The impact of Helicobacter pylori infection on iron status and gastric mucosa in adult patients with refractory iron deficiency anemia

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Abstract: Background and objectives: Iron deficiency is one of the most common nutritional problems of human race. *Helicobacter pylori* (*Hp*) infection is the main known cause of gastritis, gastroduodenal ulcer and gastric cancer. The study was designed to evaluate the role of Hp infection in refractory iron deficiency anemia (rIDA) in adults, and to determine whether *Hp* eradication is an effective therapeutic strategy to improve iron status and gastric mucosal pathology in these patients. **Methods:** The study included 53 adults with rIDA and active gastric *Hp* infection. Following standard workup for rIDA, patients were offered triple therapy for *Hp* eradication then oral iron therapy for 2 months. Reassessment of iron status was performed twice; at 3 and 6 months after initiation of iron therapy. Endoscopy, histopathological and immunohistochemical assessments were repeated in the second follow up visit. **Results:** In all studied patients, *Hp* eradication was effective and the rIDA patients achieved normal hemoglobin levels (mean; 12.52 gm/dl), transferrin saturation (mean; 30.62%), and serum ferritin (mean; 47.59 ng/ml) three months following iron therapy with significantly higher levels as compared to pre-treatment values ($p<0.001$). At the 6 months' follow up visit, minimal non significant improvement of the iron status was noted with no relapse after termination of oral iron therapy. *Hp* induced gastritis (58.5%), gastric epithelial metaplasia (18.9%) and dysplasia (22.6%) in the studied patients. *Hp* eradication resulted in histological improvement in 83.9% of cases with gastritis, 60% of cases with metaplasia and 83.3% of dysplasia cases. **Conclusion:** The study proved a significant role of *Hp* infection in the pathogenesis of rIDA and the impairment of gastric mucosa in the form of gastritis, metaplasia or dysplasia. Hp eradication improves iron status with partial improvement of gastric mucosal lesions. Screening for *Hp* infection and its early treatment are recommended in patients with rIDA to treat anemia and improve gastric mucosal lesions.

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Key words: Refractory iron deficiency anemia, *Helicobacter pylori*, gastritis, and immunohistochemistry.

1.Introduction:

Iron deficiency anemia (IDA) is a global public health problem affecting both developing and developed countries, with major consequences for human health as well as social and economic development [(1)].

Following initial diagnostic workup in patients presenting with IDA, treatment is implemented to correct anemia and prevent relapse [(2)]. In the majority of patients, oral iron treatment should be satisfactory [(3)].

Refractory iron deficiency anemia (rIDA) is defined by the lack of response to iron ingestion treatment prescribed for at least two months [(4)]. Treatment failure remains a significant problem. Poor compliance and poor choice of oral medications may explain some of these failures [(3)]. However, impaired iron absorption associated with celiac disease [(5,6)], autoimmune atrophic gastritis and *Helicobacter pylori* (*Hp*) gastritis are increasingly recognized as conditions responsible for obscure IDA refractory to oral iron treatment [(4,7)].

Intercellular attachment of epithelial cells is maintained via tight junctions and adherent junctions (AJs) and is critical for gastrointestinal epithelial homeostasis. Tight junctions help regulating cell polarity, epithelial barrier function and absorption, while AJs maintain tissue architecture, cell polarity and suppress cellular proliferation and migration [(8)].

AJs are composed of the transmembrane protein E-cadherin. Intracellularly distinct sites on cytoplasmic tail of E-cadherin interact with proteins of β-catenin [(9)]. CagA, expressed by CagA- positive strains of *Hp* interacts with E-cadherin, leading to β-catenin activation and subsequent transdifferentiation from a gastric to an intestinal epithelial phenotype [(10)].

Gastric *Hp* infection is a highly prevalent chronic infection with a worldwide prevalence of nearly fifty percent [(11)]. *Hp* is known to play a major contributory role in the pathogenesis of chronic gastritis, peptic ulcers, gastric mucosa- associated lymphoid tissue (MALT) lymphoma and gastric malignancies [(12 – 14)].

Hp infection impairs iron absorption [15] mainly via interfering with gastric acid secretion and with gastric ascorbate levels, via development of autoimmune gastritis induced by inflammation and also via dietary iron competition [16, 17].

