DNA Damage in Patients Infected by *Helicobacter pylori*

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Abstract

*Helicobacter pylori* (*H. pylori*) is considered to predispose carriers to gastric cancer but its role on gastric carcinogenesis is still unknown. The aim of this study was to investigate DNA damage by the comet assay in gastric epithelial cells from antrum and corpus in *H. pylori*-infected patients with gastritis of different degrees. *H. pylori* status, gastric histology, and DNA damage were studied in 62 *H. pylori*-infected and 18 non-infected patients, all of them non-smokers, non-alcoholics, and non-drug users. DNA damage was significantly higher in *H. pylori*-infected patients presenting gastritis than in non-infected patients with normal mucosa. A direct correlation between the levels of DNA damage and the intensity of gastritis was observed in *H. pylori*-infected patients. Association between DNA damage and age was also found. The levels of DNA damage were significantly higher in patients older than 50 years than in younger patients with the same degree of gastritis. Our results indicate that *H. pylori* infection is associated with DNA damage in gastric epithelial cells, which could be a biomarker of risk for gastric cancer in humans.

Introduction

There is growing evidence supporting an association between *Helicobacter pylori* and gastric cancer (1, 2). *H. pylori* infection induces chronic inflammation that can progress to gastric atrophy, intestinal metaplasia, and gastric adenocarcinoma (3). The mechanisms involved in this model of inflammatory-mediated carcinogenesis are beginning to be understood (4). Bacterial products and reactive species of oxygen (ROS) and of nitrogen (RNS) released at the site of inflammation can damage DNA, which may represent the early step in gastric carcinogenesis.

The ability to study DNA damage in the human stomach infected by *H. pylori* should allow more accurate identification of bacterial virulent risk factors and permit preventive strategies. DNA damage can be assessed by the single cell gel electrophoresis (SCGE) or comet assay. It is a simple and sensitive test for the investigation of DNA damage, such as double- and single-strand breaks, incomplete repair sites, alkali-labile sites, and cross-links at individual cell level (5, 6). The comet assay has been developed into a basic tool for human biomonitoring (7).

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Note: Due to low number of non-infected patients and the multiple treatments (n = 18) required for the multivariate analysis, this kind of statistical analysis was not applied on the results.

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Subject and Methods

Subjects. This study was approved by the Ethical Committee of the Medical School, Paulist State University (UNESP), Botucatu, São Paulo, Brazil, and by the National Committee of Ethics in Research, Brasília, D.C., Brazil. Informed consent to participate had been obtained from all the patients or their parents.

We studied, prospectively, 80 volunteer patients (29 males and 51 females); with mean age of 41 ± 19.7 years, and an age range of 5–81 years. All of them were non-smokers, non-alcoholics, and non-drug users. None had received any medication for at least 30 days before the study. All patients had been scheduled for upper digestive endoscopy on account of symptoms suggestive of gastrointestinal pathology.

Hartmann et al. (8) demonstrated the usefulness of the SCGE as a screening for the prediction of the outcome chromosome aberration test. Gedik et al. (9) performed a study to clarify the relationship between different markers of oxidative DNA damage, after induction of 8-oxo-7,8-dihydroguanine in DNA of HeLa cells and concluded that comet assay with formamidopyrimidine DNA glycosylase and high-performance liquid chromatography are equally efficient.

Giving the high risk for gastric cancer in *H. pylori* infection in humans, we applied the comet assay to investigate DNA damage in the gastric epithelial cells of non-infected patients and *H. pylori*-infected patients with gastritis of different degrees. Attempts were made to correlate DNA damage with gender and age.
Biopsy and Blood Collections. Biopsies were obtained during endoscopy from the lesser curvature of the antrum within 2 cm of the pyloric ring and from the corpus between 50 and 35 cm from the incisors along the greater curvature. One biopsy from each site was used for a rapid urease test (10). Two biopsies from the antrum and two from the corpus were sent for histopathological study. One sample from each site was used for evaluation of DNA damage by the comet assay and another sample from each site was used for detection of ureA by PCR. H. pylori infection was confirmed when positive results were obtained for at least two of the following tests: rapid urease test, histological analysis, carbolfuchsin-stained smear, serology, and gastric biopsy PCR for ureA. Blood samples (5 ml) were obtained from all the patients: 1 ml was used for the comet assay and 4 ml for serology.

