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Inflammatory response induced by *Helicobacter pylori* infection in lung

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Abstract

Helicobacter pylori is a microorganism that in the last years has been associated with extragastric disorders such as respiratory diseases, however, its impact on lung is partially understood. The aim of this work was to study infection impact of *H. pylori* on the inflammatory markers expression at the pulmonary level using an animal model. Infection was performed by BALB/c wild type (WT) mice orotracheal instillation with 20 µl of 1×10^8 *H. pylori* reference strain suspension once per day throughout 3 days. Inflammatory response was evaluated at 3, 7, 14, 21 and 30 days post infection. Lung was aseptically removed and pulmonary edema index values showed a significant change at 30 days of infection. Hematoxylin-Eosin (H-E) stain allowed to visualizing *H. pylori* presence in lung samples at 3 days of infection near the phagocytic cells or in the alveoli lumen. Bronchoalveolar lavage (BAL) was used for inflammatory response evaluation.

Lactate dehydrogenase values showed a gradual increase in infected animals along infection time. Protein concentrations in mg/ml from BAL increased significantly at 7 days in infected animals. Macrophages viability obtained from BAL, decreased at the first moment of infection, maintaining constant values along contamination time.

Results obtained demonstrate an inflammatory response in lung after orotracheal *H. pylori* infection and suggest that the pathogenic mechanism is strongly evidenced by tissue damage, endothelial dysfunction inflammatory mediators and markers expression at the pulmonary level.

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Introduction

Half of the world's population is infected with *Helicobacter pylori* attributable to the infection transmission between humans. All available literature regarding the prevalence of this microorganism shows lower infection rates in developed countries (around 30-40%) compared to developing countries, in which some exceed 85% (Zamani and col., 2017; Hooi et al., 2017).

H. pylori is a curved gram-negative, strict microaerophilic bacillus that colonize human stomach. The microorganism has several virulence factors such as the potent urease and mobility that allow it to enter the gastric mucosa, alter the protective mucus and penetrate between epithelial cells for establish a chronic infection. This is a prior condition for the development of several disorders such as peptic ulcer disease, atrophic gastritis, gastric adenocarcinoma, and MALT (mucosa-associated lymphoid tissue) lymphoma (Sonnenberg et al., 2010; Malfertheiner et al., 2017; Liu et al., 2019).

In addition, stomach persistence is strongly influenced by microorganism ability to elude the host's immune system. In the innate immune response evades different receptors, through the target modification and transduction suppression of recognition signalling pathways. While, in the adaptive immune response, evasion is due to the effector modulation functions of T cells (Salama et al., 2013).

In recent decades, several authors have proposed an *H. pylori* infection association in body organs and areas outside the gastroduodenal zone. The first report was made by Mendall and its collaborators, in 1994, when *H. pylori* seropositivity was associated with coronary heart disease. Different pathologies related with the microorganism include diabetes mellitus, idiopathic thrombocytopenic purpura, iron deficiency anemia, Raynaud's phenomenon, migraine, Guillen Barré syndrome and cardiovascular, liver, dermatological (rosacea, idiopathic urticaria), autoimmune (thyroiditis), neurological and pulmonary (Suzuki et al., 2011; Goni and Franceschi, 2016; de Korwin et al., 2017).

H. pylori ability to reach the respiratory tract, together with the similarity between bronchiectasis and ulcerogenesis, could explain the relationship of this bacterium with respiratory diseases. In that sense, in the ulcerogenesis produced by the microorganism, a large recruitment of neutrophils and T lymphocytes in the submucosa and release of IL-8, IL-1 and TNF-α, is observed. This allows us to propose the hypothesis of a possible pathogenic role of the microorganism in the respiratory tract.

The mechanism that relates *H. pylori* infection and respiratory diseases development is not fully elucidated. However, there are three theories that could explain this relation, as well as *H. pylori* infection possible mechanism in the pathogenesis of most extragastric diseases (Chmiela and Gonciarz, 2017). The theories are: i) *H. pylori* immune response impulse; ii) Molecular mimicry and iii) Bacteria Aspiration or inhalation in the respiratory tract (Malfertheiner et al., 2011; Deng et al., 2013; Samareh Fekri et al., 2014; Testerman and Morris, 2014; Chmiela and Gonciarz, 2017).