This study was designed to evaluate the role of *Hp* infection in rIDA in adult patients, and to determine whether *Hp* eradication is an effective therapeutic strategy to improve iron status and gastric mucosal pathology in these patients.

2.Patients and methods:

The present study was conducted in Hematology and Gastroenterology Units of Internal Medicine Department and in both Clinical Pathology and Pathology Departments of Tanta University Hospitals in the period from January 2013 to August 2014. The study was approved by Ethical Committee of the research center of Tanta University and all participants provided informed consent.

Fifty three adult patients with rIDA and gastric *Hp* infection were involved in the study. They were 19 (35.8%) males and 34 (64.2%) females, with ages at diagnosis ranged from 18 to 56 years (mean; 36.17±11.41). **Patients fulfilled the following inclusion criteria;**

- Iron deficiency anemia defined as hemoglobin (Hb) level less than 10.5 gm/dl in females and 11.5 gm/dl in males, together with serum ferritin less than 12 ng/ml and transferrin saturation less than 15%.
- Refractoriness to oral iron treatment which was defined as Hb increment of less than one gm/dl after two months of therapy at a daily dose of at least 100 mg elemental iron [4].
- Untreated gastric *Hp* infection initially diagnosed by serum Hp-IgG and stool antigen then confirmed by histopathological examination and positive urease test on gastric biopsy samples.
- Normal physical examinations and laboratory investigations including C-reactive protein (by nephelometric method from Behring Diagnostics), serum vitamin B12 (by competitive immunoassay using direct chemiluminescent technology), folic acid, liver and kidney function tests and urine analysis.
- Negative fecal immunochemical test (Hemoccult II) for occult blood in at least three samples.
- Absence of celiac disease confirmed by negative anti-tissue transglutaminase antibodies (Anti- tTG IgA and IgG) measured by Bindazyme ™ Human anti-tissue transglutaminase ELISA kits from (The Binding Site Ltd, Birmingham, UK) and by absence of villous atrophy in duodenal biopsy samples on histopathological examination.

Patients with the following criteria were excluded from the study;

- Obvious blood loss or excessive menstrual bleeding with cycles longer than 5 days or more than 2 days of heavy bleeding with formed clots.
- Conditions interfering with normal erythropoiesis as hematological disorders, malignancies, connective tissue diseases, chronic renal or hepatic disorders.
- Previous gastrointestinal surgery.
- Pregnancy or lactation.
- Frequent use of non steroidal anti-inflammatory drugs or salicylates.

Laboratory methods:

Complete blood counts were performed by an Advia – 60 cell counter (Bayer, Pennsylvania, USA) with examination of Leishman stained blood smears.

Serum iron and total iron binding capacity (TIBC) were estimated colorimetricaly using kits provided by Bio Mérieux – France. Transferrin saturation was calculated by dividing serum iron by TIBC.

Serum ferritin levels were measured by enzyme linked fluorescent assay (ELFA) test with Bio Mérieux HPY-VIDAS System (Marcy l'Etoile, France). The assay principle combines a one – step enzyme immunoassay sandwich method with a final fluorescent assay (ELFA).

Serum IgG antibodies to Hp were detected using the Immulite®2000 Hp IgG, a solid phase chemiluminescent immunometric assay (DPC Diagnostics, Los Angeles, Calif. USA).

For detection of Hp antigen in stool; specimens were transported in cool conditions, then aliquited and frozen at -70ºC. A commercial enzyme linked immunoassay kit (Premier Platinum HpSA PLUS, Meridian Bioscience, Inc, Cincinnati, OH, USA) employing monoclonal anti-Hp antibody adsorbed to 96 – well microtiter plates was used to detect Hp antigen in stool samples according to the manufacturer's instructions [18].

Urease test; the test was performed immediately on small fragments of endoscopic biopsy specimens. Briefly, each of the two biopsy specimens from antrum and body of the stomach was incubated at 37 ºC in a 2% urea broth (urea 20gm/L, phenol red 0.04 gm/L, KH_2PO_4 0.2 gm/L, Nacl 0.5 gm/L; pH 6.8) and if a change in color from yellow to pink was noted within the following 48 hours, the biopsy was deemed to be urease test positive [19].

