Low Concentrations of Zinc in Gastric Mucosa are Associated with Increased Severity of Helicobacter pylori-Induced Inflammation

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Abstract

Background: Chronic Helicobacter pylori infection is the most common cause of gastric cancer. H. pylori induces oxidative stress while zinc deficiency results in increased sensitivity to it. In Ecuador, the prevalence of gastric cancer and zinc deficiency are high. We hypothesized that zinc deficiency in Ecuadorian people would cause increased H. pylori-induced inflammation in the gastric mucosa associated with lower tissue zinc concentrations.

Methods: Three hundred and fifty-two patients with dyspepsia underwent endoscopy to obtain gastric mucosa biopsies. Diagnosis of H. pylori infection and its severity, histopathology, mucosal zinc concentration, and inflammation intensity were determined.

Results: H. pylori-infected patients with non-atrophic chronic gastritis had lower concentrations of zinc in gastric mucosa than uninfected patients with the same type of gastritis (251.3 ± 225.3 vs. 426.2 ± 279.9 ng/mg of protein; p = .016). Considering all patients, the more severe the H. pylori infection, the higher the percentage of subjects with infiltration by polymorphonuclear (PMN) cells (p = .0001). Patients with high PMN infiltration had lower mucosal zinc concentrations than patients with low PMN infiltration (35.2 ± 20.7 vs. 242.9 ± 191.8 ng/mg of protein; p = .021).

Conclusions: The degree of inflammation in H. pylori-induced gastritis appears to be modulated by gastric tissue zinc concentrations.

More than 50% of the population worldwide is infected by Helicobacter pylori [1]. Chronic H. pylori infection is associated with gastritis and peptic ulcer disease [2], and is also the single most common cause of gastric cancer [3]. Gastric cancer is the second most common cause of cancer-related mortality worldwide [4]; however, there are important regional differences in gastric cancer prevalence. Western Europe and the United States have a low incidence, whereas Japan, China, and South America have a higher incidence [3,4]. In Ecuador, gastric cancer is a serious public health problem that represents 12.7% of all cancer cases, with a prevalence of 29 cases per 100,000 inhabitants [5].

H. pylori is successful at surviving the effects of many components of the innate and adaptive host immune response [2,6,7]. H. pylori possesses many virulence factors that allow it to survive in this hostile environment, such as urease, flagella, cag type IV secretion system, and neutrophil-activating protein (NAP) [2]. The cag system allows H. pylori to transfer into host cell cytoplasm different bacterial proteins that modulate host-cell functions [8]. One of the principal functions of cag is the activation of interleukin-8 (IL-8) gene expression. IL-8 is a potent neutrophil (polymorphonuclear – PMN) chemotactic factor [9–11]. Once attracted, these PMN are activated by NAP to perform their inflammatory functions [12]. Activated PMN cells produce large quantities of reactive oxygen species (ROS) that facilitate bacteria killing.

It has been demonstrated that damage to the gastric mucosa during H. pylori infection is mainly caused by increased ROS and consequent oxidative stress [13–15]. Catalase, superoxide dismutase (SOD), thioredoxin, and glutathione reductase have been shown to serve important roles in the mucosal defense against H. pylori infection [16–19]. H. pylori-induced gastric mucosa inflammation is characterized by an increase of manganese SOD and reduction of copper/zinc SOD [17]. It has also been shown...
that after \textit{H. pylori} eradication, the gastric mucosa content of copper/zinc SOD returns to normal levels [18]. Furthermore, a recent study demonstrated that the overexpression of the antioxidant enzyme thioredoxin, in a mouse model of \textit{H. pylori} infection, not only reduces oxidative stress but also inhibits PMN chemotaxis [20]. All these findings emphasize the role of oxidative stress in \textit{H. pylori}-induced gastric pathogenesis.

Zinc is an abundant intracellular micronutrient that participates in a wide range of cellular processes [21]. Zinc deficiency results in an increased sensitivity to oxidative stress [22–24]. As zinc participates in several critical cellular functions, especially DNA repair, it has been suggested that zinc deficiency may increase the risk for cancer [23,25,26]. Zinc deficiency is a major public health problem in Ecuador [27,28]. There is evidence that zinc deficiency in Latin American people, particularly from the Andean regions as Ecuador, is due to both lack of nutritional sources of zinc in their diet and increased consumption of cereal bran containing phytates, which are strong chelators of minerals and therefore prevent zinc absorption [29]. Given the risk of zinc deficiency in Andean populations and the role of zinc in the response to oxidative stress, we hypothesized that a poor population living in an Andean region of Ecuador would have increased \textit{H. pylori}-induced inflammatory damage in the gastric mucosa associated with lower mucosal zinc concentrations.