In this way, previous study showed the potential *H. pylori* pathogenic role in the histoarchitecture of the lung using mice as animal model (Arismendi Sosa et al., 2018). The aim of this work was to study the *H. pylori* infection impact on the lung inflammatory markers expression.

Materials and methods

<u>Animals and experiment protocol</u>

BALB/c wild-type (WT) mice were used. They were keep under specific-pathogen-free condition at UNSL's bioterio, according to protocol approved by the Comité Institucional de Cuidado y Uso de Animales (CICUA). The male mice of 6–12 weeks old, and 25–30 g approximately, was provided with sterile food and water *ad libitum* during the experience. Three independent experiments were carried out with 4 four mice per group (total n=20 mice).

Bacteria and experimental protocol for *H. pylori* infection

Strain NCTC 11638 (kindly provided by Dra. Teresa Alarcón Cavero, Microbiology Service of Hospital Universitario de la Princesa, Madrid, Spain) was used for infection. *H. pylori* strain were grown in Mueller- Hinton agar (MHA) supplemented with 7% horse blood (MHA-HB) at 37 °C under microaerophilic conditions and identified by microscopy, urease, catalase and oxidase tests. A bacterial suspension of $1–5x10^8$ colony forming units for millilitre (CFU/ml) was prepared for the infection. Mice were divided in two groups, infected and control. To the infected group, 50 µl of the microorganism suspension was administered by orotracheal instillation; while to the control group it was given phosphosaline buffer (PBS 1X pH 7.4) sterile.

Lung edema

The lungs of both groups were obtained aseptically and surgically separated from the rest of the airway and heart. Each lung was blotted, weighted and finally oven dried to constant weight, at 60 °C for 48 h. From the difference in weight, an index is obtained. The index shows wet to dry lung relation in order to assess tissue edema (Folz et al., 1999).

Bacteriologic identification

The presence of *H. pylori* was tested in lung from one mouse from each group and at each time point. The lung was extracted aseptically, was fixed with Bouin's liquid, and the paraffin wads were mounted. The obtained sections were stained with Hematoxylin-Eosin (H-E) and visualized under an Olympus BX50 optical microscope connected to a digital camera. The analysis of the microphotographs was performed with the ImageJ system and the shapes similar to *H. pylori* were analysed.

Bronchoalveolar Lavage (BAL)

Mice of both groups at 3, 7, 14, 21 and 30 days after infection, were anesthetized with ketamine-xylazine and the trachea was cannulated in order to obtain the BAL. The BAL was collected using cold sterile PBS and centrifuged at 3000 rpm to perform the proper analysis.

The supernatant was used for the determination of Lactate dehydrogenase (LDH), while the the cells pellets were used for viability evaluation and cell culture.

BAL cell viability assessment

BAL cells ($0,5–1 \times 10^6$ cell/ml) were diluted in Trypan blue and counted within 3 minutes. Cell viability was further assessed by Trypan blue exclusion.

Determination of proteins in BAL

Protein concentration was determined in BAL by the method of Lowry et al. (1951) using bovine serum albumin as a standard (Gutierrez et al., 2008).

Lactate Dehydrogenase (LDH) determination

Lactate dehydrogenase (LDH) activity in BAL was determined by monitoring the LDH catalyzed oxidation of pyruvate coupled with the reduction of NAD using a commercial kit

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from Wienner Laboratory (Buenos Aires, Argentina) and following the manufacturer's instructions.

