Asian Journal of Medicine and Health

5(4): 1-9, 2017; Article no.AJMAH.34618
ISSN: 2456-8414

Helicobacter pylori Containing More Phosphorylation Sites of the CagA Protein Induces Greater Reduction of Gastric Mucins

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MFJ, DMMQ, IDA and MVC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All authors managed the analyses of the study. Author MFJ managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJMAH/2017/34618
Editor(s):
(1) John K. Triantafillidis, Associate Professor, Iasi University of Medicine and Pharmacy, Romania and IASO General Hospital, Holargos, Athens, Greece.
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(3) Jaques Waisberg, Hospital do Servidor Público Estadual, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/19977

Original Research Article

Received 2nd June 2017
Accepted 5th July 2017
Published 10th July 2017

ABSTRACT

Aims: *H. pylori* infection is associated with gastritis, pre cancerous lesions, gastric cancer and changes in the protective layer of mucus. However, to our knowledge, there are no studies in humans or experimental models that relate the infection with *H. pylori* strains containing one or three phosphorylation sites of the CagA protein with the mucins of the protective layer of mucus. The aim of this study was to research the effects of infection with *H. pylori* strains containing one or

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three phosphorylation sites of CagA protein on the MUC1 and MUC5AC mucins of Mongolian gerbils (*Meriones unguiculatus*) gastric mucosa.
Methods: Mongolian gerbils were inoculated with *H. pylori* isolates containing one or three *EPIYA C* phosphorylation sites. The control group consisted of uninfected animals receiving only Brucella broth. Gastric mucosa fragments were assessed by immunohistochemistry using rabbit monoclonal antibodies against MUC1 and MUC5AC. The fragments were then analyzed through a digital morphometric method.
Results: From the control group of non-infected animals (CTRL) to the Cag A positive locci with 1 *EPYIA C* group (CagA1Ep) and Cag A positive locci with 3 *EPYIA C* group (CagA3EP) there was a progressive and significant reduction in the expression of both MUC1 (p=0.003) and MUC5AC (p=0.003) after 45 days of infection. This reduction was more prominent in the CagA3Ep group. Similar results were obtained six months post-infection, but the reduction in the MUC5AC (p=0.001) and MUC1 (p=0.001) expression was more intense.
Conclusion: *H. pylori* strains containing more phosphorylation sites of the CagA protein are able to reduce with greater intensity the expression of MUC1 and MUC5AC.

Keywords: Helicobacter pylori; Mucin-1; Mucin-5AC; CagA protein.

1. INTRODUCTION

Mucins are high molecular weight glycoproteins synthesized by various secretory epithelial cells. In a normal human gastric mucosa the produced mucins include MUC1, MUC5AC and MUC6. The membrane-associated MUC1 is produced by foveolar cells and to a lesser extent, by the mucosal glands. The MUC5AC mucin is secreted only by the foveola epithelium cells and it is the primary constituent of the gel surface layer. The secretion of MUC6 is limited to the mucous glands [1].

H. pylori is mainly found in the gastric layer of mucus, and rarely colonizes the lower portions of the gastric mucosa [2]. It is well documented by some authors that patients infected with *H. pylori*, present a decreased amount of polymeric mucin on the surface of the gastric mucosa, with subsequent weakening of mucus layer firmly adhered to the surface [3]. Furthermore, in chronic gastritis the epithelial surface shows degenerative changes with loss of mucins and increased cell exfoliation [4]. Moreover, the different degrees of gastric diseases caused by *H. pylori* are closely related to their genetic background and their polymorphism index [5,6]. The main difference in the gene content of the different strains of *H. pylori* is the presence or absence of a 40 kb locus, called *Cag PAID* (Cag pathogenicity island), which is present in *H. pylori* strains distributed in different parts of the world [7-9]. This locus is composed by about 27 to 31 genes, among these, is the Cag 26 gene, which encodes a highly pathogenic protein called CagA; and the genes Cag7, Cag10 and Cag12, which encode a secretion factor type IV (T4SS) that translocates CagA into the interior of the gastric epithelial cells [10].