Endoscopy and histopathological assessment:

All patients were subjected to upper endoscopy after 6 hours fasting, under sedation. Four endoscopic biopsies were obtained; two from the body and antrum of the stomach and two from the second and third parts of the duodenum. Biopsy fragments were placed in formalin, fixed in paraffin and stained

using hematoxylin – eosin and modified Giemsa together with immunohistochemistry for E-cadherin (Rabbit polyclonal antibody Abcam 15148), and β-catenin antibodies (Rabbit polyclonal antibody Abcam 16051), which was performed by the standard biotin-streptavidin-peroxidase method.

Immunohisochemical expression of E-cadherin is characterized by an intense and homogenous immunoreactivity of the epithelial cells' membranes. Regarding the immunoreactions interpretation, cases are either uniformly positive (+) when over 90% of the cells are immunostained at membranous level, heterogeneous (±) when from 10% to 90% of the cells are immunopositive at membranous and cytoplasmic levels, and negative (-) when less than 10% of the cells are immunopositive. Aberrant immunoreactions are either negative or heterogeneous [20]. The expression of β-catenin was classified as positive when more than 70% of cell membranes are stained, otherwise as negative and if more than 10% of cells showed cytosol or nuclei staining classified as ectopic. Both negative and ectopic expressions are considered abnormal [21].

Patients' evaluation, treatment and follow up:

After being diagnosed as having both rIDA and gastric *Hp* infection, all studied patients received one week triple Hp eradication regimen of therapy (consisted of Omperazol 20 mg x 2/day, Amoxycillin 1 gm x 2/day and Clarithromycin 500 mg x 2/day). Eight weeks after completing the eradication therapy, the response was evaluated by testing stool samples for *Hp* antigen. Negative results (optical density <0.140 values) mean proper eradication and if still positive, patients were shifted to 2 weeks quadruple regimen with addition of Levofloxacin 250 mg x 2/day till the results of *Hp* stool antigen turned negative.

All patients who respond to *Hp* eradication therapy (53 patients) received oral iron therapy in the form of Ferrous sulfate at a daily dose of 500 mg for a period of two months.

Reassessment of iron status was performed twice, at 3 months and 6 months after the initiation of iron therapy. Endoscopy, histopathological and immunohistochemical assessments were repeated at the second follow up visit (6 months from initiation of iron therapy).

Statistical analysis:

Results were expressed as mean ± SD and as percentages. For variables that had measurements before and after therapy, we used paired t- test. For all analysis we used the SPSS version 20 for windows statistical package. *P*- value <0.05 was considered statistically significant.

3.Results:

The study involved 19 (35.8%) males and 34 (64.2%) females with ages at diagnosis ranged from 18 to 56 years (mean; 36.17±11.41). No statistically significant difference was noted between males and females regarding age (p=0.602).

At diagnosis Hb levels ranged from 7.9 to 10.4 gm/dl with a mean value of 9.24 ± 0.74. Hb was significantly higher (p=0.003) in males than in females (Table 1).

Table (1): Comparison between males and females regarding age and Hb levels pre-treatment.

	Age (years)		Hb (gm/dl)	
	Males	**Females**	**Males**	**Females**
Range	19 – 53	18 – 56	7.9 – 10.4	7.9 – 10.1
Mean ± SD	35.26±10.90	37.08±12.75	9.64±0.73	9.02±0.66
t. test	0.276		9.684	
***p*. value**	0.602		0.003	

Response to *Hp* triple therapy was documented by a negative repeat of stool *Hp* antigen and negative urease test on gastric mucosal biopsy samples. In all treated patients *Hp* eradication was effective, and this was accompanied by improvement of gastritis on histological examination of endoscopic gastric mucosal biopsy.

Response to oral iron treatment as assessed by Hb levels, transferrin saturation and serum ferritin is described in tables (2 – 4) and figure (1).

Three months following initiation of oral iron therapy, Hb levels ranged from 10.8 to 14.4 gm/dl with a mean value of (12.52± 0.95) with a highly significant elevation as compared to pre-treatment levels (p<0.001). While 6 months after therapy, the mean value of Hb levels was 12.81± 0.99 with no significant difference between levels assessed at 3 and 6 months post therapy (Table 2).

Oral iron therapy significantly elevated mean transferrin saturation in studied patients from 11.26% (pre-treatment) to 30.62% three months after therapy (p<0.001). On the 6 months' assessment, transferrin saturation wasn't significantly different compared to the 3 months' level.

