ROLE OF HELICOBACTER PYLORI IN REFRACTORY IRON DEFICIENCY ANAEMIA

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ABSTRACT

Background: The role of helicobacter pylori (H. pylori) infection in the development of iron deficiency anaemia has been the focus of attention during the last decade. Confirmation of the relationship between H. pylori infections and iron deficiency anaemia has not confirmed the pathophysiologic mechanisms involved in this phenomenon.

Aim of the present work: was to study the levels of fasting gastric acidity (free and total) as well as the level of tumor necrosis factor alpha in refractory iron deficiency anaemic male patients seropositive for H. pylori infection versus those with seronegativity for H. pylori infection. Also, we tried to find the underlying pathophysiologic mechanism for iron deficiency anaemia observed in these patients.

Methods: This study was conducted on 30 adult male patients having iron deficiency anaemia and gastroduodenitis. They were subdivided into 2 groups of matched age and haemoglobin value. Group I was seropositive for H. pylori infection and refractory to iron therapy. These patients did not receive prior treatment for eradication of H. pylori infection while group II was seronegative for H. pylori infection and was considered a control group. Patients with active bleeding or previous medical problem were excluded from the study. All patients and controls in the present study were subjected to the following at presentation: careful history taking and thorough clinical examination, complete blood picture, reticulocytes %, assessment of serum iron, total iron binding capacity, serum ferritin, IgG antihelicobacter antibody and tumor necrosis factor-alpha (TNF-α), stool for occult blood and measurement of gastric acidity (total and free) by chemical method. Upper endoscopy was done and multiple biopsies were taken and tested for expression of cytotoxin associated gene A (cag A) by polymerase chain reaction (PCR).

Results: results revealed statistically significant higher values of free and total gastric acidity as well as tumor necrosis factor-alpha levels in H. pylori seropositive compared with H. pylori seronegative group. Among H. pylori seropositive group, higher value of TNF-α level was observed in H. pylori cagA positive (7 patients) than cagA negative patients (8 patients). Also, haemoglobin values were inversely correlated with tumor necrosis factor-alpha levels.

Conclusion: From this study, we can conclude that elevated serum tumor necrosis factor (TNF-α) in H. pylori seropositive group may be one of the underlying pathophysiologic mechanism for iron deficiency anaemia observed in these patients.

Key words: H. pylori, TNF-α, cag A, iron deficiency anaemia.

INTRODUCTION

The role of helicobacter pylori (H. pylori) infection in the development of iron deficiency anaemia has been the focus of attention during the last decade. Epidemiologic studies have indicated that H. pylori seropositivity is associated with low serum ferritin and haemoglobin levels compared with seronegative controls in adults and children.1

These findings have been supported by a few case reports in which eradication of H. pylori resulted in improvement of iron deficiency anaemia in patients resistant to iron replacement therapy. It has also been reported that eradication of H. pylori may result in improvement of anaemia even without iron supplementation.1

Confirmation of the relationship between H. pylori infections and iron deficiency anaemia has not confirmed the pathophysiological mechanisms involved in the phenomenon.1 Two main hypotheses have been proposed to explain the association between H. pylori infection and iron deficiency anaemia. The first hypothesis is sequestration of iron by antral H. pylori infection. A previous study showed that iron was diverted away from the bone marrow in patients with H. pylori infection and iron deficiency anaemia.1 The second hypothesis is that H. pylori-related changes in the gastric physiology result in iron deficiency anaemia. H. pylori gastritis decreases gastric acidity and the ascorbic acid content of gastric juice, both of which may decrease nonheme iron absorption. It was demonstrated that both pangastritis and pangastritis-induced hypochlorohydria were more prevalent in adult patients with H. pylori who had anemia than in those who did not have anemia.1

Aim of the Work

The aim of the present study was to compare the levels of fasting gastric acidity (total and free) as well as the level of tumor necrosis factor alpha in refractory iron deficiency anaemic male patients seropositive for H. pylori infection versus those with seronegativity for H. pylori infection. Also, we tried to find the underlying pathophysiological mechanism for iron deficiency anaemia observed in these patients.
METHODS

This study included 30 adult male patients having iron deficiency anaemia and gastroduodenitis. All patients presented with dyspeptic symptoms or epigastric pain for at least 2 months. They were subdivided into 2 groups of matched age and haemoglobin value. The mean value of age in group I was 28 ± 7.64 years while in group II was 26.4 ± 7.73 years (t= 0.57, p=0.573). Group I was seropositive for H. pylori infection. These patients did not receive prior treatment for eradication of H. pylori infection while group II was seronegative for H. pylori infection and was considered a control group. Patients with active bleeding or having other medical problems such as cardiac, hepatic or renal diseases were excluded from the study.