The virulence of some *H. pylori* strains is increased due to the phosphorylation of tyrosine residues of a sequence of five repeated amino acids, located in the carboxyl terminal portion of the CagA protein, called EPIYA. The polymorphism in this region involves changes in the sequence of repeated amino acids resulting in modified EPIYA A, B, C and D [5,11]. Thus, *H. pylori* strains with more number of EPIYAS have a higher degree of CagA phosphorylation, and therefore are associated with more severe chronic gastritis and the emergence of intestinal metaplasia and gastric cancer in humans [12]. Recently, we found that Mongolian gerbils infected with *H. pylori* strains containing three *EPIYA-C* sites developed a more intense chronic gastritis and changes in the numbers of G and D cells, as observed in human disease [13,14].

Some papers relate mucin content with *H. pylori* infection, both in humans and experimental models [15-17]. However, there is no study relating the content of specific mucins to infection by strains of CagA positive *H. pylori* with more than one phosphorylation site. To our knowledge, there are no studies quantitatively evaluating the expression of MUC1 and MUC5AC in healthy Mongolian gerbils. Thus, the aim of this research was to evaluate the immunohistochemical expression of MUC1 and MUC5AC mucins in Mongolian gerbils, which were healthy or infected by *H. pylori* strains containing one or three CagA EPIYA C phosphorylation sites, by a digital morphometric method.

2. MATERIALS AND METHODS

2.1 Animals, Experimental Design and Inoculation of *H. pylori*

The animals were kept under natural ventilation and mechanical exhaust, subjected to natural day and night cycles, with free access to filtered water and standard rat chow (Labina®, Purina, Brazil) and handled in accordance to the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG).

The animals were divided into three groups: a control group of non-infected animals (CTRL), a Cag A positive locci with 1 *EPYIA C* (CagA1Ep) group and Cag A positive locci with 3 *EPYIAS C* (CagA3Ep) group. Each group consisted of 16 animals. Eight of these animals were euthanized 45 days after the infection, and the remaining 8 after 6 months of the infection. The animals of this research were used in two previous studies [13,14]. Prior to inoculation, the animals were fasted for eight hours and lightly sedated with Isoflurane (Abbott, São Paulo, SP, Brazil). The inoculum of 0.8 ml of bacterial suspension containing 109 CFU / ml in sterile Brucella broth or only Brucella broth (CTRL group) was administered by intragastric gavage three times at 48 hour intervals. Four hours after inoculation, the animals were given standard rat chow (Labina®, purine, Brazil) and water ad libitum. Further details about the isolation of HP lines, DNA extraction and sequencing can be obtained in our previous study [13].

2.2 Necropsy and Immunohistochemical Reactions to MUC1 and MUC5AC

An abdominal incision was made, the stomach removed and opened along the greater curvature and the mucosa of the gastric body and antrum were exposed. Then the stomach was distended, washed with saline solution and the antral fragments of approximately 2.0 x 0.5 centimeters were collected along the small and large curvature to histological processing. Then, these fragments were fixed in 10% buffered formalin for a period of 48 hours. After this procedure, the fragments were dehydrated, diaphanized, infiltrated, and embedded in paraffin and sections of 4 micrometers thick were obtained for staining and immunohistochemical reactions. The sections were deparaffinized and hydrated in alcoholic solutions of decreasing concentrations and washed in phosphate buffered saline (PBS) pH 7.2. Antigenic recovery was performed with Dako Retrieval Solution (Dako, USA) at 100°C for twenty minutes for both MUC1 and MUC5AC producing cells. The endogenous peroxidase activity was supressed by incubating the sections in a solution containing 3.5% H2O2 (30vv) in 200 ml of PBS solution for twenty minutes, and the nonspecific binding sites were blocked by incubating sections with diluted goat serum (1:40) for forty minutes. For the identification of MUC1 and MUC5AC mucins, anti-MUC 1 and anti-MUC5AC monoclonal primary antibodies were used (Santa Cruz Biotechnology, Santa Cruz, USA. VU4H5 and H-160 clones, respectively) diluted in 0.1% BSA solution at concentration of 1:80. Subsequently, goat anti-mouse and goat anti-rabbit secondary antibodies for MUC5AC and MUC1(dilution, 1:50) were respectively used for one hour at room temperature. The stain was detected using a solution containing 0.05% diaminobenzidine in 0.2% H_2O_2 (40 v/v) for three minutes. As a negative control, the primary antibody was replaced with PBS in some antral sections of control gerbils. Histological antral sections of control gerbils were also used as positive controls. The sections were counterstained with Harris hematoxylin (diluted in distilled water to 60%) and mounted in entelan.

