Determination of Enzymatic Antioxidant in Iraqi Patients with Chronic Gastritis

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Abstract

Infection of the gastric mucosa with *Helicobacter pylori* is strongly associated with chronic gastritis, peptic ulcer and gastric cancer. *Helicobacter pylori* virulence factors include a variety of proteins that are involved in its pathogenesis, such as VacA and CagA. Another group of virulence factors is clearly important for colonization of *H.pylori* in the gastric mucosa. These include urease, motility factors (flagellin), and Superoxide dismutase (SOD). Because of this organism's microaerophilic nature and the increased levels of reactive oxygen in the infected host, we expect that other factors involved in the response to oxidative stress are likely to be required for virulence. Superoxide dismutase is a nearly ubiquitous enzyme among organisms that are exposed to toxic environments. In this study, we measured the SOD in serum of 80 patients complain from chronic gastritis and infected with *H.pylori*. 37 patients infected with *H.pylori* have the CagA gene, and 13 patients are not and also measured the SOD in 30 control groups that not infected with *H.pylori*. Serum level of SOD was significantly ($p<0.05$) higher in patients with chronic gastritis compared to controls. Also significantly higher ($p<0.001$) in patients with chronic gastritis infected with *H.pylori* positive CagA than patients infected with *H.pylori* negative CagA.

Key words: chronic gastritis, *H.pylori*, CagA, SOD

الخلاصــة

إصابة الغشاء المخاطي للمعدة ببكتريا ال *Helicobacter pylori* تترافق مع التهاب المعدة المزمن والقرحة المعدية وسرطان المعدة .عوامل الضراوة الخاصة بهذه البكتريا تتضمن أنواع من البروتينات التي تشارك في أحداث في المراضية وهي ال CagA و VagA . مجموعة أخرى من عوامل الضراوة تكون مهمة في تكوين المستعمرات في الغشاء المخاطي المعدي منها اليوريز و عامل الحركة وأنزيم ال Superoxide dismutase (SOD) الذي يتواجد في الكائنات التي تتعرض إلى وسط سام . في هذه الدراسة تم قياس مستوى الـ SOD في مصل 80 مريض كانوا يعانون من التهاب المعدة المزمن ومصابين ببكتريا ال *H.pylori* منهم 37 مريض مصابين ال *H.pylori* الحاملة لل CagA و13 مريض مريض مصابين ال *H.pylori* لكن غير حاملة لهذا الجين . وكانت مجموعة السيطرة تتكون من 30 شخص بحالة صحية سليمة وغير مصابين بالبكتريا .وجد أن مستوى الـ SOD يزداد معنوياً ($p<0.001$) في المرضى المصابين بالتهاب المعدة المزمن مقارنة بمجموعة السيطرة ويزداد معنويا في مرضى التهاب المعدة المزمن المصابين ببكتريا ال *H.pylori* والحاملة للـ CagA ($p<0.05$) مقارنة بمرضى التهاب المعدة المزمن المصابين بالبكتريا ولكن غير حاملة للـ CagA

Introduction

The gastric pathogen *Helicobacter pylori* is a curved, microaerophilic proteobacterium that has been implicated as a causal agent of peptic ulcers and a risk factor for adenocarcinoma (1,2, 3,4,). During the infections, disease symptoms may or may not occur, though gastric inflammation is apparently ubiquitous. The pathogenesis of *H.pylori* relies on its persistence in surviving a harsh environment, including acidity, peristalsis, and attack by phagocytic cells and their released reactive oxygen species (5). Several potential virulence factors derived from *H.pylori* are considered to attract or activate neutrophils and mononuclear cells., an immunodominant 120-140 KDa antigen termed cytotoxic associated antigen (CagA), the CagA positive strain cause more server inflammations(6).

