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Histological Studies in Animal Model of *Helicobacter pylori* Infection Treated with Attacin A Antimicrobial Peptide

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Abstract

Recent years have seen an upsurge in the demand for alternative treatment agents due to the advent of antibiotic-resistant strains of *Helicobacter pylori* (*H. pylori*). Small peptides belonging to the class known as antimicrobial peptides (AMP) are an essential component of the innate immune systems of many different types of organisms. The goal of this study to detect histological changes in rat animal models infected with *H. pylori* and treated with the antimicrobial peptide Attacin A (AttA) and its effectiveness as anti-*H. pylori*. The purpose of this research was to determine the efficacy of the AttA against *H. pylori* by observing any histological alterations that occurred in an *H. pylori*-infected rat model after treatment with AttA. Three groups of rats (normal control and *H. pylori*-infected and *H. pylori*-infected and treated with AttA). Stomachs were examined histologically to evaluate the AttA therapeutic activity. In conclusion, the results of the current study showed the effect of the AttA on reducing the severity of the infection of *H. pylori* and reducing the tissue damage in the lining of the stomach of rats.

KEYWORDS
Antimicrobial peptide, Attacin, *Helicobacter pylori*, Histopathology, Stomach, Rat

INTRODUCTION

From ancient times, people have been aware of microorganisms and have employed a wide range of responses to them. There was likely an initial effort to combat infectious diseases with natural antibiotics. The need to treat bacteria that haven't seen before and the advent of drug-resistant species, have pushed research to find new antibiotics and modify older ones. Many different types of antibiotics, both natural and synthetic, have been discovered and used for this purpose, each having its own unique history, mode of action, structural makeup, and therapeutic window. Some of these compounds kill bacteria by interfering with their ability to produce nucleic acids, while others disrupt protein synthesis. Most antibiotics have unique features due to a ring present in their structure. Antibiotics offer many benefits, but they also have certain downsides that prevent them from being used in every circumstance (Dehghan Esmatabadi *et al*., 2017).

Among the human pathogens known for causing gastritis and gastroduodenal ulcers is *Helicobacter pylori* (*H. pylori*) (Dinat *et al*., 2023). Several different diseases and conditions may be brought on by *H. pylori*, a Gram-negative spiral bacteria (Retnakumar *et al*., 2022). Studies have shown that it is a key contributor to peptic ulcer disease, which includes both stomach and duodenal ulcers (Hawkey *et al*., 2022). The bacterium *H. pylori* has a significant role in the development of gastroduodenal illness (Dinat *et al*., 2023). Comprehending pathophysiology and creating novel treatment techniques rely heavily on the use of experimental animal models (Goo *et al*., 2009).

The pathophysiology of *H. pylori*-associated problems may be better-understood thanks to in vivo investigations employing a variety of animal models. Consistent evidence linking stomach issues in several animal models, including Mongolian gerbils, transgenic mouse models, guinea pigs, and other species, including non-human primates, has led to their widespread adoption (Arismendi Sosa *et al*., 2018).

Rodents are highly regarded as a model due to the striking parallels between their physiology and that of the human thymus, bone marrow, and lungs, as well as the human innate immune and complement systems (Giese and Marx, 2014). They share several features with humans, including a delayed kind of hypersensitivity, a major histocompatibility complex, and the production of IFN- as well as multiple homologs of human group 1 cluster of differentiation (CD) 1 protein (Stair *et al*., 2023). Because they are not predisposed to infection with Helicobacter spp., rats make a useful infection animal model in which to study the impact of several virulence determinants on pathogenicity (Whary *et al*., 2015).

Standard triple therapy has lately been revealed to have diminishing efficacy as a treatment for H pylori. Antimicrobial medication resistance is a leading cause of treatment failure. To combat the growing problem of drug-resistant *H. pylori*, innovative treatment approaches need to be deployed without delay (Suzuki *et al*., 2019). In this way, antibiotics might be chosen

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based on the *H. pylori* strain's sensitivity to the drug. Developing treatments for infections caused by bacteria that are resistant to the antibiotic clarithromycin is a top goal of the World Health Organization (WHO) (Savoldi *et al*., 2018).