\section*{Subjects and Methods}

\subsection*{Study Design and Population}

The study was performed in Hospital Pablo Arturo Suarez, in the city of Quito, Ecuador. This hospital serves a low-income population that is mainly of Spanish-Indian (mestizo) background. Subject enrollment took place from 2000 to 2003. The study design and protocol were approved by the Bioethical Committees of the Corporación Ecuatoriana de Biotecnología and that of the Pan American Health Organization (PAHO).

Patients of either sex seeking care in the hospital aged 35–69 years old with a diagnosis of dyspepsia and who were willing to provide written informed consent were eligible for participation. Exclusion criteria included previous treatment for \textit{H. pylori} in the last 6 months, regular use of local or systemic antimicrobial agents (e.g. antibiotics and bismuth), and the presence of other severe diseases (cancer, cardiac, renal or respiratory failure).

After enrollment, study participants underwent endoscopy to obtain gastric mucosa biopsies. From these biopsies the diagnosis of \textit{H. pylori} infection and its severity, histopathologic changes, zinc concentration, and intensity of inflammation were determined.

\section*{Definitions}

\subsection*{Dyspepsia}

Dyspepsia was defined as the presence of epigastric pain shortly after eating or continuous epigastric pain with nausea and/or vomiting and/or abdominal discomfort.

\subsection*{Histologic Gastritis}

The histologic observations were classified according to the International Workshop that assessed and revised the Sydney System for the reporting of gastritis with slight variations [30]. Chronic gastritis was determined by the presence of mononuclear cells (lymphocytes, plasma cells, and macrophages). Histologic diagnoses were classified as: normal, non-atrophic chronic gastritis, atrophic chronic gastritis, intestinal metaplasia, or dysplasia. If PMN cells were present, the gastritis was defined as chronic active gastritis. PMN infiltration was graded as mild, moderate, and severe according to the Sydney System.

\subsection*{\textit{H. pylori} Colonization}

Colonization by \textit{H. pylori} was determined by the presence of characteristic short bacilli as evidenced by Giemsa staining either in the crypts of the mucosa or attached to the epithelium. Three categories of \textit{H. pylori} infection were determined [30]: 1. mild, if a low number of bacteria was present; 2. moderate, if higher number of dispersed bacteria was present; and 3. severe, if small colonies or aggregated bacteria were present.

\subsection*{Endoscopic Procedures and Laboratory Evaluations}

During the gastroscopy nine biopsies were obtained: three from the incisura-antrum (1, 2 and 3), three from the greater curve of the antrum (4, 5, and 6), and three from the corpus (7, 8, and 9). Biopsies 1, 2, 3, 4, 7, and 9 were used to identify \textit{H. pylori} and for histopathology. Biopsies 5, 6, and 8 were used to measure tissue zinc concentrations.

\subsection*{Histopathology}

A total of 12 slides per subject were studied. Six slides were stained with Giemsa to identify \textit{H. pylori}. The other six slides were stained with hematoxylin-eosin for histopathology. For quality control, an experienced observer who was blinded to the first reading performed a second reading of the slides. In the case of disagreement between both observations, the diagnostic criteria of the second observer (MD) prevailed.
Determination of Zinc Concentration in Gastric Tissue

Zinc mucosal concentrations were determined in the United States Department of Agriculture, Grand Forks Human Nutrition Research Center in North Dakota. The biopsies were preserved at −20 °C until analysis. Three mucosal samples for each individual were pooled and solubilized in sodium hydroxide/sodium dodecyl sulfate solution for 24 hours at room temperature. Five hundred microlitres of solubilized cells was added to 2 mL of nitric acid. The mixture was processed in the AIM500 block digester (A.i. Scientific, Saxonburg, PA, USA) as follows: 100 °C for 35 minutes, 120 °C for 120 minutes, 130 °C for 90 minutes, and 145 °C for 140 minutes. This cycle was repeated once. When samples were dry and cool, 1 mL of hydrogen peroxide was added. The samples were again processed in the AIM block digester under the same conditions as above. Before analysis, 2 mL of 5% nitric acid was added and incubated at room temperature for 1 hour. Every sample was analyzed in the PerkinElmer Optima 3300 DV Inductively Coupled Argon Plasma Atomic Emission Spectrometer (Perkin Elmer, Wellesley, MA, USA). Biopsies were coded, and the researcher performing zinc determinations was blinded to the histopathologic diagnosis. The zinc mucosal concentration was expressed in ng/mg protein. Protein concentration was determined on the solubilized samples by the bicinchoninic acid method of Smith et al. [31].