The stomach gastritis associated with *Helicobacter pylori* infection stimulates the generation of reactive oxygen species (ROS) by the inflammatory cells present in the mucosa (7, 8, 9). An increase in ROS directly correlated with bacterial load (10). In addition to internally generated reactive oxygen species, the successful pathogen must also deal with reactive oxygen species that are generated by phagocytic cells of the host immune response. (11, 12). Protection of cells against ROS is accomplished through the activation of oxygen-scavenging enzymes such as SOD, catalase and glutathione peroxidase have been identified (13). Superoxide dismutase is a nearly ubiquitous enzyme among organisms that are exposed to toxic environ

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The single SOD of *Helicobacter pylori*, encoded by the *sodB* gene, has been suspected to be a virulence factor for this pathogenic microaerophile, but mutations in this gene have not been reported previously (14). The mechanisms for the detoxification of reactive oxygen species are of particular interest in *H.pylori*. Despite the fact that this organism is an obligate aerobe, it is unable to grow in atmospheric concentrations of oxygen. microaerophilic organisms, like *H.pylori*, are particularly vulnerable to the detrimental effects of oxygen and oxidative stress (15). Nevertheless, they do possess some of the enzymatic machinery needed to eliminate or minimize toxic oxygen-derived products. Organisms that grow in toxic environments must have mechanisms to handle reactive oxygen species (e.g., superoxide anions, peroxides, and hydroxyl radicals) that are by-products of oxygen metabolism (16, 17). Of these genes, only *katA* (catalase) mutants have been characterized (18). Although catalase-defective mutants are no different from the parent in their binding to epithelial cells, this enzyme may be important in detoxification of reactive oxygen species produced by the host immune response. Genes encoding an akyl hydroperoxide reductase, a thiol-specific peroxidase, and other potential detoxification enzymes were identified, but mutations in these genes have not been reported or characterized (14, 19). Impairment in this important host cell defense mechanism would greatly reduce the ability of the gastric to epithelial cells to tolerate an environment high in ROS, such as would be present with the chronic gastritis associated with *H.pylori* infection. Disturbance of the oxidant-antioxidant balance in the stomach might greatly increase the risk of cell death or DNA damage, from ROS (20, 21). The aim of this study to investigate the relation between the *H.pylori* with CagA- positive and CagA - negative strains and the production of SOD in patients with chronic gastritis (HP+) and healthy control group (HP-).

Materials and Methods

Eighty subjects (48 male and 32 female; mean age 51.7), were referred to the gastrointestinal endoscopy unit at Al-Yarmook Teaching Hospital, non of whom had received non steroidal anti- inflammatory drugs, within previous three months, participated in this study. Endoscope fining in the patients were as follows: normal mucosa and no *H.pylori* infected (30 subjects) and 50 patients with chronic gastritis without ulcer. Biopsy specimens were taken from the antrum of all subjects in this study, by using the same size forceps, from similar topographical sites at each endoscopy; biopsies were fixed in 10% formal buffer saline for histological examination. Blood samples were taken from all subjects and the serum were stored at -20 Ċ until be used.

Histology

The biopsy specimens were embedded in paraffin and stained with haematoxylin – eosin (HandE) and Giemsa stained for *H.pylori* determination and diagnostic as chronic gastritis. In situ hybridization (ISH) for detection of *H.pylori* / CagA gene. In situ hybridization (ISH) is a technique makes use of the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell. For detection of this markers , the biotinylated DNA probe hybridize to the target sequence (*H.pylori* / CagA mRNA sequence) then a streptavidin-AP (streptavidin-alkaline phosphatase) Conjugate is applied followed by addition of the substrate promo-chloro – indolyl – phosphatel / nitro-blue tetrazolium (BCIP/NBT) which yield an intense blue – black signal appears at the directly specific site of the hybridized probe. This strepteividin – Ap conjugate like the biotinylated probe provides raid and highly sensitive detection method. The use of Biotin – Labeled DNA probe for *H.pylori* / CagA (8 μg/10015 ML) litter dd H2O . Probe size: 349 bp (Maxim Biotech, Inc., U.S.A).