Antimicrobial peptides (AMP) are a subset of peptides that play an important role in innate immunity by preventing pathogen growth through mechanisms such as blocking the synthesis of nucleotides and proteins, preventing the formation of pores in the cell membrane, and modulating the immune response of the host (Roque-Borda *et al*., 2022). AMPs are attractive options for alternative therapy for antibiotic-resistant infections because they are broad-spectrum antimicrobial agents against which most bacteria develop slowly. This makes them ideal for treating illnesses that have become resistant to antibiotics (Bugli *et al*., 2022). AMP has been maintained under sterile conditions apart from a wide range of flora and wildlife, including fish, plants, and amphibians.

Rapidly killing bacteria and possessing wide antibiotic action are also characteristics of AMPs. In particular, they are immune to the effects of more conventional antibiotic resistance mechanisms (Bradshaw, 2003; Hase *et al*., 2003). The fact that AMPs interact preferentially with microbial cells while being non-toxic to mammalian cells at antibacterial doses is one of the most essential characteristics of these molecules. Intriguingly, antimicrobial activity is the primary biological function of AMPs; however, recent research has uncovered some novel functions of these molecules, such as wound healing, the neutralization of endotoxins, immunomodulatory activities, and anti-neoplastic properties. This is even though antimicrobial activity is the primary biological function of AMPs (Diamond *et al*., 2009; Zhang *et al*., 2015)

Innate immune systems rely on AMPs as key effector molecules to combat pathogens such as bacteria, viruses, fungi, and parasites (Müller *et al*., 2008). These peptides are effective because of their mode of action, which often includes non-specific membrane contacts, which makes it difficult for infections to evolve long-term resistance (Costa *et al*., 2011). As a first line of defense against invading pathogens and to stimulate immunological responses, AMPs of animal origin are present in the outer and inner epithelia, in phagocytic cells, and in bodily fluids of all animals, from invertebrates to humans (Christophers and Schröder, 2022).

The first publication describing the discovery of a novel insect AMP family termed Attacin, which was discovered in the gigantic silk moth Hyalophora cecropia, was published in 1983 (Buonocore *et al*., 2021). After immunizing pupae with the bacteria Enterobacter cloacae, several isoforms with molecular masses between 20 and 23 KDa were isolated from the hemolymph. The Attacin A peptide was almost non-toxic to porcine red blood cells, allowing it to reach various target organs normally. These characteristics of Attacin A peptide made it a promising candidate for use in medicine and agriculture. The detection of biological activity of the Attacin A peptide successfully demonstrated that it was active against several Gram-negative bacteria (Wang *et al*., 2010).

The goal of this research was to focus on the histological examination of AMP-AttA as an inhibitor of *H. pylori* in epithelial cells of a stomach rat model.

MATERIALS AND METHODS

Bacteria

Helicobacter pylori bacteria was provided by the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt. The *H. pylori* were initially grown on freshly prepared blood agar plates with the addition of trimethoprim (5 mg/L), vancomycin (8 mg/L), and polymyxin B (10 mg/L). The plates were incubated at 37 ◦C in a microaerophilic atmosphere in a cell culture incubator (5% O2, 10% CO2) for 3 to 6 days (Saha *et al*. 2022).

Tested Antimicrobial peptide

Antimicrobial peptide Attacin A was purchased from Science and Technology Centre with Greater or equal to 85% purity, 35-224 full-length protein, and Mwt 23,239 Da. Recombinant protein powder amino acids sequence denoted as QVLGGSLTSN PAGGADARLD LTKGIGNPNH NVVGQVFAAG NTQSGPVTTG GT-LAYNNAGH GASLTKTHTP GVKDVFQQEA HANLFNNGRH NLDA-KVFASQ NKLANGFEFQ RNGAGLDYSH INGHGASLTH SNFPGIGQ-QL GLDGRANLWS SPNRATTLDL TGSASKWTSG PFANQKPNFG AGLGLSHHFG.