Macrophage cytotoxicity from BAL

Macrophages obtained from BAL, were centrifuged at 3000 rpm and cultured in 96 wells plates with DMEM medium. Cell cytotoxicity tests were performed using the MTT assay with the cell proliferation reagent MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma), as described previously. The MTT tetrazolium ring is cleaved only by active mitochondria, yielding purple formazan crystals whose amount directly correlates with the viable cell count. At 16 h post-infection, 10 µl of a 5 mg/ml MTT solution was added into each well, and the plates were incubated at 37°C. The purple formazan crystals were dissolved by adding 100 µl of MTT solubilization solution, and the absorbance at A_{570} was spectrophotometrically measured with a reference wavelength of A_{690}. Three wells per experimental condition were counted in three independent experiments.

Statistical analyses

All experiments were repeated at least three times. Statistical differences were tested by analysis of variance (ANOVA) or by unpaired Student's test. All data are expressed as the mean ± S.E.M. (Standard Error of Mean). A probability of $p < 0.05$ was considered statistically significant.

Results

Pulmonary edema marker evaluation

Pulmonary edema marker was measured to probe pulmonary capillary permeability. The inflammation was evaluated at different times of infection, by indexes that determine the lung wet/dry ratio. In the early stages of infection, no significant difference was observed; however, after 21 days edema increased but the difference is not significant. Edema was significantly higher at 30 days in the infected group respect to control group (Fig. 1).

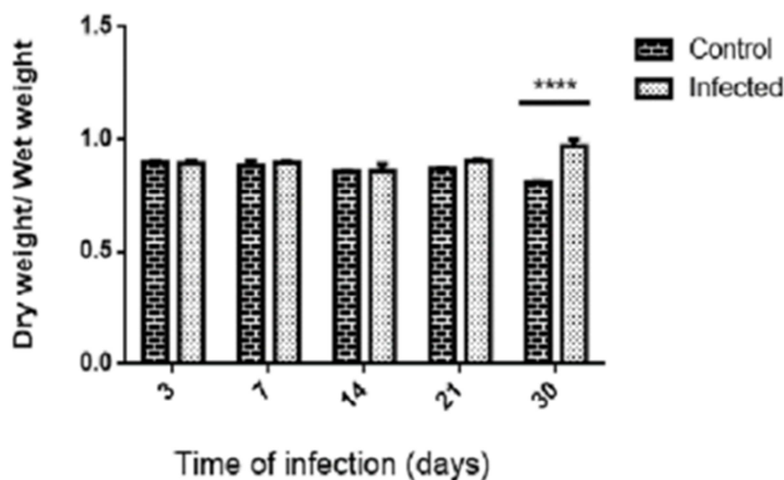


Figure 1: Pulmonary edema index. The degree of tissue permeability was not modified in the lungs of the infected group respect to the control, in the first days of infection (3, 7 and 14). However, there was a slight increase after 21 days of treatment and showed significant difference at 30 days after infection (****: p <0.0001).

Bacteriologic examination

H. pylori presence in samples of lung after infection were visualizing at 3 days of infection, where characteristic curved bacilli of *H. pylori* near the phagocytic cells or in the lumen of the alveoli are detected (Fig.2).