2.3 Morphometric Analysis of Immunohistochemical Expression of MUC1 and MUC5AC

For evaluation of MUC1 and MUC5AC expression, the area of immunohistochemical staining in the antral mucosa of all animals of the CTRL, CagA1EP and CagA3EP groups was measured in both periods of infection. A total area of $9x10^5$ μm^2 of the antral mucosa was scanned by a microcamera Q-Color3 (Olympus, Tokyo, Japan) for each animal. The area was calculated with the use of algorithms sequences built in the KS300 software coupled to a Carl Zeiss image analyzer (Oberkochen, Germany) [18]. Through the segmentation of images all brown pixels related to immunohistochemical markers were selected for creation of binary images and the subsequent calculation of the area (μm^2). The MUC1 and MUC5AC positive area of the control animals was considered as a normal expression pattern of these mucins in healthy Mongolian gerbils. The data were compared among the different groups and periods of infection by the two tailed Mann-Whitney U test (GraphPad Prism 6.01).

3. RESULTS

A brown cytoplasmic staining in the gastric epithelial cells identified positive immunohistochemical reactivity for both MUC1 and MUC5AC. Positive immunoreactive cells for MUC5AC were located predominantly in the apical portion of the foveolar epithelium and in the basal portion of the glands, usually showing a less intense stain between the two portions, with regions of negative marking. The MUC1-producing cells were found throughout the entire mucosal thickness, but usually with a lower marking intensity than MUC5AC mucin (Figs. 1a, b). The omission of the two primary antibodies did not produce a positive immunohistochemical reaction (Figs. 1c, d).

After 45 days of infection, there was a significant decrease in both MUC1 and MUC5AC expression in the CagA1Ep group (MUC1: 209,761 ± 13,740 μm^2; MUC5AC: 326,888 ± 11.298 μm^2) (p=0.003) and CagA3EP group (MUC1: 76,599 ± 16,709 μm^2; MUC5AC: 97,358 ± 18,724 μm^2) (p=0.003) when compared to the control group (MUC1: 322,024 ± 15,137 μm^2; MUC5AC: 424,140 ± 13,423 μm^2). This reduction was more prominent in the CagA3Ep group (p=0.003) (Figs. 2 a,b,c,d and 3a).

After six months of infection, there was also a significant decrease in MUC1 and MUC5AC expression in the CagA1Ep group (MUC1: 110,296 ± 9,667 μm^2; MUC5AC: 164,447 ± 8,111 μm^2) (p=0,001) and CagA 3Ep group (MUC1: 30,153 ± 7,972 μm^2; MUC5AC: 38,844 ± 8,714 μm^2) (p=0.001) when compared to the control group (MUC1: 288,529 ± 16,137 μm^2; MUC5AC: 441,149 ± 36,353 μm^2). The decrease was more pronounced in CagA3Ep group than in the CagA1Ep group (p=0.001) (Figs. 2e,f,g,h and 3b).

Comparing the infected animals that were euthanized 45 days post-infection to the infected animals that were euthanized six months post-infection, there was a reduction in the expression of both MUC1 (p=0,001, for both groups) and MUC5AC (p=0.03 and p=0.02 for CagA1Ep and CagA3Ep groups, respectively). No significant changes were observed between the CTRL groups in the two analyzed periods (p = 0.20 and p = 0.33 for MUC5AC and MUC1, respectively).