Animals

Nine Albino rats (about 7 weeks old and weight, 120 to 150 g) were divided into 3 groups of three rats each. At the National Research Centre in Egypt, animals were administered injections and received treatment. They were kept in polypropylene cages with stainless steel mesh tops in a well-ventilated area. The room was maintained at 23 degrees Celsius with a relative humidity of 45 to 60 percent and a light-dark cycle of 12 hours. During the experiment, all the animals had unrestricted access to running water and the same diet. Before being dosed, the animals were acclimated to the laboratory environment by being maintained in their cages for at least 7 days.

Rat Models of H. pylori infection and AttA Treatment

The *H. pylori* gastric infection was produced according to the O'Rourke and Lee (2003) protocol, in which the rats were treated by oral gavage with 1 mL 5×108 CFU/mL) of bacterial solution used to inoculate each animal twice a day for 7 days. Each animal was given a day off in between each inoculation. The rat has to be in a fasting condition for at least four hours before each injection, and it should only have access to sterile water. During the process of inoculation, the animals are not anesthetized.

Rats were randomly divided into 3 groups. Group I served as the control group which received 2 ml of distilled water daily. Group II was the *H. pylori*-infected group, inoculated as mentioned above. Group III represented the infected animals and was treated with AttA which was administrated intraperitoneally with a daily dose of 3 mg/kg for ten days (Braunstein *et al*. 2004). At the end of the experimental period, rats were sacrificed, and the stomachs were removed and fixed in 10% formalin saline. The Ethics committee of Scientific Research, Suez University, Suez, Egypt approved this research (Approval no 152223).

Histological analysis

For histological examination, sections of the stomach tissue were taken from all of the animals. The samples of tissue were first fixed in a 10% formalin saline solution, then dehydrated using increasing concentrations of alcohol, cleaned in xylene for four hours, and then embedded in paraffin at 58 degrees Celsius before being arranged in blocks. The slices, each measuring 5 micrometers in thickness, were cut using a manual microtome, then stained with hematoxylin and eosin, and examined using a

light microscope (Williams *et al.* 2016).

The degree of gastritis severity and *H. pylori* density were investigated microscopically, and scored as normal (0), mild (1), moderate (2), or severe (3) gastritis utilizing the Sydney grading in measuring lymphocyte infiltration, glandular atrophy, and *H. pylori* existence (Hassan *et al.* 2016).

Statistical analyses

Histopathological scoring presented here was analysed statistically using One-way ANOVA test in SPSS V.20.0 (SPSS Institute Inc., Cary, NC). When $p < 0.05$, the data are considered statistically significant.

RESULTS

The current results revealed that stomach histology in rats experimentally infected by *H. pylori* displayed severe disruption to the surface epithelium and edema of the submucosal layer with leucocyte infiltration (Figures 1C and 1D). *H. pylori* and treated with AttA revealed the normal architecture of stomach tissue with apparently normal mucosa (Figure 1E and 1F).

Regarding Gastric histology scores in the present investigation; Figure 2 summarizes the followings: the control group's gastric histology was normal with intact surface mucosal and submucosa epithelium with an average distribution of parietal cells, but the HP group's histopathology revealed significant ulcer and gastric inflammation reaching muscularis mucosa and widespread *H. pylori* colonization. Histopathological alterations were mitigated in the HP + AttA group compared to the HP group, particularly in terms of inflammatory cell infiltration. Nearly all the stomach tissues in the HP + AttA group exhibited a mild scattered infiltration of inflammatory cells. Likewise, *H. pylori* colonization was reduced in the HP + AttA group.

DISCUSSION

Antibiotics are used to treat a broad spectrum of infectious disorders. However, the rise of antibiotic-resistant bacterial species has led to a high rate of death (Lamberte and van Schaik 2022). Therefore, if adequate actions are not adopted immediately, antibiotic resistance will have severe repercussions. The growth of antibiotic-resistant bacteria strains has raised the demand for research and development of antimicrobial compounds.