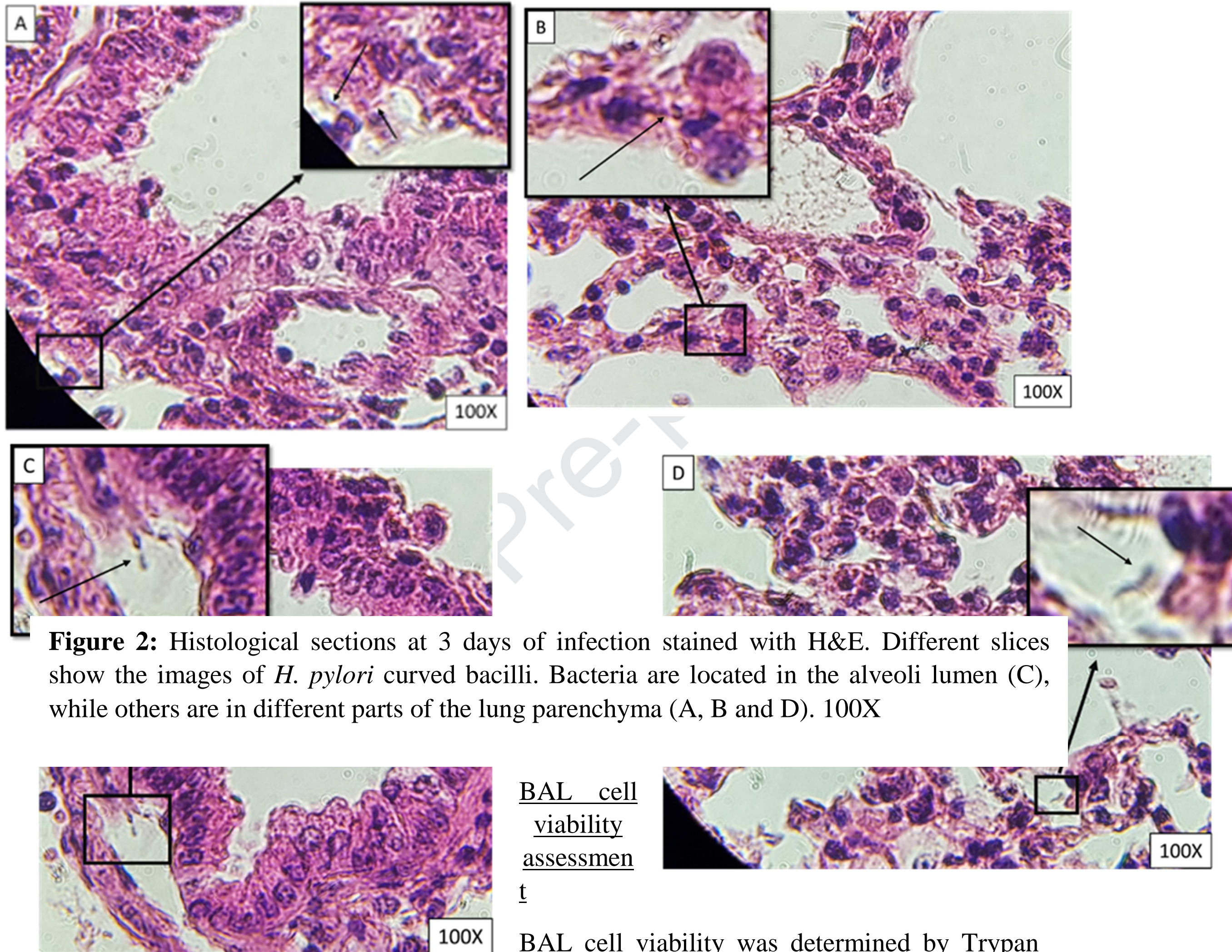


Figure 2: Histological sections at 3 days of infection stained with H&E. Different slices show the images of *H. pylori* curved bacilli. Bacteria are located in the alveoli lumen (C), while others are in different parts of the lung parenchyma (A, B and D). 100X

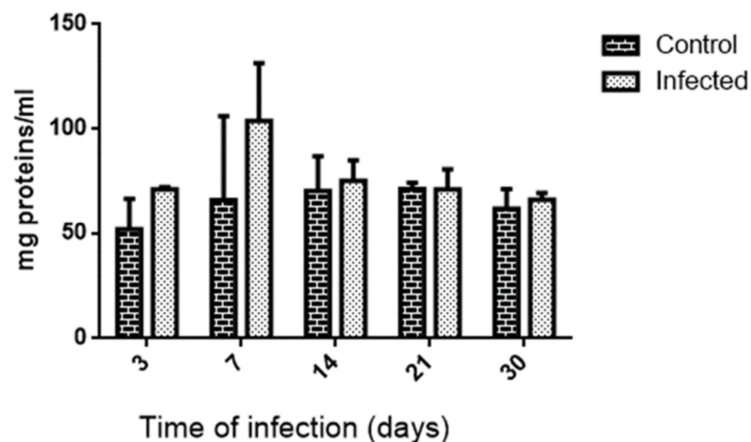
BAL cell viability assessment

BAL cell viability was determined by Trypan blue exclusion immediately after cell isolation. Cell viability showed no significant difference in the values obtained for days 3, 7 and 14 days after infection. However, at 21 and 30 days after infection, percentage of viable BAL cells obtained from infected animals decreased compared to the control ($p < 0.01$) (Fig. 3).