All patients in group II received treatment with proton pump inhibitors for 4-6 weeks followed by oral iron therapy for at least 3 weeks. Minimum rate of response which should be rise of 20 g/L in haemoglobin values every 3 weeks was not attained in all patients. So, triple therapy for H. pylori eradication using a proton pump inhibitor (omeprazole 20 mg) with amoxicillin (1 g), and clarithromycin (500 mg) was then initiated twice daily for 2 weeks followed by oral iron therapy with good therapeutic response.

All Patients and Controls in the Present Study were subjected to the following at presentation:
- Careful history taking and thorough clinical examination.
- Reticulocytes %.
- Determination of serum iron colorimetrically (BIO-MERIEUX, France), total iron binding capacity (Biodiagnostics, Egypt).
- Determination of serum ferritin by ELISA (DRG International, Inc. USA).
- Serum ferritin concentration of < 50 µg/L may be associated with lack of storage iron in patients with anaemia of chronic disease.
- Test for presence of occult blood in stool (Acon laboratories, Inc., USA).
- Measurement of gastric acidity (total and free) by chemical method.

Technique: Few drops of Topfer's indicator were added to 5 mL of gastric juice. Titrate against 0.1 N-NaOH until the color changed from pink to salmon pink (orange yellow). Stop titration, take reading of burette (X1) which corresponded to all free HCl present in 5 mL gastric juice and was neutralized by 0.1 N-NaOH. Few drops of phenolphthalein indicator were added to the content of beaker and titration was continued until color changed to just pink. The reading of burette from the start (X2) was taken. It corresponded to all total acidity that was present in 5 mL gastric juice and was neutralized by 0.1 N-NaOH. Repeat the experiment and determine the mean values of X1 and X2.

Calculation:
- Free acidity: (all HCl in gastric juice) = mean X1 x 100/5 mL 0.1 N-NaOH %.
- Total acidity: (free HCl, combined HCl and other organic acids) = mean X2 x 100/5 0.1 N-NaOH %.
- Quantitative determination of plasma tumor necrosis factor-alpha by ELISA. It is a solid phase sandwich enzyme linked immunosorbent assay. The kit was supplied by Diaclone Research Company, Besacon, France. Its value was expressed as pg/mL.\(^(8)\)
- Hepatic and renal function tests.\(^(9)\)
- Quantitative determination of serum IgG antihelicobacter antibody measured by enzyme immunoassay technique (Biocheck, Inc, Foster City, USA).\(^{(10)}\)
- Upper gastrointestinal endoscopy was performed after overnight fast for all patients using "Olympus video endoscopy EVIS 100" and passed down to second part of the duodenum.\(^{(11)}\)
- During endoscopy, gastric aspirate was collected and biopsies were taken from the antrum from all patients. Biopsies were tested by hp fast for color change which indicates presence of urease (Medical Instruments Corp, France).
- Detection of Cytotoxic Associated Gene A (Cag A) by Polymerase Chain Reaction (PCR):

Two biopsy specimens were dipped into a small screw capped tube containing 1 mL of brain heart infusion broth supplemented with 10% human serum and 3 sterile glass beads.\(^{(12)}\) The tube was then vortexed at high speed for 2 minutes. 200 mL quantities of the tissue suspension were inoculated onto two plates: selective Dent's agar and Columbia blood agar. The inoculated plates were incubated at 37°C under microaerophilic conditions using campy gas pack (oxoid) for 4-6 days. Colonies that exhibited characteristic morphology were identified as H. pylori if they rapidly hydrolyze urea, produced catalase and oxidase and were gram-negative spiral rods.\(^{(12,13)}\)
- Positive cultures were subjected to PCR for cag A detection.

A-DNA extraction\(^{(14)}\): H. pylori colonies were digested by proteinase K (25 ng/mL) incubated at 70°C for 2 hours. DNA was extracted with 1 volume of phenol-chloroform isoaamyl alcohol 25:24:1 (Sigma) and then precipitated with 2.5 volumes of absolute ethanol at -20°C for 2 hours. The DNA was centrifuged at 4°C for 15 minutes and the pellet was resuspended in 20 mL of distilled water.