Table (2): Hb levels pre-treatment and post-treatment.

Hb (gm/dl)	Pre-treatment	After 3 months	After 6 months
Range	7.9 – 10.4	10.8 – 14.4	11.3 – 14.8
Mean ± SD	9.24±0.74	12.52±0.95	12.81±0.99
	Pre vs. 3m.	**Pre vs. 6m.**	**3m. vs. 6m.**
t. test	19.71	21.12	1.54
***p*. value**	<0.001	<0.001	0.127

Table (3): Transferrin saturation pre-treatment and post-treatment.

Transferrin saturation (%)	Pre-treatment	After 3 months	After 6 months
Range	8 – 14	21 – 38	26 – 37
Mean ± SD	11.26±1.79	30.62±2.93	31.03±2.54
	Pre vs. 3m.	**Pre vs. 6m.**	**3m. vs. 6m.**
t. test	41.06	46.65	0.77
***p*. value**	<0.001	<0.001	0.443

Serum ferritin levels in all studied patients before treatment were less than 12 ng/ml with a mean value of 5.81 ± 3.42. These levels significantly elevated 3 months after oral iron therapy (47.59 ±11.71) with mild but not significant increase 6 months following therapy (p=0.357).

Table (4): Serum ferritin levels pre-treatment and post-treatment.

Ferritin (ng/ml)	Pre-treatment	After 3 months	After 6 months
Range	1.5 – 11.5	29.1 – 72.4	31.2 – 81.9
Mean ± SD	5.81±3.42	47.59±11.71	49.86±13.42
	Pre vs. 3m.	**Pre vs. 6m.**	**3m. vs. 6m.**
t. test	23.17	24.95	0.92
***p*. value**	<0.001	<0.001	0.357

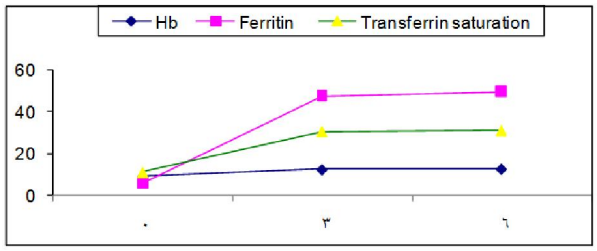
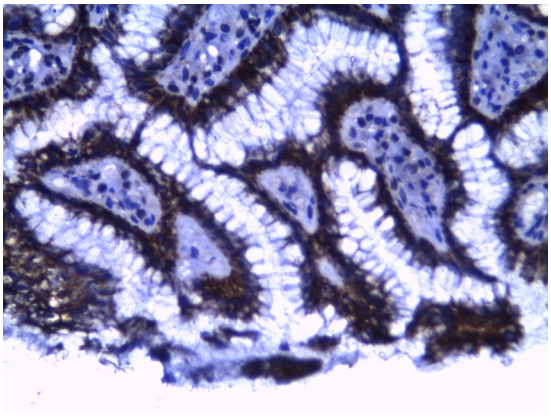


Figure (1): Significant increase (paired t. test <0.001) in Hb level (gm/dl), serum ferritin (ng/ml) and transferrin saturation (%) 3-months after oral iron therapy with non significant variation at the 6-months' visit.

Resolution of IDA was observed in all the 53 *Hp* eradicated patients. In all of them, rIDA recovery was observed at the 3-months follow up visit and there was no relapse at the 6-months' visit. Patients maintained normal Hb, ferritin and transferrin saturation levels 4 months after discontinuation of oral iron therapy.



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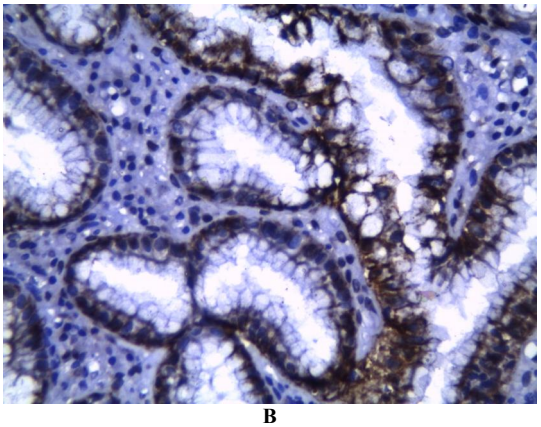