Figure 3: Cell viability. The results were expressed as a percentage of viable cells obtained from the BAL. The first times of infection (3, 7 and 14 days) showed no significant difference for the viability of BAL cells of infected mice with respect to controls. At 21 and 30 days after infection, the viability of the cells obtained from infected mice decreased when compared to the control group. At day 30, the percentage of viable cells was significantly higher in control mice compared to infected mice (**: $p < 0.01$).

Protein determination in BAL

Proteins are an indicator of changes in cell permeability and provide information about potential tissue damage caused by infection. Protein concentrations in mg/ml were determined in BAL obtained from control and infected animals. At day 3 it increased slightly and reached a maximum values at 7 days post-infection. Although there was a decrease in the infected mice protein concentration on day 14, while no significant differences in lavage protein concentration were observed until 30 days after infection (Fig. 4).



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Lactate dehydrogenase (LDH) determination

In infectious processes, another cell damage indicator is the release of enzyme Lactate dehydrogenase (LDH). Along infection time control group showed normal enzyme values, while in infected animals a gradual values increase was observed and progressed with values close to 400 U/I at 21 and 30 days after infection (p <0.001) (Fig. 5).

Figure 4: (concentrations obtained at ntrol mice. An increase in ference is not significant.

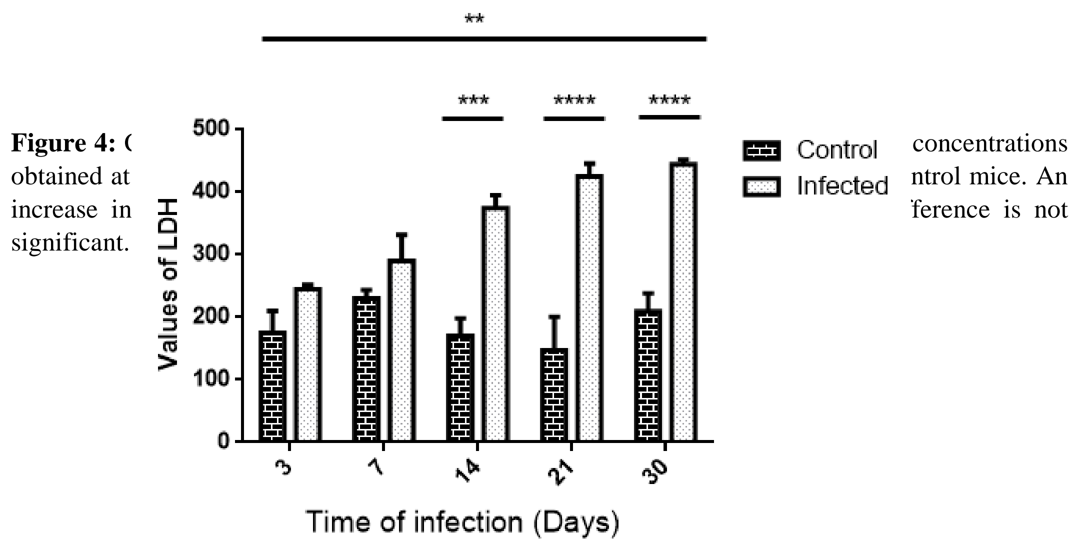

Figure 5: Lactate dehydrogenase (LDH) values. The graph shows a gradual increase in LDH values in infected animals as the infection time elapses, while LDH values for control animals remain within normal range (100-240 U / I) (****: p <0.0001; ***: p <0.001; **: p <0.01).

In vitro macrophage cytotoxicity

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Macrophage viability during the infection was measured through MTT test. Macrophages obtained from infected and control animals lungs were grown in 96-well plates for 24 hours, and then MTT solution was added, incubated for 4 hours and absorbance was measured at 550-570 nm. Results showed that macrophages viability diminished at the first time of infection (day 3), while, a non-significant increase was observed throughout the rest of the infection days at constant values.

