolated cytotoxin cytotoxic prenatally. The study conclusively demonstrates that these methods provide strong and effective protection against allergic asthma, not only in the first generation of offspring but also in the second generation. Exposure to *H. pylori* significantly impacts the diversity and composition of the gastrointestinal tract microflora in carriers and their offspring. The development of the fetal immune system can be influenced by maternal nutrition, exposure to microbes, smoking, and other environmental variables through the epigenetic mechanism. DCs promote the differentiation of primary T cells into Th-2 cells or T-regs.[45,46]

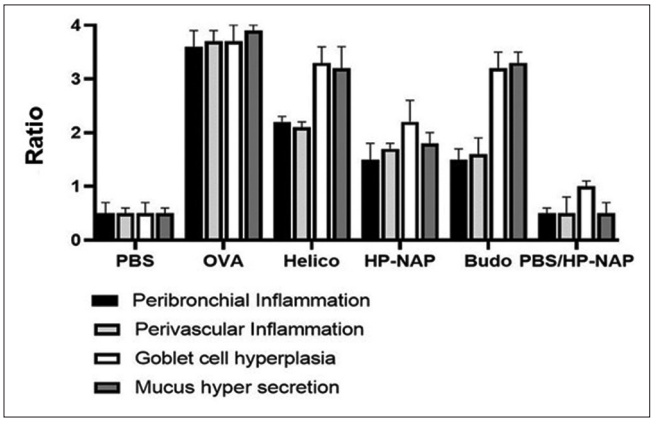

Figure 3: Results of histopathology. In mice that received *H. pylori* and budesonide, the amount of perivascular and per bronchial inflammation decreased significantly compared to the asthmatic group, but mucus secretion and goblet tuber hyperplasia were not significant. However, in the group that received HP-NAP, the amount of per bronchial inflammation, perivascular inflammation, goblet cell hyperplasia, and the amount of mucus secretion decreased significantly compared to the asthmatic group. Also, the reduction of per bronchial inflammation and perivascular inflammation in the group that received HP-NAP was not significant compared to the groups receiving *H. pylori* and budesonide, but the reduction of goblet cell hyperplasia and the amount of mucus secretion were significant

Recent research has definitively shown that *H. pylori* affects DCs to promote immunological tolerance and enhance the protective response against allergic asthma. This effect is primarily mediated by T-regs.[47,48] Karakullukcu *et al*.[28] discovered that none of the 92 children between the ages of 3 and 8 years with asthma had *H. pylori* in their stomachs, in contrast to the 18 children who tested positive for the bacteria. This finding demonstrates the absence of a significant association between *H. pylori* and asthma in young children.

Zhou *et al*.[29] confirmed the regulatory role of HP-NAP in countering the Th-2 inflammatory response and its protective effect against asthma in male children. The study involved administering pure HP-NAP to mice via IP injection and inhalation, resulting in a significant reduction in eosinophil increases in the lungs of mice with OVA-induced asthma. Pure HP-NAP treatment resulted in a decrease in BALF eosinophil count in mice. Furthermore, asthmatic mice treated with pure HP-NAP exhibited decreased levels of IL-13 and IL-4, whereas the levels of IFN-γ and IL-10 increased compared to the control group. Pretreatment with pure HP-NAP has the potential to alleviate OVA-induced asthma in mice by decreasing the serum level of IgE. The utilization of pure HP-NAP presents a novel approach to the prevention or treatment of asthma. Liu *et al*.[49] proposed that the efficacy of the IL-4 receptor (sIL-4R) in treating asthma can be enhanced based on their findings from phase I/II clinical trials. To investigate whether HP-NAP could improve the therapeutic efficacy of IL-4 in asthma, the researchers confidently initiated their study by constructing a plasmid called pcDNA3.1-sIL-4R-NAP (PSN). The HP-NAP peptide is produced by this plasmid, which also encodes the murine forms of NAP and sIL-4R. It significantly reduces airway inflammation, inhibits the production of OVA-specific IgE antibodies in the bloodstream, and restores the balance between Th-1 and Th-2 immune responses. Notably, the PSN plasmid has been demonstrated to be more effective