Statistical Analysis

Data entry and management was carried out using Epi-Info software version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). Statistical analyses were carried out using the software SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

Descriptive statistics for histologic evidence of *H. pylori* infection, histopathologic diagnoses, presence of PMN in biopsies, and zinc concentration in gastric mucosa, were calculated. The mean zinc concentration in gastric mucosa was compared between *H. pylori*-positive and -negative biopsies, between *H. pylori* infected patients with non-atrophic gastropathy and uninfected patients with nonatrophic gastritis, and between the degrees of PMN infiltration in gastric mucosa. In addition, the relationship between severity of *H. pylori* infection and gastric infiltration by PMN was evaluated. Differences of means and percentages were evaluated by Student’s *t*-test and chi-squared test, respectively. A significance level of ≤ 0.05 was accepted.

Data of zinc concentration in gastric mucosa were not normally distributed. Non-parametric analysis produced the same results as those of parametric.

Table 1 Histopathologic diagnosis in Ecuadorian *H. pylori*-infected patients with clinical gastritis

<table>
<thead>
<tr>
<th>Histopathologic diagnosis</th>
<th>n = 328 %</th>
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</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>0</td>
</tr>
<tr>
<td>Non-atrophic chronic gastritis</td>
<td>48.5</td>
</tr>
<tr>
<td>Atrophic chronic gastritis</td>
<td>22.9</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>24.7</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Results

Three hundred and fifty-two subjects were enrolled. Although biopsies were obtained from 352 patients for histopathology, *H. pylori* colonization and for determination of mucosal zinc concentration, only 338 determinations of zinc concentration are included in the analysis because the initial attempt to locally process 14 samples failed. Of all patients, 70% were female (245/352) and 93.2% of patients (328/352) had histologic evidence of *H. pylori* infection.

Histologic Diagnosis

Twenty-four out of 352 patients were uninfected by *H. pylori*. From those infected patients, nearly half (48.5%) had non-atrophic chronic gastritis. Atrophic chronic gastritis and metaplasia were seen in nearly one fourth of the patients. In contrast, dysplasia was rarely encountered (Table 1).

Zinc Concentration in Gastric Biopsies

*H. pylori*-infected patients with non-atrophic chronic gastritis (n = 154) had lower zinc concentrations in gastric mucosa than uninfected patients with similar histologic findings (n = 11) (251.3 ± 225.3 vs. 426.2 ± 279.9 ng/mg of protein; *p* = .016). When all subjects were analyzed (n = 338), those patients with presence of *H. pylori* in their gastric mucosa had relatively lower concentrations of zinc in gastric mucosa in comparison to patients without *H. pylori* infection. This difference was not statistically significant (256.4 ± 214.7 vs. 321.2 ± 269.8 ng/mg of protein; *p* = .179).

Presence of Polymorphonuclear Cells in *H. pylori*-Infected Patients

The correlation between severity of *H. pylori* infection and gastric infiltration by PMN cells was evaluated in all patients. We found that the more severe the *H. pylori* infection, the higher the percentage of subjects with PMN infiltration (*p* = .0001) (Table 2).
Table 2 Comparison between the degree of H. pylori infection and presence of polymorphonuclear (PMN) cells in gastric biopsies

<table>
<thead>
<tr>
<th>Degree of H. pylori infection</th>
<th>Presence of PMN cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>25% (26/96)</td>
</tr>
<tr>
<td>Moderate</td>
<td>59% (82/136)</td>
</tr>
<tr>
<td>Severe</td>
<td>74% (72/96)</td>
</tr>
</tbody>
</table>

χ² = 46.68, p = 0.0001.