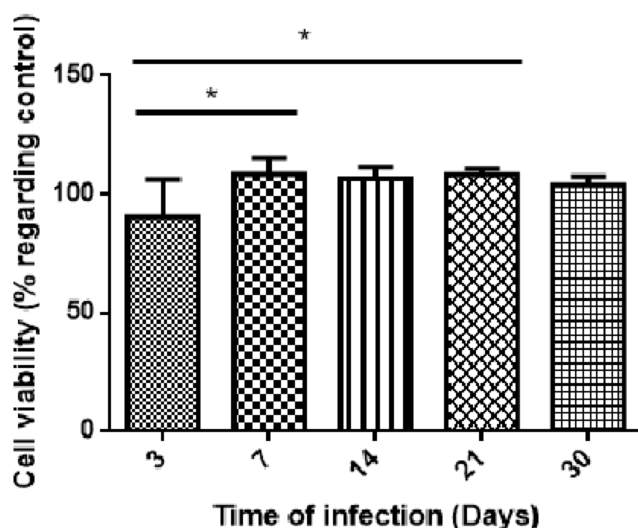

Figure 6: Cytotoxicity assay. This assay is frequently used to investigate cytotoxicity. Graph shows cell viability relationship between control and infected animal macrophages. A decrease is observed on the third day of infection, while in the rest of the time viability is not modified between controls and infected cells (*: p <0.05).

Discussion

Gastric mucosa has been recognized as a natural niche of *H. pylori*, which is infecting approximately 50% of the world's population. Microorganism ability to adhere in the gastric mucosa epithelium or internalize within these cells, induces pathologies such as gastritis, peptic ulcers, MALT-type lymphoma and even cancer. However, there is an increase in scientific articles that associate these microorganisms with different extragastric disorders, such as autoimmune, neurological, skin, hematological, gynecological and vascular diseases. Also, it has also been associated with respiratory disorders, such as chronic obstructive disease (COPD), bronchioectasia, asthma, lung cancer and chronic bronchitis, in which microorganism seroprevalence is high (Malfertheiner et al., 2011; Shams-Hosseini and col., 2011; Chmiela et al., 2018; Larussa et al., 2018). It is well known that the gastrointestinal location of *H. pylori* is attributed to its "protective advantages" against highly acidic environments, however, it is still unknown if it would have any advantage in both upper and lower the respiratory tract. All available information about *H. pylori* infection in the lung is based on epidemiological studies or case studies, in which there is a discrepancy, linked to the difference in patient characteristics and the diversity of methods used in the bacteria detection. In addition, most studies have been aimed to demonstrate association between *H. pylori* and respiratory diseases as well as understanding its pathogenesis, hypothesizing that the gastric infection of *H. pylori* initiates a systemic immune response that has a lung impact of susceptible patients (Kreuter et al., 2016).

Present study demonstrates an inflammatory response in lung after orotracheal *H. pylori* infection. In this situation, inflammation index obtained by comparing the weight difference between control and infected lungs was used as a marker of pulmonary edema. Results showed that *H. pylori* infection in mice lungs did not cause water mobilization towards the organ stroma, in the first days of infection. While, at 21 days, a small increase was determined compared to controls, being more noticeable at 30 days after infection. This would indicate an increase in capillary permeability as a result of pulmonary edema. Other studies conducted in

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mice infected with *H. pylori* showed an increase in the lung inflammatory index 24h after infection. However, these studies were not performed with a follow-up kinetics as in our work Chen et al. (2017). On the other hand, Feng et al. (2016) also obtained an increase in the inflammatory index in mice infected with *Pseudomonas aeruginosa* 72 hours after infection. In contrast, mice infected with the murine pneumonia virus showed, for the first days of infection, similar values between the control and infected groups, with an increasing on day 6 and maximum value at day 8 after infection (van den Berg and col., 2014).