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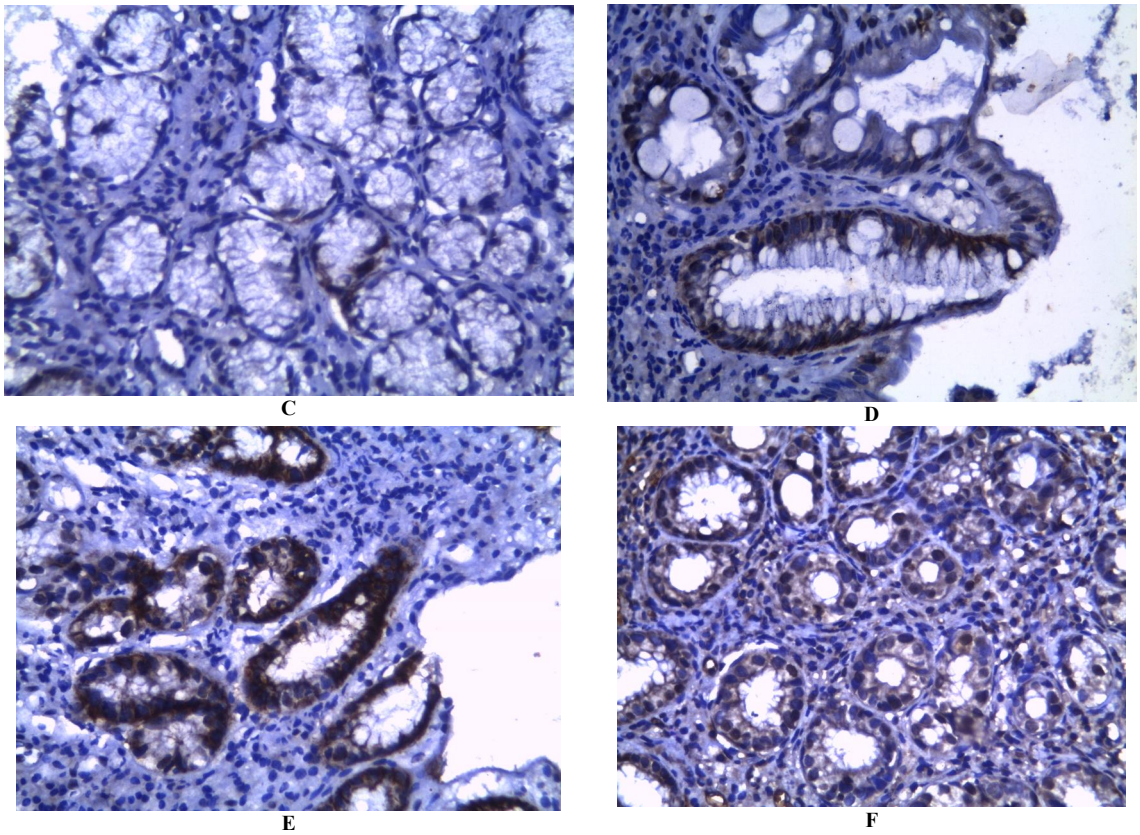

Figure (2): Immunohistochemical staining of E-cadherin and β-catenin in gastric mucosa (x400). (A); membranous expression of E-cadherin in chronic gastritis. (B); membranous expression of E-cadherin in metaplasia. (C); aberrant negative expression of E-cadherin in metaplasia. (D); cytoplasmic and nuclear expression of β-catenin in metaplasia. (E) aberrant cytoplasmic and membranous expression of E-cadherin in dysplastic glands. (F) nuclear expression of β-catenin in dysplastic glands.

Histopathological results:

All cases at diagnosis were *Hp* positive proved by Geimsa stain, *Hp* colonized in antrum mucosa. Histologically, 31 cases (58.5%) showed chronic gastritis with inflammation in the superficial gastric mucosa and intense lymphoplasmacytic infiltrate admixed with neutrophils.Besides follicular gastritis with lymphoid infiltrate up to formation of lymphoid follicles and atrophic gastritis with thinning of mucosa and flattening of ruga could be detected.

12/53 (22.6%) cases showed dysplastic changes in the form of atypical cytologic features and architectural derangement independent of the degree of inflammation, and 10/53 (18.9%) cases showed epithelial metaplasia in the form of columnar cells with brush border alternating with goblet cells.