Due to their physiologic similarities to humans, rats were employed in this investigation as experimental animals (Barré-Sinoussi and Montagutelli, 2015). Mice and rats have been utilized as animal models of *H. pylori*-induced infection for a very long time until recently (Werawatganon, 2014).

H. pylori infection induce stomach ulcers, and oxidative stress plays a key part in the etiology of different disorders including gastric ulcer (Bhattacharyya *et al.*, 2014). This investigative study demonstrated that experimental *H. pylori* infection of rats induces severe disruption of stomach mucosa, which results in many

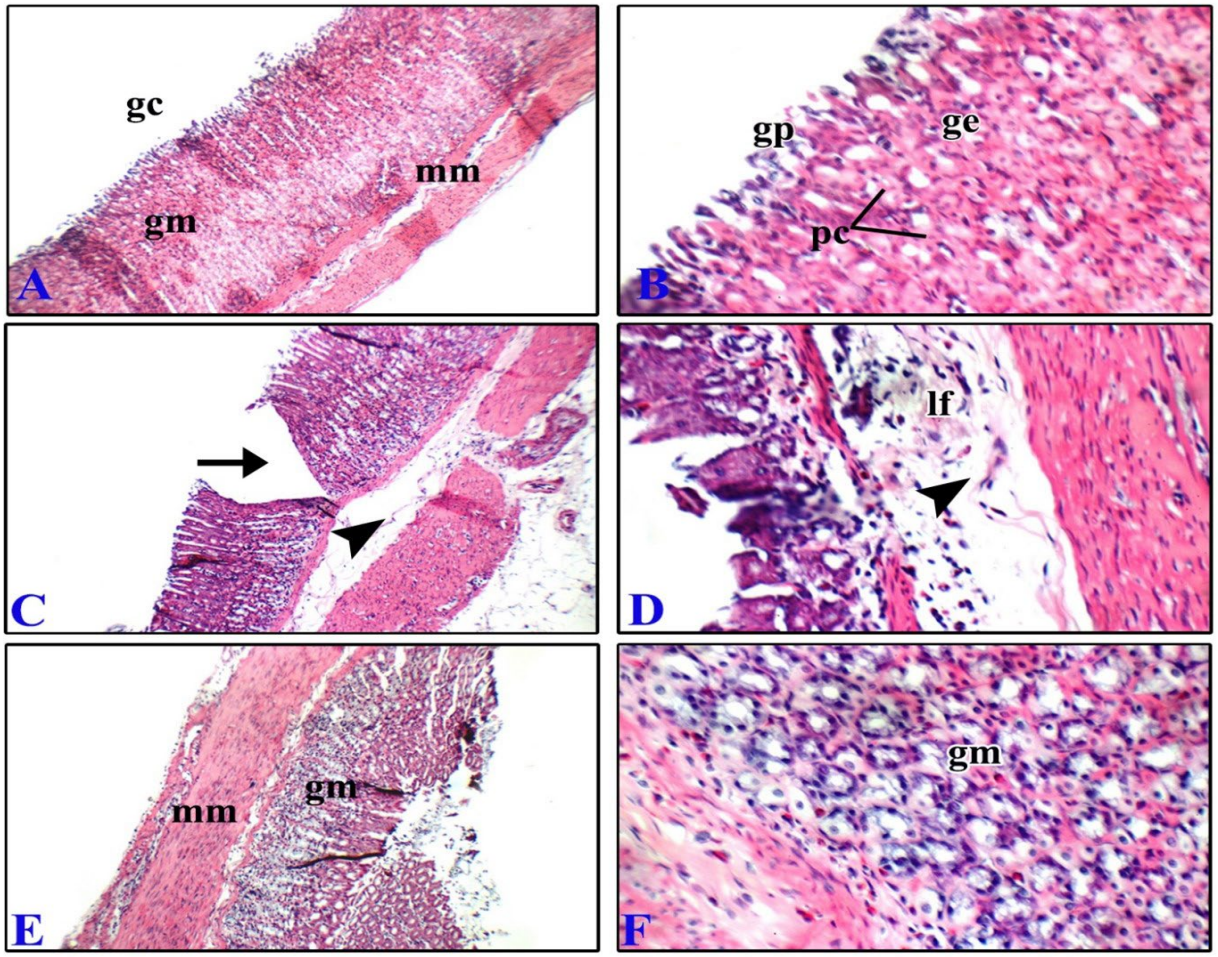

Fig. 1. Gastric histopathology in rats with *H. pylori* infection and treated with AttA. (A) The control group showed normal histological architecture, Gastric cavity (gc), Muscolaris mucosae (mm), and Gastric mucosa (gm) (200X). (B) Magnification of stomach mucosa in the control section showing Gastric epithelium (ge), Gastric pits (gp), and parietal cells with normal count and distribution (400X). (C) The *H. pylori*-infected group displayed edema (head arrow in the submucosa and disruption to the mucosal epithelium (arrow). (D) Magnification of stomach mucosa in *H. pylori* infection group indicating edema (head arrow) and inflammatory cell infiltration in (lf) (400X). (E) *H. pylori* infection group treated with AttA showing normal histological architecture Muscolaris mucosae (mm), Gastric mucosa (gm) (200X) (F) Magnification of stomach mucosa in *H. pylori* infection group treated with AttA showing apparently normal mucosa (gm). (400X).