Previous studies show that once *H. pylori* infection in the lung occurred, microorganism could only be recovered from the tissue seven days post infection, but not in the next days. This was associated with a decrease in bacterial load during the process of lung atrophy observed on days 21 and 30 after infection (Arismendi Sosa et al., 2018). Lung atrophy creates a hostile environment for *H. pylori*, in which it cannot persist and therefore, is not possible to recovered it from tissue lung.

Additionally, it has been reported that when *H. pylori* is phagocytized by macrophages, enters the phagosome and a megasome is formed. Bacteria contained within both organelles have a cocoid appearance, which are viable, but not cultivable. Thus, the microorganism only has been recovered from macrophages after 24 h of infection (Borlace et al., 2011; Arismendi Sosa et al., 2018). In coincidence, in our study, the results obtained from the Hematoxiline - Eosine staining analysis at day 3 after infection, bacilli with helical morphology characteristic of *H. pylori* are observed located in the proximity of phagocytic cells. Although, at long time of infection we observed a greater presence of active macrophages, but no bacteria were observed after 7 days of infection in the analyzed tissue.

Gutierrez et al., (2008) demonstrated that infection with *Y. enterocolitica* induces an increase in BAL protein values of infected mice at 21 days, while in the first days of infection the increase is not significant (Gutiérrez y col., 2008). In concordance, our results showed a not significant increase after 3 and 7 days' infection with *H. pylori* in proteins, in infected mice with respect to the control. Although there is a decrease in protein concentration in infected mice at 14 days, which remains constant until 30 days. This indicated that while other markers account for the inflammatory process, this particular infectious process does not produce extravasation of proteins, at the alveolar space.

In order to evaluate cell damage, enzyme lactate dehydrogenase activity was determined. In this experimental model, BALs obtained from both control and infected mice was used to determine LDH activity. Gradual increase in the values, along the infection time, were observed, with values close to 400 U/I at 21 days after infection, almost double value obtained for the control group. Comparing with the lung response after *Y. enterocolitica* infection, it was an increase in LDH´s values at 21 days, between 100 to 200 times more to controls (Gutiérrez et al., 2008). Both bacteria (*Y. enterocolitica* and *H. pylori*) increase the response at approximately the same time of infection, although the responses to *H. pylori* are more exacerbated. Therefore, in both experimental lung mouse models, cell damage increases along time infection, indicating that the lung parenchyma injury exacerbates as time progresses. In addition, studies in patients with gastroesophageal reflux and suffering from idiopathic pulmonary fibrosis; showed high levels of LDH in both serum and BAL samples. This leads to the hypothesis that there is a possible association between both pathologies (Vukovac et al., 2014).

Previous studies in BAL obtained from *H. pylori* infected mice showed a slight decrease in the viability of macrophages on the third day of infection, which is maintained in the following infection days and decreases significantly after 30 days. Additionally, the presence of lymphocytes and few PMNs was observed (Arismendi Sosa et al., 2018). These results are consistent with Gutiérrez et al. (2008) who observed in C57BL/6 wild type (WT) mice infected

with *Y. enterocolitica* that cell viability decreased slightly at 3 days' post-infection, with a significant decrease at 21 days. The viability of this defense cell declines after *H. pylori* is swallowed and phagocytized. Future studies would be needed to understand cell viability loss processes factors whichever bacteria are responsible.

Results obtained suggest that *H. pylori* pathogenic mechanism is strongly evidenced by lung tissue damage. This knowledge is crucial to understanding how inflammatory mononuclear and phagocytic cells participate in the host antimicrobial defense and cause inflammatory damage, considering that they could be a possible target for pharmacological therapy.

Conclusion

Helicobacter pylori infection impact on the respiratory system represents a field with unfinished data and pending responses. This is the first works, using an animal model, whose results reveal bacteria pathogenic potential evidenced by tissue damage, endothelial dysfunction inflammatory mediators and markers expression at the pulmonary level.

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Highlights

- *Helicobacter pylori*´s infection in lung showed edema pulmonary.
- The concentration of proteins from bronchoalveolar lavage showed significant values at 7 days.
- Values of lactate deshidrogenase from BAL were high in the infected group.

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