Scoring

Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in both positive test tissues. A scoring system that includes evaluation of the staining percentage of stained gastric cells was employed for the expression of CagA gene of *H.pylori*. Counting the number of the positive cells in the gastric tissue which gave a blue-black nuclear staining under the light microscope. The extent of the ISH signaling the cells of the examined tissue was determined in 10 fields under high power microscope (40X). In each field, the total of examined cell was about 100 cells per field and this gives a total number.

Measurement of superoxide dimidiate (SOD)

For the quantitative determination of superoxide dismutase in whole blood. This produce is suitable for manual use RANDOX, Cat. No. SD 125 Mixed substrates, Buffer, xanthine oxidase and standard. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react

with 2 (4- iodophonyl) – 3- (4- nitrophenol) -5-phanyltetrazolium chloride (I.NT) to form a red fermazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Xanthine $\longrightarrow$ **Uric acid + O_2**

I.N.T $\longrightarrow$ **formazan dye**

$O_2 + O_2 + 2H$ $\longrightarrow$ **$O_2 + H_2O$**

Statistical analysis

Statistical analysis was performed using ANOVA test to determine whether the means were equal among three groups – i.e. CagA-, CagA+ and controls, p value of < 0.05 was considered statistically significant.

Results

The expression of CagA was detected by in situ hybridization technique. From 50 patients complaining chronic gastritis and infected with *H.pylori* who were tested for CagA, 37 (74%) were found to be positive CagA and 13 (26%) patients have CagA – negative (Table 1, Figure 1).The mean level of SOD increased significantly in patients infected with *H.pylori* CagA positive strains p<0.001 when compared with healthy subjects and patients infected with *H.pylori* CagA negative (Table 2,Figure 2).The difference in SOD level between with *H.pylori* that have CagA positive or CagA negative is statistically significant p<0.01.

Table (1): Expression of CagA mRNA in *H.pylori*– positive patients with chronic gastritis by (ISH).

	CagA status	No.	(%)
***H.pylori* positive**	positive	37	74
	negative	13	26
	Total	50	100

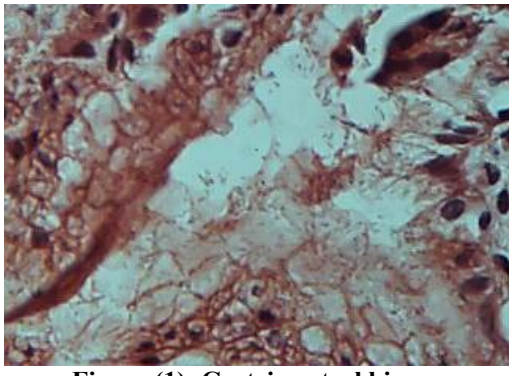

Figure (1): Gastric antral biopsy specimen from stomach infected with *H.pylori* that appear curved or round (Giemza stain) (100X).

Table(2): The serum level of SOD in patients infected with *H.pylori* and CagA status.

Group	Mean ± S.E SOD	S.D.	*P* Value	F Test
Control (Hp-)	177.70 ±2.91	9.20		18.56
Hp+ CagA-	* 284.00 ± 14.22	44.95	<0.05	
Hp+ CagA+	** 477.00 ±54.71	73.84	<0.001	

No significant difference $p > 0.05$

* Significant at the 0.05 levels

** Significant at the 0.001 level

Hp: *H.pylori*

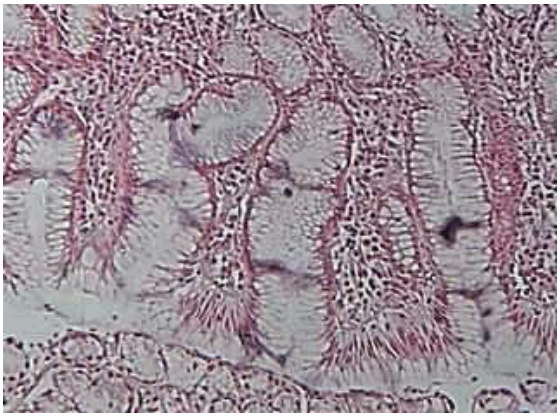

Figure (2): Detection of CagA, in patients with gastroduodenal disease by in situ hybridization. staining of CagA mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from patients with antral gastritis shows positive CagA by hybridization signals.