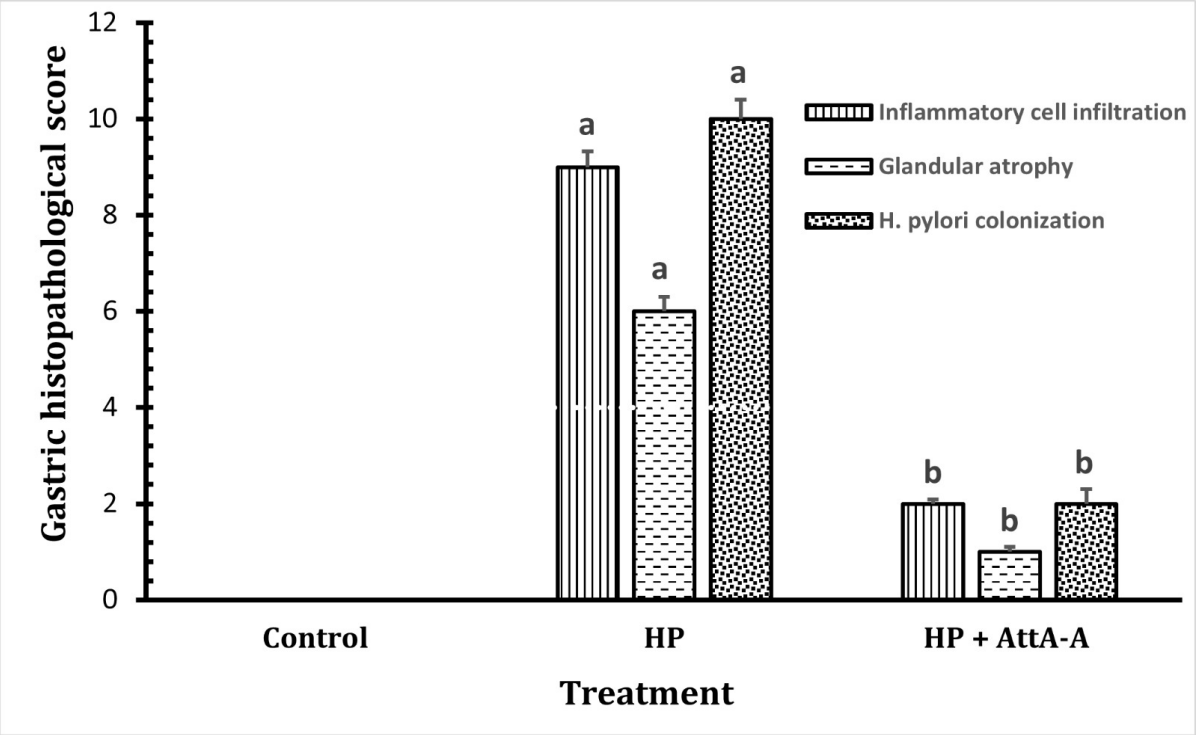

Fig. 2. Gastric histopathological score.
a: Significantly different (P<0.05) compared with the control group, b: Significantly differentl (P<0.05) compared with the HP group,

histopathological changes including mononuclear cell infiltration and edema. Similar results have been reported by several previous studies (Wyatt, 1995; Lee and Kim, 2015).

H. pylori infections are becoming increasingly difficult to treat due to drug resistance. In *H. pylori*, treatment resistance has been linked to changes in the bacteria's chromosomes, according to a study published in Nature. It has been found that *H. pylori* medication resistance varies with geographic origin and pharmacological variability (Torres-Debat *et al*., 2009).

Insects and other species manufacture chemicals called AMPs to defend themselves against harmful bacteria and other microorganisms (Li *et al*., 2012). Insect AMPs have been revealed to exhibit anti-inflammatory, anti-cancer, and other therapeutic characteristics (Xia *et al*., 2018). Insect AMPs are consequently a valuable source of novel therapeutic medicines for the treatment of infectious illnesses (Sahoo *et al*., 2021).

In the present study, the acute *H. pylori* infection caused damage to the stomach wall as well as the occurrence of necrosis and leucocytic infiltration, and edema. Lymphocyte and plasma cell infiltration of the lamina propria, edema, hyperemia, and epithelial hemorrhage spreading from the lamina propria to the surface of the mucosa are all hallmarks of the inflammatory reactions and pathological state of the mucosa caused by *H. pylori* (Slomiany *et al*., 2000; Mansfield *et al*., 2003). Stomach tissue specimens from infected animals treated with antimicrobial peptide Attacin A treated group revealed effectively repaired cells damaged by the *H. pylori* infection. Repair of tissue was evident in histological pictures of the rat stoma, with significant regrowth of glandular epithelia and infiltration of inflammatory cells into intestinal folds compared to the