Histopathology. Samples from the gastric mucosa were fixed in 10% formalin for 24 h, dehydrated in alcohol and xylene, and embedded in paraffin. Sequential 3–5 μm sections were obtained and stained with H&E for routine histology. The slides were blindly examined under a light microscope by two pathologists. Gastritis was classified according to Sydney’s system as mild gastritis, moderate, or severe gastritis (11). The presence of H. pylori was determined by carbolfuchsin-stained sections.

Serology. The presence of anti-H. pylori antibodies in the serum of patients was determined by H. pylori-One Step test (INLAB-Diagnostica, Sao Paulo, Brazil).

DNA Extraction. DNA extraction was performed as described by Fox et al. (12). DNA was quantified by measuring absorbance at 260 nm (Genesys 5—Spectronic Instruments, New York, NY).

ureA Detection. Genomic DNA was amplified using a set of synthetic oligonucleotide primers (Life Technologies, Sao Paulo, Brazil): HPU1 (5′-GCCAATGGTAAAT-CTCCTTAATTGTTTTAC-3′) and HPU2 (5′-CTCCTTAATTGTTTTAC-3′). The PCR amplification was performed according to Clayton et al. (13). DNA extracted from biopsies with negative results in the other three analyses (urease test, serology, and histopathology) was used as negative control. As a positive internal control of the PCR reaction, we have used DNA extracted from biopsies with positive results in the other three analyses. The samples were tested twice.

Preparation of the Single Cell Suspension. Epithelial cells from gastric mucosa biopsies were isolated as described by Pool-Zobel et al. (14). Briefly, each sample was pooled and incubated with 5.5 mg proteinase K (Life Technologies) and 3 mg collagenase I (Life Technologies) in 3 ml of HBSS (Life Technologies) for 45 min at 37°C to liberate the cells, that were resuspended in 10 ml of HBSS. The resulting suspensions were centrifuged at 800 rpm for 5 min and the supernatant was discarded. Aliquots of 50 μl were stained with 150 μl methyl green and checked for the yield of cells released from the biopsy. Suspensions of 0.6–4 × 10⁶ cells were obtained per biopsy.

Leukocyte Content in the Single Cell Suspension. Leukocyte contamination was assessed in samples of the cell suspensions. Aliquots of 100 μl were dropped into a slide, fixed with acetone, and stained with H&E. The slides were examined blindly for the levels of leukocyte content.

Cell Viability. Cell viability, 78–96% with mean of 89%, was determined using the fluorescein diacetate (Sigma)/ethidium bromide (EtBr) (Sigma Chemical Co, St. Louis, MO) assay according to Strauss (15). Briefly, a freshly staining solution was prepared: 30 μl fluorescein diacetate in acetone (5 mg/ml), 200 μl EtBr in phosphate buffer saline (200 μg/ml), and 4.8 ml PBS. Then, 25 μl of the single cell suspension were mixed with 25 μl of the staining solution, spread onto a slide, and covered with a coverslip. Viable cells appeared green-fluorescent, whereas red-stained nuclei indicated dead cells. At least 200 cells were counted per sample.

Determination of DNA Damage. The alkaline comet assay in the single cell suspensions from the gastric mucosa was performed according to Singh et al. (16), with some modifications (17). Briefly, 15 μl of the single cell suspension (≥2 × 10⁶ cells) were embedded in 0.5% low-melting-point agarose (Sigma) and spread on agarose-precoated microscope slides. Slides were immersed overnight at 4°C in freshly prepared cold lysing solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-lauryl sarcosine (pH 10), with 1% Triton X-100, and 10% DMSO added fresh; all these reagents were supplied by Sigma). Subsequently, the cells were exposed to alkali buffer (1 mM EDTA and 300 mM NaOH, pH ≥ 13.4), at 4°C, for 40 min to allow DNA unwinding and expression of alkali-labile sites. In the same solution, electrophoresis was conducted at 4°C, for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 40 μl EtBr (20 μg/ml), and analyzed in a fluorescence microscope (Axioplan II—Zeiss, Oberkochen, Germany), under green light at 400×, using an image analysis system ( Comet Assay II—Perceptive Instruments, Suffolk, United Kingdom). Two hundred randomly selected cells (100 from each of two replicate slides) were evaluated from each sample and the mean of the tail moment was determined. Tail moment according to Comet Assay II—Perceptive Instruments is defined as “the product of DNA in the tail and the mean distance of migration in the tail. It is calculated multiplying tail intensity/sum comet intensity by tail center of gravity – peak position.” Under this method, the extent of DNA migration is related to the level of DNA damage in each cell, creating the so-called image of comets (Fig. 1).
A whole-blood sample from each patient was assayed under the same test conditions to obtain tail moments of peripheral blood leukocytes as controls for the cell suspensions of the gastric mucosa.