Presence of Polymorphonuclear Cells and Gastric Mucosa Zinc Concentration in Patients with H. pylori Infection

Lower zinc concentrations were seen in patients with PMN in their gastric mucosa (n = 169) than in patients without PMN infiltration (n = 147) (232.45 ± 192.36 vs. 283.84 ± 235.48 ng/mg of protein; p = .034). Furthermore, patients with moderate PMN infiltration (n = 161) had lower zinc concentrations when compared to patients with low PMN infiltration (n = 161) (35.2 ± 20.7 vs. 242.9 ± 191.82 ng/mg of protein; p = .021) (Fig. 1).

Discussion

We found that relatively low gastric mucosal zinc concentrations in all H. pylori-infected patients were correlated with increased infiltration by PMN cells and, consequently, increased inflammation. Furthermore, H. pylori infection was correlated with a significant reduction in the concentration of zinc in gastric mucosa of patients infected with this bacterium and having non-atrophic chronic gastritis.

H. pylori-associated inflammation is recognized as an important feature of gastric carcinogenesis [32]. Furthermore, the virulence of infecting H. pylori and the stomach environment also play a role in determining the outcome of this infection. One of the mechanisms by which H. pylori induces inflammation is by generation of ROS by infiltrating PMN cells [18]. Zinc deficiency induces oxidative stress in mammalian cells with consequent oxidative DNA damage [23,25,26]. These mechanisms suggest an explanation for our findings. H. pylori infection together with lower zinc concentration in gastric mucosa would induce increased oxidative stress, which would be associated with increased inflammation. This would explain our finding of statistically significant lower concentrations of zinc in gastric mucosa in patients with non-atrophic chronic gastritis and H. pylori infection, compared to those without infection.

In response to H. pylori infection, gastric epithelial cells produce the chemokine IL-8 [33], which induces chemotaxis and transepithelial migration of PMN cells. Neutrophils recruited to the site of inflammation generate ROS. Our findings demonstrate a direct correlation between the severity of H. pylori infection and the degree of infiltration by PMN. Furthermore, we found that the degree of infiltration by PMN cells is correlated inversely with the zinc concentration in gastric mucosa. This would suggest that patients with low gastric mucosa zinc concentrations have increased inflammation caused by augmented infiltration of PMN cells.

Our hypothesis was that the Ecuadorian population, with high prevalence of zinc deficiency as characterized by plasma zinc concentrations [29,34], may have a higher risk of increased inflammatory damage by H. pylori infection. Our study is the first to report zinc concentrations in gastric mucosa. As zinc is essential for the function of numerous intracellular enzymes, zinc concentration in gastric mucosa might be a good indicator of zinc status. However, a potential issue is the absence of plasma zinc determination in our study. Although it is still controversial whether or not plasma zinc concentration is the best marker to evaluate zinc status, plasma zinc concentration is nevertheless widely used as a measure of zinc status. Determination of plasma zinc concentrations in our study would have allowed us to correlate with that of gastric mucosa to have a better idea about zinc status.

An additional limitation to our study is the lack of bacterial culture or urease test to confirm infection by H. pylori. However, the Giemsa stain has a sensitivity of 91%, specificity of 100%, and accuracy of 95% [35]. Another limitation was the small number of non-infected subjects.
This is a difficult issue to solve due to the overwhelming amount of H. pylori-infected subjects among those with dyspepsia in Ecuador [5].

The observation that H. pylori infection in the presence of low zinc concentrations in gastric mucosa results in increased PMN cell infiltration and augmented inflammation suggests a possible explanation for the increased incidence of gastric cancer in developing countries, such as Ecuador, where zinc deficiency and H. pylori infection are important public health problems. It has been shown that zinc supplementation inhibits H. pylori-induced gastric mucosal oxidative inflammation in gerbils [36,37]. Furthermore, in a small randomized clinical trial, a zinc compound in combination with triple therapy (lamsoaprazole, amoxicillin, clarithromycin) significantly improved the cure rate for H. pylori [38]. However, this study did not evaluate histopathologic changes related to H. pylori infection or eradication. Taken together, we propose that zinc deficiency is associated with increased PMN infiltration in gastric mucosa and consequent inflammation. As inflammation is the main mechanism of H. pylori-associated gastric carcinogenesis, we suggest that zinc supplementation be considered for future studies as an adjunctive approach to the treatment and eradication of H. pylori.

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