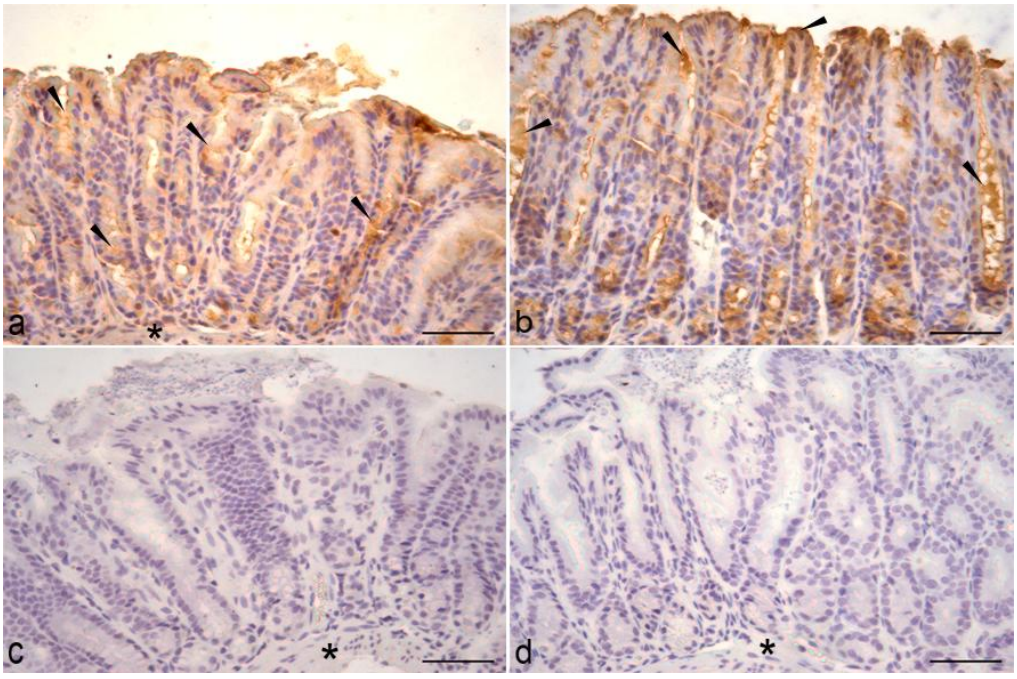

Fig. 1. Immunohistochemical reaction for MUC1 and MUC5AC mucins in the antral mucosa of the control group gerbils. Muscularis mucosa (*). (a) Positive reaction for MUC1 (arrowheads); (b) Positive reaction for MUC5AC (arrowheads); (c) Negative control to immunohistochemical reaction for MUC1; (d) Negative control to MUC5AC immunohistochemical reaction. Counter-staining with Harris hematoxylin. Scale bar: 50 micrometers