**Statistical Analysis.** The statistical analysis consisted of the application of Kruskal-Wallis, seeking the comparison between the individual mean tail moment in groups with normal mucosa and gastritis of different degrees. Kruskal-Wallis test was used for comparison of individual mean tail moment of gastritis by gender and age. The significance was set at 5%. Logistic regression analysis was used to examine the relationship between DNA damage and *H. pylori* infection, histopathology, gender, and age. The results are presented as odds ratios (OR) with 95% confidence intervals (CI). Significance was set at *P* < 0.05.

### Table 1. Relationship between DNA damage by the comet assay (mean ± SD of tail moment) and degree of gastritis in *H. pylori*-infected patients

<table>
<thead>
<tr>
<th>Histological status</th>
<th>N</th>
<th>Tail moment (mean ± SD)</th>
<th>Blood leukocytes</th>
<th>Gastric mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antrum</td>
<td>Corpus</td>
</tr>
<tr>
<td>Normal mucosa¹</td>
<td>18</td>
<td>0.38 ± 0.48</td>
<td>1.35 ± 1.05</td>
<td>1.50 ± 1.20</td>
</tr>
<tr>
<td>Mild gastritis²</td>
<td>31</td>
<td>0.56 ± 0.49</td>
<td>3.35 ± 2.57</td>
<td>3.23 ± 2.46</td>
</tr>
<tr>
<td>Moderate gastritis²</td>
<td>20</td>
<td>0.61 ± 0.77</td>
<td>5.28 ± 3.16</td>
<td>3.70 ± 3.77</td>
</tr>
<tr>
<td>Severe gastritis²</td>
<td>11</td>
<td>0.98 ± 0.73</td>
<td>8.32 ± 6.93</td>
<td>6.63 ± 4.93</td>
</tr>
</tbody>
</table>

Note: Significant differences: (¹) compared to normal mucosa (*P* = 0.0059); (²) compared to normal mucosa (*P* = 0.0029) and (³) compared to mild gastritis (*P* = 0.0489); (⁴) compared to normal (*P* = 0.0002); (⁵) compared to mild gastritis (*P* = 0.0149); (⁶) compared to normal mucosa (*P* = 0.0078); (⁷) compared to normal mucosa (*P* = 0.0256); (⁸) compared to normal (*P* = 0.0002); and (⁹) compared to mild gastritis (*P* = 0.0438).

¹Not infected by *H. pylori*.
²Infected by *H. pylori*.

**Fig. 2.** Association between DNA damage by the comet assay and gender according to degree of gastritis. Columns, mean of tail moment; bars, SD. For male in the antrum: normal mucosa versus moderate or severe gastritis (*P* = 0.0277 and *P* = 0.0204), respectively, and mild gastritis versus moderate or severe gastritis (*P* = 0.0355 and *P* = 0.0159). For male in the corpus: normal mucosa versus moderate or severe gastritis (*P* = 0.0347 and *P* = 0.0287, respectively) and mild gastritis versus moderate or severe gastritis (*P* = 0.0456 and *P* = 0.0389). For female in the antrum: normal mucosa versus mild or moderate or severe gastritis (*P* = 0.0204, *P* = 0.0418, and *P* = 0.0470), respectively.
Results

The prevalence of H. pylori infection was 62 of 80 (77.6%). The histological analysis showed the following results: 31 of 80 (38.8%) with mild gastritis, 20 of 80 (25%) with moderate gastritis, and 11 of 80 (13.8%) with severe gastritis. Intestinal metaplasia was found in seven patients with gastritis and gastric atrophy in four patients. All 62 of these patients were infected by H. pylori, and 18 of 80 (22.4%) with normal mucosa were not infected by H. pylori.