After *Hp* eradication, there were histologic improvements in 26/31(83.9%) gastritis cases with decreased inflammatory cells and decreased lymphoid follicles, while 5/31 cases showed increased inflammation. Besides 10/12(83.3%) cases of dysplasia showed improvement, while 2/12 (16.7%) cases showed no changes. 6/10 (60%) cases of intestinal metaplasia showed decreased goblet cells, while 4/10 (40%) cases showed no changes.

Immunohistochemical results:

In cases of chronic gastritis, E-cadherin and β-catenin stained intensely in a membranous distribution throughout the epithelium which reflects the normal location of this molecule of intercellular adhesion (Fig. 2A). While in cases of metaplasia and dysplasia; areas of metaplasia showed normal membranous distribution of staining for E-cadherin in 6/10 (60%) cases (Fig.2B), while 4/10 (40%) showed aberrant negative expression (Fig. 2C). Membranous β- catenin expression was found in 8/10 (80%) cases while cytoplasmic and nuclear β-catenin expression was shown in 2/10 (20%) cases of metaplasia (Fig. 2D).

Glands with dysplasia showed aberrant cytoplasmic and membranous E-cadherin expression in 8/12(66.7%) cases (Fig. 2E) and 4/12(33.3%) cases showed nuclear expression of β-catenin (Fig. 2F).

After treatment, E-cadherin and β-catenin expression returned membranous in all cases of intestinal metaplasia, while in cases of dysplasia only 2 /12(16.6%) cases returned membranous staining.

4. Discussion:

Since gastric acidity and ascorbate play a critical role in solubilization and reduction of iron for subsequent duodenal and jejunal absorption, the achlorhydria associated with *Hp* gastritis may explain not only the circumstances aggravating IDA but also the poor response of such patients to oral iron treatment [2 ,22].

The present study involved 53 adult patients with active *Hp* infection and coexistent gastritis (58.8%), gastric mucosal dysplasia (22.6%) or metaplasia (18.9%). All studied patients had refractory IDA with negative standard diagnostic work-up for exclusion of causes of rIDA. Hemoglobin levels at diagnosis had a mean value of 9.24 gm/dl, with mean transferrin saturation of 11.26% and depleted iron stores manifested by low serum ferritin (5.81 ng/ml).

Following *Hp* eradication, all previously rIDA patients achieved normal Hb levels (mean; 12.52 gm/dl) within 3 months from initiation of oral iron therapy. Further non significant elevation of Hb levels was achieved at 6 months' follow up visit (mean; 12.81 gm/dl). This was accompanied by a significant increase in transferrin saturation from 11.26% pre-treatment to 30.62% after 3 months, then 31.03% 6 months after initiation of iron therapy and serum ferritin levels from 5.81 ng/ml pre-treatment to 47.59 ng/ml after 3 months, then 49.86 ng/ml 6 months after oral iron therapy.

The present study showed that the statistically significant increment from baseline to end-point in Hb levels, transferrin saturation and serum ferritin with Hp eradication therapy which contrast sharply with the pre-treatment refractoriness to oral iron therapy is the strongest evidence supporting a cause and effect relationship between Hp infection and rIDA in this group of patients.

Similar results were obtained in a study of 84 adult patients in whom the effect of Hp eradication on iron refractory or iron dependent anemia of unknown origin was studied [23].

The role of *Hp* in the causation of IDA is of considerable current interest. Major population surveys conducted over diverse geographic areas [24 – 27] indicate that Hp positivity is associated with an increased prevalence of iron deficiency. Hp was also implicated in several studies as an important cause of IDA in patients refractory to oral iron treatment and a favorable response to Hp eradication was reported [28 – 31].

A number of possible mechanisms may explain the relation between Hp gastritis and IDA. Previous study [32] has shown that gastric acidity and ascorbate content both of which are critical for normal iron absorption [22, 33], are adversely affected by *Hp* infection and that Hp eradication results in normalization of gastric pH and ascorbate content. Hp suppresses acid secretion through induction of interleukin-1β and tumor necrosis factor- α that are potent inhibitors of gastric parietal cells function [34, 35]. Hp also, causes loss of acid secreting parietal cells through induction of apoptosis [36]. In addition, occult gastrointestinal bleeding caused by *Hp* may also play a significant role in the development of rIDA [37].