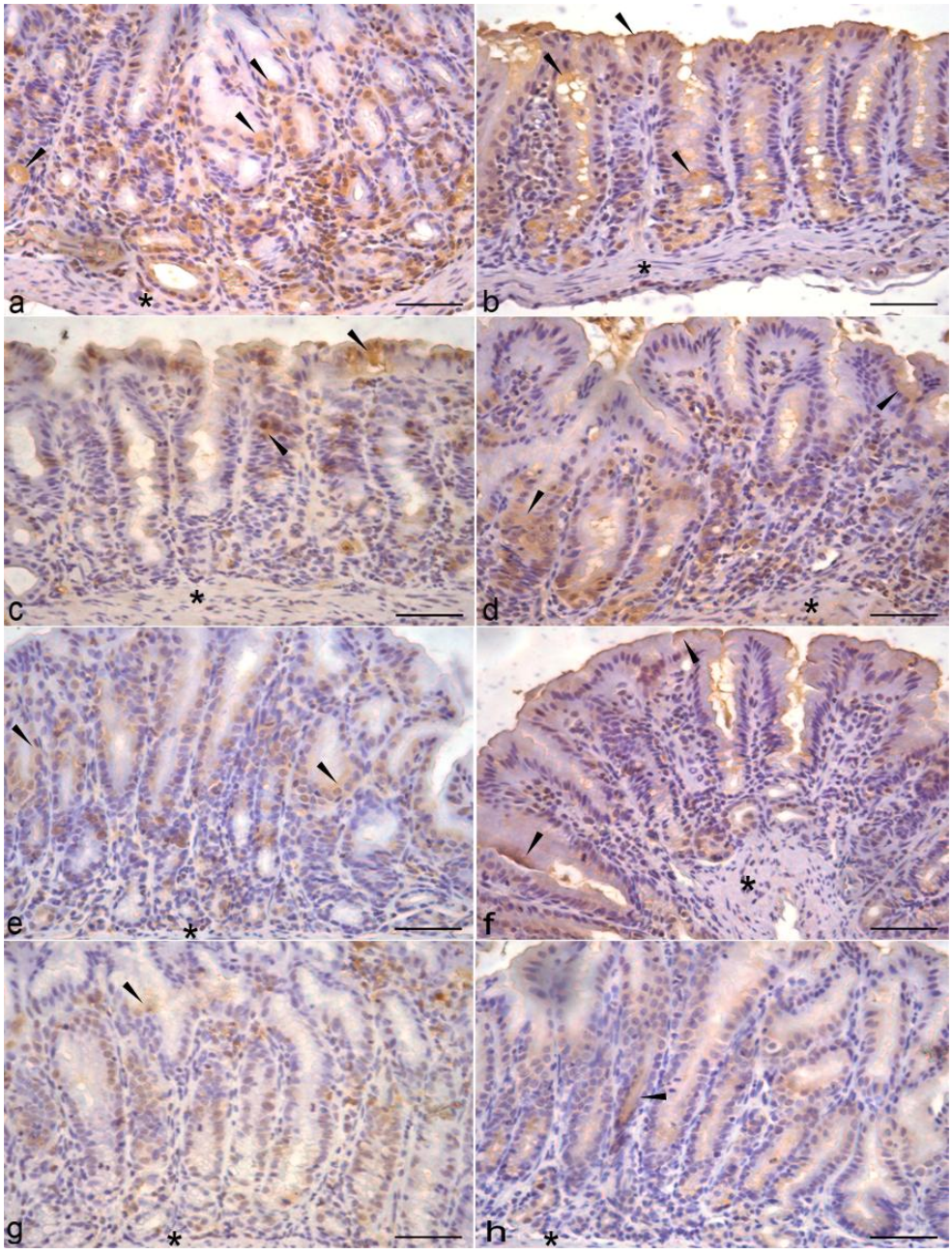

Fig. 2. Photomicrographs of immunohistochemical reactions for MUC1 and MUC5AC mucins in antral mucosa of gerbils infected with CagA positive *Helicobacter pylori*, with one or three *EPIYA-C* segments. Muscularis mucosa (*). (a) Positive reaction for MUC1 (arrowheads) - CagA1Ep group - 45 days of infection; (b) Positive reaction for MUC5AC (arrowheads) - CagA1Ep group - 45 days of infection; (c) Positive reaction for MUC1 (arrowheads) - CagA3Ep group - 45 days of infection; (d) Positive reaction for MUC5AC (arrowheads) -CagA3Ep group - 45 days of infection; (e) Positive reaction for MUC1 (arrowheads) - CagA1Ep group - six months of infection; (f) Positive reaction for MUC5AC (arrowheads) - CagA1Ep group - six months of infection; (g) Positive reaction for MUC1 (arrowheads) - CagA3Ep group - six months of infection; (h) Positive reaction for MUC5AC (arrowheads) - CagA3Ep group - six months of infection. Counter-staining with Harris hematoxylin. Scale bar: 50 μm