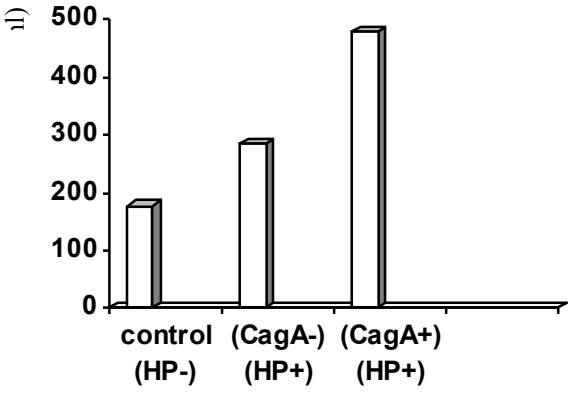

Figure (3): The serum level of SOD in patients infected with *H.pylori* and CagA status.

Discussion

Cytotoxine associated antigen – positive strain was significantly higher ($p<0.001$) in chronic gastritis patients than in control group. It can be seen that 74% of patients who have *H.pylori* infection have CagA positive strains. *H.pylori* strains were positive for the CagA 74.4% of Costa Rica patients [22]. Other study reported the prevalence of CagA was more than 80% among patients with chronic gastritis [23]. Increasing of CagA mRNA among those patients may explain the role of CagA positive *H.pylori* in the development of gastritis [24]. The mechanisms by which CagA modify the activity of epithelial cells is explaining by serving as scaffolding protein able to interact and modify the function of a variety of molecules involved in cell to cell interaction, cell motility, and proliferation [23, 25].The differences between the results, possibly by using different methods to assess the expression of CagA positive *H.pylori* in patients with gastritis, such as ELISA methods, PCR that the correct design of primers is very important , the different sets of CagA primers give different results, and this will attributed to divergence in the primer target sequences [26, 27].*H.pylori* infection of the gastric mucosa is associated with abundant inflammatory response; this bacterium is capable of stimulating oxidative bursts from noutrophils [28].Gastric tissue from *H.pylori* infected persons contains more ROS than normal tissue and there is a direct correlation between bacterial and the amount of ROS in the gastric mucosa [29, 30].This study supports the direct correlation between ROS and gastric mucosal damages in patients infected with *H.pylori* which can increase the susceptibility of gastric epithelial cells to ROS – associated cell injury. The increase of SOD and activity in patients with *H.pylori* – CagA positive strains is probably responsible for the increased survival of these cells. The generation of intracellular ROS on the presence of CagA positive *H.pylori* strains is possible explanation for the increases in activity of ROS – scavenging enzymes. That *H.pylori* induces the production of intracellular ROS; this increase in ROS in gastric cells was enhanced by increasing the concentration of *H.pylori* and inhibited by use of antioxidant[22].There are more ROS in the gastric mucosa of patients infected with *H.pylori* CagA positive strains, is significantly difference from patients with *H.pylori* CagA negative strains. As a response to increase formation of ROS the antioxidant enzyme are increase in these cells, suggest that gastric epithelial cells have a higher concentration of these enzymes (SOD and catalase) so they will be able to avoid lethal injury in patients infected with CagA positive strains than in patients infected with CagA negative strains [13].Gastric cells infected with CagA-positive *H.pylori* strains have higher catalase level, catalase enzyme convert hydrogen peroxide to H2O and oxygen molecule. Accumulations of hydrogen peroxide reduce the activity of SOD; these conversions of hydrogen peroxide protect the cells from sudden exposure to superoxide, giving them a survival advantage [13, 14].The presence of ROS along with the reduction of antioxidants, such as vitamin C in the gastric mucosa of people infected with *H.pylori* potentially increases the risk of oxidant – related cellular injury and DNA damage, change in the levels of cellular ROS-scavenging enzymes induced by *H.pylori* may further increase this risk of developing gastric cancer from ROS in patients infected with *H.pylori* [31].

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