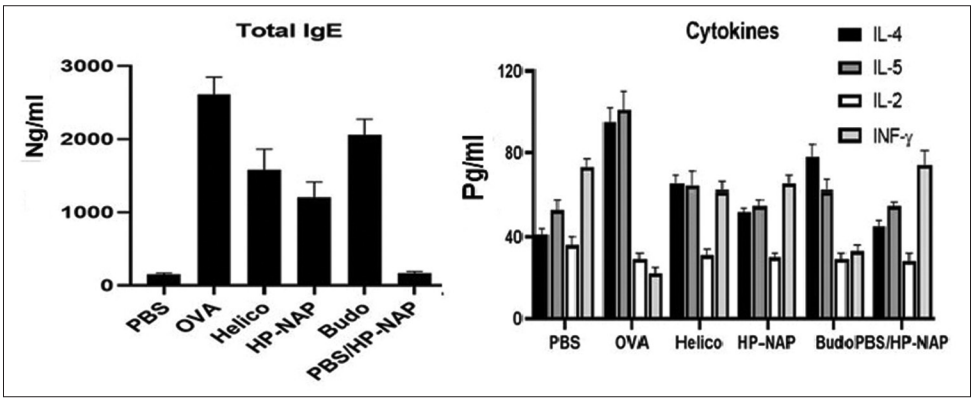

Figure 4: Cytokines and IgE. The levels of IL-2, IL-4, IL-5, INF-γ, and also IgE were measured in all groups. IL-4 and IL-5 were increased in the asthma group and treatment with HP-NAP could control these cytokines. IL-2 and INF-γ were decreased in the asthma group and treatment with HP-NAP could enhance these cytokines. Total IgE was increased in the asthma group compared to the healthy group. Total IgE was decreased significantly in treated groups compared to the asthma group.

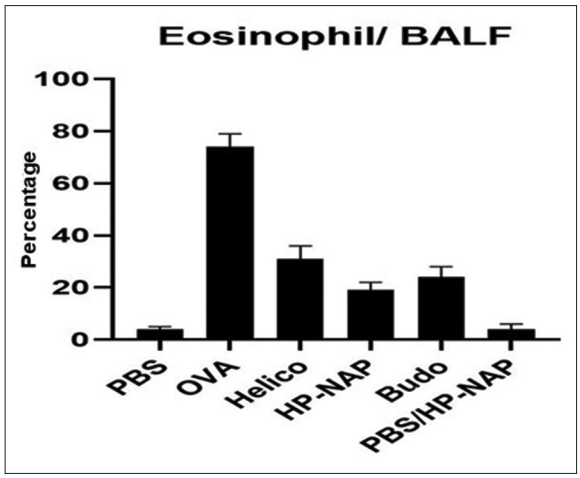

Figure 5: The eosinophil counting. After counting the number of eosinophils in the BALF of mice, their number in the samples treated with *H. pylori* and HP-NAP and the budesonide drug compared to the asthmatic samples decreased significantly. The number of eosinophils in the samples receiving *H. pylori* compared to the drug Budesonide has no significant difference; the number of eosinophils in the sample receiving HP-NAP has a significant difference compared to *H. pylori*

in treating asthma than the IL-4-generating plasmid. This research highlights the potential therapeutic uses of HP-NAP in treating asthma. HP-NAP has been found to contribute to bacterial defense and host inflammation by stimulating innate immune cells, such as neutrophils, monocytes, and mast cells, to trigger oxidative stress and inflammatory responses.[50,51] Several studies indicate that HP-NAP, acting as a significant Th1-promoting virulence factor, hinders the release of Th2 cytokines in both humans and animals, potentially decreasing allergic asthma. Research confidently suggests a negative correlation between *H. pylori* infection and childhood asthma. *H. pylori* is believed to offer protection against allergic asthma by influencing the immunological response, possibly through HP-NAP.[28] Further research is needed to fully understand the potential therapeutic uses of HP-NAP in treating asthma.[52]

Pure HP-NAP has more potent effects compared to *H. pylori* and budesonide. It significantly decreases IgE levels in the blood, acting as an immunomodulatory agent. It suppresses IL-5 and IL-4 while enhancing the levels of IL-2 and IFN-γ to hinder the buildup of eosinophils and mucus in the airways. This therapy is an effective treatment for preventing and treating allergic asthma. Warrants further investigation for additional clinical trials aimed at developing medication or a vaccine to manage and treat allergic disorders, including allergic asthma.

Conclusion

Pure HP-NAP has stronger effects compared to *H. pylori*. It significantly reduces the serum level of IgE, reduces IL-5 and IL-4, and increases the levels of IL-2 and IFN-γ. Additionally, it prevents the accumulation of eosinophils and mucus in the airways. HP-NAP can be used as a useful therapeutic agent for the prevention and treatment of allergic asthma. Clinical studies must be conducted to develop medicines or vaccines for controlling and treating allergic diseases, especially allergic asthma.

Ethics approval

All methods were performed after the approval of the ethics committee with code IR.IAU.Z.REC.1401.069

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

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