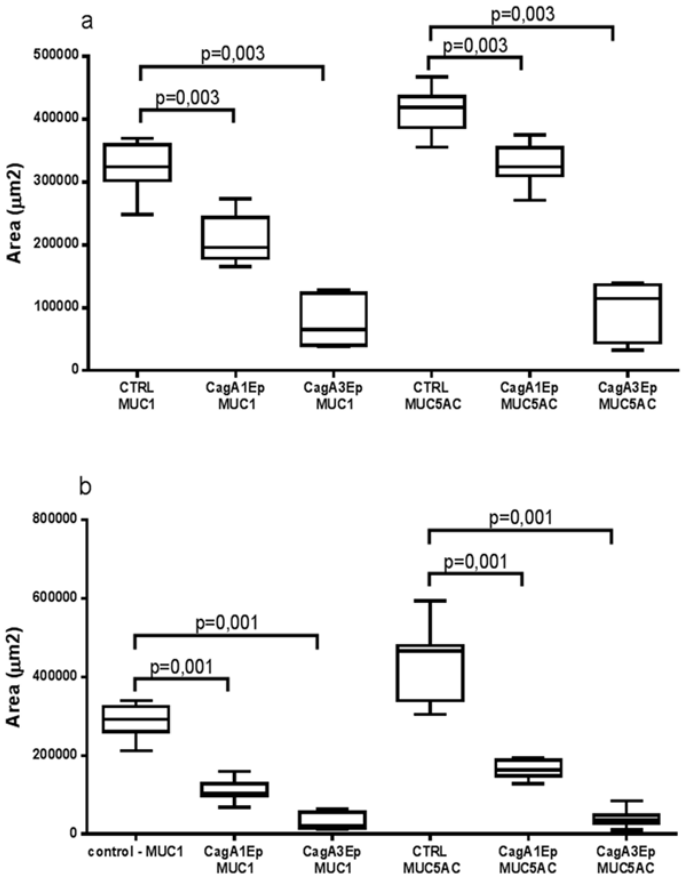

Fig. 3. MUC1 and MUC5AC areas of the antral gastric mucosa of gerbils infected with *H. pylori* strains producing CagA protein, containing one or three *EPIYA-C* phosphorilation sites, and of non-infected animals after 45 days (a) or six months (b) post infection. Each group consists of eight animals. The upper and lower boundaries of the boxes represent the percentage of 75% and 25%, respectively. The horizontal line throughout the box shows the median and the filled boxes indicate the maximum and minimum data value. Data were analyzed by the two-tailed Mann-Whitney U test

4. DISCUSSION

Several authors have demonstrated that infection by *H. pylori* interferes with the gastric mucin content [15,17,19]. However, this is the first study to demonstrate reduction in the gastric mucin expression of gerbils infected with CagA positive *H. pylori* strains containing one or three *EPIYA C* phosphorylation sites using an accurate morphometric digital method.

When *H. pylori* was still known as *Campylobacter pyloridis,* it was found that infection with this bacterium is able to interfere with the gastric mucus layer, leading to its reduction [20]. However, the mechanisms involved in these alterations are not fully understood and there is no consensus about the results obtained for the probable alterations in the gastric mucins composition [21].

In our study *H. pylori* isogenic strains isolated from a single patient with gastric cancer were used. We observed a reduction in the expression of two gastric mucins during different periods of infection. From the CTRL group to the CagA1Ep and CagA3EP groups there was a progressive and significant reduction in the expression of both MUC1 and MUC5AC after 45 days of infection. This reduction was more prominent in CagA3Ep group. Similar results were obtained six months post-infection, but the reduction of the MUC5AC and MUC1 expression was more intense. The findings about the reduction of

mucins associated with *H. pylori* infection are supported by the work of other authors [4,14,17,19]. Navabi et al. [19] showed a reduction in the MUC1 production and a decreasing trend in the production of MUC5AC of the gastric antrum of mice infected with *H. pylori* [19]. The reduction of MUC5AC expression was also progressively higher in human advanced gastric carcinoma when compared to metaplasia and dysplasia, suggesting that mucin alterations could be considered as markers of the gastric mucosa malignant transformation [16]. Also in mice infected with *Helicobacter felis*, it was observed a loss of MUC5AC, which was correlated with gastritis and occurred before the onset of atrophy and intestinal metaplasia [20].

H. pylori seems to interfere in the MUC1 and MUC5AC glycosylation process, favoring the interaction of bacterial adhesins with the gastric epithelial surface and contributing with the bacteria colonization [17]. Furthermore, *H. pylori* releases urease, which degrades urea and releases ammonia, raising the gastric pH. This increase in pH results in a decrease in the mucus layer viscoelasticity making it more fluid, which facilitates the mobility of the bacteria through this layer and its adhesion to gastric epithelial cells [21]. The VacA cytotoxin is another product of *H. pylori* that is involved in the mucin reduction. Beil et al. [20] demonstrated that positive CagA *H. pylori* strains that produce vacA cytotoxin caused a significant inhibition of the mucin synthesis when compared to cagA positive strains that do not produce this toxin. Negative CagA strains did not interfered in the mucin synthesis.