The analysis of leukocyte content of cell suspensions prepared from six samples of gastric biopsies with gastritis demonstrated less than 12% of leukocyte content with mean of 8.7%.

Table 1 presents the relationship between DNA damage (tail moment) and the degree of gastritis in H. pylori-infected patients. The levels of DNA damage in gastric epithelial cells were significantly higher in H. pylori-infected patients with gastritis irrespective of the intensity of the inflammation, in antrum and corpus, than in non-H. pylori-infected patients with normal mucosa (P = 0.0001 and P = 0.0003, respectively). In the antrum, significant differences were found between mild compared to moderate and severe gastritis. In the corpus, there was a significant difference between mild and severe gastritis. Data from blood leukocytes showed that the mean tail moments of blood leukocytes were significantly lower than those from epithelial cells of the gastric mucosa (Table 1). Blood leukocytes were used as controls for the inflammatory cells, because they might be a confounding factor in the comet assay. We have found that the mean tail moments of blood leukocytes were significantly lower than those from epithelial cells of the gastric mucosa.

Figure 2 summarizes the relationship between DNA damage (tail moment) and gender according to the degree of gastritis. In the gastric antrum, significant differences were found between males and females with moderate and severe gastritis, and in the corpus, only with moderate gastritis. Significant differences were found in the following cases: (a) in males: normal mucosa compared to moderate and severe gastritis; and mild gastritis compared to moderate and severe gastritis; (b) in females (only in the antrum): normal mucosa compared to mild, moderate, and severe gastritis.

Figure 3 shows the association between DNA damage (tail moment) and age according to the degree of gastritis. In the antrum, patients with moderate or severe gastritis and older than 50 years showed higher levels of DNA damage when compared with those younger. In the gastric corpus, patients with moderate gastritis older...
than 50 years showed a higher level of DNA damage than in those younger. A significant difference was also found in normal mucosa of patients 5–17 years old when compared to those older than 50 years.

Figure 4 shows the association between DNA damage (tail moment) and gender according to age. Male patients 5–17 years old presented significantly lower levels of DNA damage in both antrum and corpus when compared with the older groups. For female patients, the levels of DNA damage were significantly higher in the groups older than 50 years when compared to the group 5–17 years old.

The association between *H. pylori* infection, histopathology, gender, and the risk for high DNA damage levels was assessed using multiple logistic regression models. As performed by Smith et al. (18), in the antrum, the comet tail moment was dichotomized to high or low by the median (2.55) of all patients studied. The same logistic regression analyses was also performed for the corpus (high, tail moment > 2.20; and low, tail moment < 2.20).

The multiple logistic regression models evidenced positive associations between high DNA damage levels, *H. pylori* infection, histopathology, and age, in antrum and corpus. We did not find significant association between high DNA damage levels and gender.

According to the analysis, in the antrum, *H. pylori*-infected patients presenting gastritis have approximately 8.40 (95% CI = 1.98–35.55) times more chance to be classified as higher level of DNA damage than those non-infected with normal mucosa. For each increase of degree of gastric inflammation, *H. pylori*-infected patients presented 1.76 (95% CI = 1.06–2.95) times more chance to be classified as higher level of DNA damage than those non-infected with normal mucosa. Age also presented association with high DNA damage levels, for each increase of 1 year, the risk of high DNA damage levels increased 1.03 (95% CI = 1.004–1.06) times.

Regarding high DNA damage levels in corpus, the results were similar to the antrum. *H. pylori*-infected patients presenting gastritis have approximately 6.45 (95% CI = 2.52–16.53) times more chance to be classified as higher level of DNA damage than those non-infected with normal mucosa and for each increase of degree of gastritis, the *H. pylori*-infected patients presented 1.53 (95% CI = 1.08–2.15) times more chance to be classified as high DNA damage levels than those non-infected with normal mucosa. As in the antrum, for each increase of 1 year, the risk of high DNA damage levels increased 1.02 (95% CI = 1.004–1.04) times.