B-DNA amplification\(^{(15)}\): Amplification was performed in a final volume of 50 µL of PCR mixture containing 50 mM KCl, 10 mM tris, 200 mM deoxy nucleotide triphosphate, 30 pmol of each primer. 0.1 µg of povine serum albumin, 2.5 U of AmpliTaq (Promega) and 20 ng of template DNA. Amplification was performed on a PCR system.

thermocycler (Tehne progene) with the following cycling profiles: 94°C for 45s, 50°C for 45s and 72°C for 45s for 35 cycles and then extension at 72°C for 10 min. The H. pylori cag A primers DZ3 (5'-AGTAAGGAGAAACATGA) and Roo9 (5'-AATAAGCCTTAGCTTITTT-GCAAATC) were derived from the sequenced cagA gene, giving an amplified product of (135 bp).

**C-Detection of PCR products** (16): The amplified products were purified by electrophoresis on 1.4% agarose gel (sigma) at 80 volts and then visualized after ethidium bromide (0.5 μg/mL) staining.

**Statistical Analysis:**
The data were analyzed using SPSS computer program (SPSS for windows release 9.0; spss, Inc). Data were compared using Student's t test. The correlation between variables was evaluated with Pearson's correlation. Data are shown as mean (x) ± standard deviation (SD)

**RESULTS**
Table I showed the laboratory parameters of the studied groups. Both groups had comparable values of haemoglobin, serum iron, total iron binding capacity and serum ferritin levels. The mean values of serum iron and total iron binding capacity were 43.53 ± 11.45 μg/dL and 478.73 ± 39.48 μg/dL respectively for group I and 42.4 ± 10.75 μg/dL and 456.33 ± 29.39 μg/dL respectively for group II (t=0.29, p=.782) for serum iron and (t=1.76, p=.089) for total iron binding capacity. There was statistically significant higher values of free and total gastric acidity as well as tumor necrosis factor-α levels in H. pylori seropositive compared with H. pylori seronegative group. Among H. pylori seropositive group, higher value of TNF-α levels was observed in H. pylori cag A positive (7 patients, 44.5 ± 4.25) than cagA negative patients (8 patients, 35.5±11).

Table II showed the correlation between the different studied clinical and laboratory parameters in group II (seropositive for H. pylori infection). The studied parameters were age, haemoglobin value, serum ferritin, total and free gastric acidity and tumor necrosis factor-α level. Haemoglobin values were inversely correlated with tumor necrosis factor-α levels (r=-0.644, p=0.01).

### Table I: Laboratory parameters of the studied groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (Seropositive patients)</th>
<th>Group II (Seronegative patients)</th>
<th>t-test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>10.74 ± 9.9</td>
<td>10.29 ± 1.15</td>
<td>1.14 (p=0.26)</td>
</tr>
<tr>
<td>Serum ferritin (ng/mL)</td>
<td>24.8 ± 13.22</td>
<td>30.8 ± 12.44</td>
<td>1.28 (p=0.21)</td>
</tr>
<tr>
<td>Gastric acidity (0.1 N-NaOHa %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>80.13 ± 31.21</td>
<td>29.93 ± 10.75</td>
<td>5.89* (p&lt;0.01)</td>
</tr>
<tr>
<td>Free</td>
<td>33.4 ± 7.41</td>
<td>14.33 ± 4.73</td>
<td>8.396* (p&lt;0.01)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>39.43 ± 6.699</td>
<td>22.2 ± 4.89</td>
<td>8.049* (p&lt;0.01)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation (SD)
P is considered significant if <0.05

### Table II: Correlation between clinical and laboratory parameters in group I (H. pylori seropositive group)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Haemoglobin (g/dL)</th>
<th>Serum ferritin (mg/mL)</th>
<th>Total gastric acidity (0.1 N-NaOHa %)</th>
<th>Free gastric acidity (0.1 N-NaOHa %)</th>
<th>TNF-α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p value</td>
<td>r</td>
<td>p value</td>
<td>r</td>
</tr>
<tr>
<td>Age (years)</td>
<td>.435</td>
<td>.105</td>
<td>.702**</td>
<td>.004</td>
<td>.004</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>-</td>
<td>-</td>
<td>.288</td>
<td>.298</td>
<td>.048</td>
</tr>
<tr>
<td>Serum ferritin (mg/mL)</td>
<td>.288</td>
<td>.298</td>
<td>-</td>
<td>-</td>
<td>-.408</td>
</tr>
<tr>
<td>Total gastric acidity (0.1 N-NaOHa %)</td>
<td>.048</td>
<td>.864</td>
<td>-.408</td>
<td>.131</td>
<td>-</td>
</tr>
<tr>
<td>Free gastric acidity (0.1 N-NaOHa %)</td>
<td>-.034</td>
<td>.903</td>
<td>-.385</td>
<td>.157</td>
<td>.763**</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>-.644**</td>
<td>.010</td>
<td>-.097</td>
<td>.731</td>
<td>.333</td>
</tr>
</tbody>
</table>