Besides the bacterial inherent factors, inflammatory phenomena seem to be significantly involved in the reduction of gastric mucin. In chronic gastritis caused by *H. pylori*, the epithelium surface shows degenerative changes with loss of mucins and increased cell exfoliation. These degenerative changes are sometimes accompanied by atrophy, while the lamina propria becomes edematous and with the presence of inflammatory cells [4]. Neutrophilic proteases released during gastritis caused by *H. pylori* may contribute to the collapse of the mucins structure [3].

Some studies evaluated the possibility of different cytokine profiles produced during *H. pylori* infection to interfere with mucin expression. IL-1β as well as IL-6 were able to increase the gastric mucin expression of epithelial cells in vitro, whereas IFN-γ potentiated the inhibitory effect of the *H. pylori* lysate [22]. Different authors have observed that *H. pylori* infection induces a prominent IFN-γ synthesis, with significant increase in the number of T cells positive for this cytokine [23,24]. Besides bacterial inherent factors, a higher concentration of IFN-γ could act as a helping factor in mucin reduction.

Some authors have shown that infection of humans with *H. pylori* CagA positive strains containing a high number of EPIYA C phosphorylation sites is associated with more severe chronic gastritis and an increased risk of developing intestinal metaplasia and gastric cancer [25,26]. Our group has recently demonstrated that Mongolian gerbils infected with *H. pylori* strains containing three *EPIYA-C* sites have a more intense gastric inflammation than the animals infected with strains containing only one *EPIYA-C* site [13]. It is known that the most virulent strains contain Cag PAI (cag pathogenicity island), responsible for encoding the T4SS. When the CagA protein is injected inside the gastric epithelial cell through T4SS, the protein is phosphorylated on its tyrosine residues located in a region of five repeated amino acids named *EPIYA* (Glu, Pro, Ile, Tyr, and Ala) A, B, C and D located in its carboxy-terminal portion. The CagA phosphorylation results in a bond with a protein tyrosine phosphatase SHP2. This CagA-SHP2 complex activates intracellular protein kinases of the MAPK family, the ERK1 / 2, inducing transcription of proinflammatory, anti-apoptotic and mitogenic genes [27,28]. Furthermore, the more intense CagA phosphorylation, the greater its binding force with protein SHP2 [29]. Thus, it is believed that the greater the number of EPIYA phosphorylation sites of CagA, the greater will be the inflammatory cytokines activation, resulting in a more exuberant inflammatory response. In fact, animals experimentally infected with *H. pylori* containing *EPIYA C* with 3 phosphorylation sites, produced a more intense gastritis than the CagA1Ep group [13].

From the Ctrl groups to the CagA1Ep and CagA3Ep groups it was possible to demonstrate a significant and progressive reduction in the MUC1 and MUC5AC contents in both periods of infection. This can be attributed to the influence of bacterial toxicity factors that interfere with MUC1 and MUC5AC synthesis and also to the inflammatory process. These findings are of great scientific and clinical relevance, since it

was demonstrated that *H. pylori* strains containing more phosphorylation sites of CagA induces a greater decrease in one of the gastric mucosa major defense barriers, predisposing a higher contact with toxic substances and pathogens, favoring a more severe gastritis and the appearance of precancerous lesions and gastric cancer. However, further work will be necessary to evaluate the effect of reducing the expression of MUC1 and MUC5AC in the development of precancerous and neoplastic lesions caused by *H. pylori* for a longer period of infection.

We concluded that *H. pylori* strains containing more *Epiya-C* phosphorylation sites of CagA induce a greater decrease of MUC1 and MUC5AC in Mongolian gerbils gastric mucosa than strains containing less phosphorylation sites.

5. CONCLUSION

H. pylori strains containing more phosphorylation sites of the CagA protein are able to reduce with greater intensity the expression of MUC1 and MUC5AC.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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