Barabino (2002), [38] hypothesized that gastritis increased levels of neutrophil-derived lactoferrin, and since *Hp* has a lactoferrin-binding protein receptors, *Hp* infection would result in increased iron losses related to bacterial turn-over.

The relation between *Hp* infection and autoimmune gastritis (AG) is intriguing [39]. *Hp* infected subjects have circulating IgG antibodies directed against epitopes on gastric mucosal cells. AG is mediated by CD4+ T-cells reactive to H^+K^+ - ATPase, and *Hp* probably trigger the autoimmune process by molecular mimicry [17].

Although eradication of *Hp* restores acid secretion and proper iron absorption even in patients with severe atrophy, cure of autoimmune gastritis by *Hp* eradication is exceptional [40].

We investigated gastric biopsies histologically and with Geimsa stain before and after eradication of *Hp* infection. It was found that 8 months after eradication therapy 83.9 % of cases with chronic gastritis showed improvement, besides improvement in 83.3% of cases with gastric mucosal dysplasia and 60% of cases with metaplasia.

The reason why damage to gastric mucosa is highly variable may be explained by observations indicating that genetic variability of host factors, as well as the composition of iron- repressible outer membrane proteins of *Hp*, may determine the clinical expression of the disease and represent important new insights into the mechanism and severity of *Hp* infection [41, 42].

Failure to achieve complete remission of gastric mucosal lesions by *Hp* eradication in some patients does not necessarily argue against the role of *Hp* in the pathogenesis of autoimmune gastritis, but more likely indicates that a point of no return may be reached beyond which the autoimmune process may no longer require the continued presence of the inducing pathogen [43]. Some studies in adults have shown that it can take many months, possibly years to resolve gastritis after *Hp* eradication [44, 45].

We also investigated E-cadherin – β-catenin complex immunohistochemically due to their role in maintaining gastric epithelial tight junction that maintain absorption of iron as well as suppression of cell proliferation and migration [8]. In this study, we found that in all cases of chronic gastritis, E-cadherin and β-catenin were of membranous staining pattern

before and after treatment, while 40% of cases with metaplasia showed aberrant negative expression of E-cadherin and 20% showed cytoplasmic and nuclear expression of β-catenin which completely returned membranous after treatment. 66.7% of cases with dysplasia had aberrant expression of E-cadherin and 33.3 % of cases showed nuclear expression of β-catenin. After treatment there were improvements in only 16.6% of cases with dysplasia and in all cases of metaplasia.

Lazar *et al.* (2008) [20], showed that chronic gastritis areas and intestinal metaplasia have showed a normal immunostaining membranous pattern. The aberrant E-cadherin expression was observed in their study in 35.5% of epithelial dysplasia. A study by Ning Zhou *et al.* (2002) [46], showed that there were decreased E-cadherin and β-catenin expression in dysplasia that raises the possibility of changes in the E-cadherin - β-catenin complex occurring at an early stage in the neoplastic process.

Nuclear expression of β-catenin in dysplasia could be explained by Kamiya *et al.* (2007) [10] who demonstrated that *Hp* (CagA type) interacts with E-cadherin and causes destabilization of the E-cadherin - β-catenin complex, resulting in cytoplasmic/ nuclear accumulation of β-catenin and also proved that Hp CagA +ve strain has a role in multi-step gastric carcinogenesis. Association of CagA with E-cadherin, which results in impaired cell–cell interaction and at the same time deregulated activation of β-catenin signal, may play an important role in promoting pathological changes that precede transformation of gastric epithelial cells and metaplasia formation [10].

A period of eight months following Hp eradication is enough for treatment of rIDA and returning near normal gastric mucosa in most of studied cases with chronic gastritis or with epithelial metaplasia while in cases with dysplasia, treatment for longer time should be investigated.

In conclusion; the present study proved a significant role of *Hp* infection in the pathogenesis of rIDA, together with impairment of gastric mucosa in the form of gastritis, metaplasia or even dysplasia. *Hp* eradication improves iron status in previous rIDA patients even after discontinuation of oral iron therapy. The partial improvement of gastric mucosal lesions obliged us to recommend screening for *Hp* infection with early treatment to prevent not only iron deficiency but also any gastric lesion which may be irreversible or precancerous in some cases.

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