Correlation is significant at the 0.05 level.
P<0.05 was considered significant.

DISCUSSION

H. pylori gastritis decreases gastric acidity.\(^{(1)}\) Gastric colonization by H. pylori also induces a transient period of achlorhydria. Factors contributing to altered gastric secretion during H. pylori infection include urease activity, a soluble protein of 46 kDa and certain fatty acid components of H. pylori lipopolysaccharide. H. pylori induced achlorhydria may also be mediated by interleukin-1 which has been shown to inhibit gastric acid secretion in rats.\(^{(17)}\) Gastric acidity increased after cure of H. pylori pangastritis.\(^{(18)}\)

In the present study, the level of total and free gastric acidity was significantly higher in H. pylori seropositive group when compared with H. pylori seronegative group. This may be explained by the presence of duodenitis in the studied patients. H. pylori infection that follows an antral predominant pattern leads to an inflammatory state in which higher levels of tumor necrosis factor-alpha and other cytokines are produced. These stimulate gastric acid production directly by increasing gastrin release from G cells and inhibit somatostatin production by D cells. This leads to a net increase in gastric acid secretion which leads to an increased acid load in the duodenum, overwhelming the mucosal defense.\(^{(19)}\)

After elimination of H. pylori gastritis in duodenal ulcer patients, acid secretion does not decrease to normal levels. Thus increased gastric acid secretion rates in duodenal ulcer patients cannot be attributed to H. pylori gastritis alone. It is likely that cytokines derived from inflammatory cells within the gastric antrum are responsible for hypergastrinaemia, either by augmenting antral G cell function or suppressing antral D cell function.\(^{(20)}\)

Recent reports suggest that H. pylori infection can affect iron homeostasis;\(^{(21,22)}\) although the mechanisms by which H. pylori cause iron deficiency is still unclear.\(^{(21)}\) Our study like other studies had shown that iron deficiency anemia refractory to oral iron therapy improved with H. pylori treatment alone or hastened along with iron supplementation.\(^{(21)}\) Thereby establishing a causal role for H. pylori infection in the development of iron deficiency anaemia.\(^{(21)}\) It is not clear if this beneficial effect results from removing H. pylori-specific infectious effects, eliminating some other infectious pathogen, or reducing the total infectious burden.\(^{(23)}\)

TNF-α is an inflammatory cytokine that has been implicated in the suppression of erythropoiesis.\(^{(24)}\) In vitro studies suggest that TNF-α can inhibit the production of erythropoietin.\(^{(25)}\) In the present study, there was statistically significant elevation in TNF-α in seropositive group when compared with seronegative group. In addition, there was an inverse correlation of statistically significant value between serum TNF-α levels and haemoglobin values and in H. pylori seropositive group.

H. pylori leads to mucosal increases in many proinflammatory and immunoregulatory cytokines, and also increases in members of the chemokine group of peptides. The stomach has a large surface area which is nearly 700 cm\(^2\) and continuous spill-over of locally produced cytokines into the blood stream is a possibility. There are conflicting data on circulatory proinflammatory cytokine levels in patients with H. pylori infection.\(^{(26)}\)

In one study, there was no difference in the mean circulatory levels of TNF-α in both H pylori-positive without systemic diseases and H. pylori negative groups. Some others reported elevated serum TNF-α levels in patients with Cag A (cytotoxin associated gene A) positive H. pylori infection.\(^{(27)}\) There is also evidence of a role of Cag A-positive H. pylori infection in iron-deficiency anaemia.\(^{(28)}\)

Conclusion:
From this study, we can conclude that elevated serum tumor necrosis factor in H. pylori seropositive group may be one of the underlying pathophysiologic mechanism for iron deficiency anaemia observed in these patients.

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