positive control group. It has been demonstrated that Attacin A contains Gly-rich antimicrobial peptides, which are well-known for their actions in preserving cellular integrity and protecting cells from harm (Gennaro *et al*., 2002). In contrast to the vast majority of other antimicrobial peptides, members of this class are very effective against Gram-negative bacterial species such as H.pylori, and they do this via a non-lytic method. More and more evidence suggests that once within the cytoplasm, the glycine and/or leucine-rich peptides bind to and disrupt the action of certain molecular targets important to bacterial development, leading to cell death. Owing to their mechanism of action, these peptides have the potential to be used in drug discovery (Buonocore *et al*., 2021).

The results of statistical analysis using ANOVA showed that the administration of the antimicrobial peptide Attacin A to experimentally induced infection of *H. pylori* rat model revealed significant alleviation of leucocyte infiltration as well as mucosal epithelial degeneration and glandular atrophy.

Tsakas and Marmaras (2010) explained the relationship between the immune response mediated by AMPs and acquired immunity to specific pathogens in insects, which depends on complex regulatory mechanisms. The lytic effect of AttA on pathogen membranes is due to its interaction with those membranes (Yu *et al*., 2016). The toroidal pore, carpet, and barrel stave modes of action are among the postulated mechanisms of action (Juárez-López *et al*., 2022). This combination of factors prevents substrates from moving between the bacterial cell and its external environment, hence reducing the cell's metabolic activity.

CONCLUSION

AttA peptide significantly slow the spread of *H. pylori* in the treated group compared to the infected/untreated group. Treatment with Attacin A in an infected group attenuate histological alterations of stomach tissue in infected albino rats.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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