**Discussion**

In the present study, 77.6% of the patients were infected by *H. pylori*. This frequency is similar to those reported in other Brazilian studies (19, 20).
Regarding DNA damage in the gastric epithelial cells, our results showed significantly higher values in patients infected by *H. pylori* and presenting gastritis than in non-infected patients with normal mucosa. This is in contrast with the findings reported by Everett et al. (21, 22), probably due to different methods used to assess DNA damage. These authors examined the percentage of cells with comet morphology and we examined the tail moment of the comets by image analysis software. The tail moment is defined as the product of tail DNA/total DNA by the tail center of gravity, which provides better information about the extent of DNA damaged cells (5).

Two other relevant methodological issues should be addressed. First, because DNA damage is associated with cell death, it is critical to distinguish between them. Dead cells have typical images on the comet assay referred to as DNA “clouds” which are made of very low molecular weight DNA fragments resulting from apoptosis (Fig. 5). They differ from true comets because they have no detectable head with nearly all DNA in the tail. In the present study, DNA “clouds” were not counted in the SCG preparations. Second, the comets were not from contaminating inflammatory cells. Distinguishing cell populations is crucial, because the DNA damage status may differ between different cell types. In a recent study on DNA damage in urothelial cells from smokers, Gontijo et al. (23) have found significant differences in DNA migration (tail of comets) between transitional and inflammatory cells. In the present study, we used a whole-blood sample from each patient as a control for the inflammatory cells, because they are a confounding factor in the comet assay. We have found that the mean tail moments of blood leukocytes were significantly lower than those from epithelial cells of the gastric mucosa.

We have found a positive correlation between DNA damage and degree of gastritis, meaning that DNA damage increases with increased intensity of gastritis. These results could be explained as a consequence of *H. pylori* infection, the primary histopathological hallmark of which is the infiltration of the gastric mucosa by inflammatory cells (24). These cells synthesize large amounts of free radicals and aldehydes, which can induce DNA damage in the epithelial cells of the gastric mucosa. Although for the most part the targets for DNA damage are postreplicative cells, it is possible that some stem cells could be affected by oxyradical overload leading to mutations (4, 25). Bacterial products, such as urease and other substances secreted by *H. pylori*, could also induce DNA damage (26–30), leading to somatic mutations and posttranslational modifications of key cancer-related proteins (4).

Although the multiple logistic regression models did not show significant associations between high DNA damage levels and gender, the high DNA damage levels in patients infected by *H. pylori*, observed in our study, may help account for the 2:1 prevalence of intestinal gastric cancer in men (31). It is known that men express more gastrin than women (32). This hormone, which enhances epithelial cell proliferation in the gastrointestinal tract (33), would facilitate the fixation of DNA damage into mutation, thus increasing the carcinogenic potential in men. Moreover, the similar levels of DNA damage in women, irrespective of the degree of gastritis, point to a possible protective hormonal factor for women (31).

We have observed great interindividual variability in DNA damage in patients with the same histological diagnosis. The clinical and pathological heterogeneity of the *H. pylori* infection suggests that the relationship between specific bacteria strain and host susceptibility plus environmental co-factors, such as diet, could be responsible for the differences on the levels of DNA damage. Also, there can be an adaptive response by the host against oxidative damage (34) or differences in the repair-system efficiency.

Relationship between DNA damage and age was also found in this study. The higher levels of DNA damage observed in patients older than 50 years could be related to the cumulative effect of genotoxins (35, 36) with age, because younger patients with gastritis of the same degree presented lower levels of DNA damage. *H. pylori*-infected patients, mainly males, with chronic gastritis for decades could present persistent induction and accumulation of DNA damage like patients with genomic instability syndromes, which present accumulation of DNA damage due to mutations in the repair system genes (37). This could lead to increased risk for developing gastric cancer.

Overall, the results of the present study indicate that *H. pylori* infection is associated with DNA damage in gastric epithelial cells, which could be a biomarker of risk for gastric cancer in humans.

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