ANTICANCER RESEARCH *25*: 4763-4768 (2005)

Relationship between Eradication of *Helicobacter pylori* and Gastric Mucosal Superoxide Dismutase Activity

RITA FARKAS[1], LASZLO PRONAI[1], ZSOLT TULASSAY[1] AND LASZLO SELMECI[2]

[1]Second Department of Medicine, H-1088 Budapest, Szentkiralyi u. 46.;
[2]Department of Cardiovascular Surgery, Semmelweis University, Faculty of Medicine, H-1122 Budapest, Varosmajor u. 68., Hungary

Abstract. *Background: Helicobacter pylori (HP) is the main pathogenic factor in the development of gastritis and gastric cancer. Superoxide-dismutase (SOD) is a key enzyme of mucosal antioxidant protection. In the presence of HP there is a significant increase of SOD activity in the antrum. Changes in gastric mucosal SOD activity were detected in response to eradication treatment of HP infection. Patients and Methods: Biopsies were taken from 13 patients upon gastroscopy performed prior to and 88.3±12.6 days after treatment. The activity of SOD was determined by spectrophotometry. Results: The activity of SOD in the gastric mucosa decreased significantly following the successful eradication, whereas in the corpus activity did not change significantly. Conclusion: In the presence of HP there is an oxidative stress in the gastric mucosa triggered by the bacterium. It may represent the final common path of HP carcinogenesis. Successful eradication treatment prevents the production of reactive oxygen metabolites.*

Gastric adenocarcinoma is one of the most common malignancies: it is the fourth most common cancer and the second most common cause of cancer deaths worldwide. Among predisposing factors and conditions for the development of gastric cancer, genetic, geographical, ethnic and socioeconomic factors have been implicated. The most important risk factors are alcohol consumption, smoking and increased salt and nitrate intake (1). In contrast, an adequate intake of fresh fruits and vegetables is associated with decreased risk (2).

Correspondence to: Prof. Laszlo Selmeci, Department of Cardiovascular Surgery, Semmelweis University, Faculty of Medicine, H-1122 Budapest, Varosmajor u. 68., Hungary. Fax: (36-1)-458-6746, e-mail: laszlo.selmeci@excite.com

Key Words: Antioxidants, eradication treatment, gastric mucosa, gastritis, *Helicobacter pylori*, reactive oxygen metabolites, superoxide dismutase.

Helicobacter pylori (HP) has been identified in the pathogenesis of chronic active gastritis and peptic ulcer disease and is epidemiologically linked to gastric cancer and MALT lymphoma. Numerous studies have shown that patients with HP infection have up to six times increased risk of developing gastric cancer when compared with healthy subjects (1).

In most subjects, HP infection is asymptomatic and its clinical outcome is variable. Sequential changes of gastric mucosa take place before neoplasia develops. The metaplasia, at the beginning of the process, resembles the small intestinal mucosa. In more advanced stages, the metaplastic cells display a "colonic" phenotype. The precancerous process displays a slow progression to more advanced lesions (2).

Oxidative stress may be a crucial mechanism in the chain of preneoplastic events. Chronic gastritis induced by HP is characterized by considerable neutrophil infiltration into the gastric mucosa, without mucosal invasion of bacteria. These phagocytes appear to be a primary cause of the damage to surface epithelial layers and probably contribute to the pathogenesis associated with persistent HP infections. HP stimulates human neutrophils to produce pro-inflammatory cytokines and increases expression of inducible nitric oxide synthase (iNOS) and production of reactive oxygen metabolites (ROMs). Through oxidative burst, ROMs may damage DNA and induce changes in the epithelial cell cycle (2).

Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), may prevent the cellular damage induced by the oxidative stress. In theory, the elevated production of ROMs in the mucosa increases SOD production. Opposing forces of oxidation and antioxidation interact either to induce or prevent neoplastic transformation. (2) SOD is well known for scavenging superoxide radicals, subsequently protecting cells from oxidative injury, and for maintaining tissue homeostasis (3).

In the absence of eradication, HP infection tends to be lifelong, because of the ineffective immune response in clearing the bacteria. Several trials have shown reduction in

the progression of gastric preneoplastic lesions by administering anti-HP therapy. Eradication of HP and/or dietary supplementation with beta-carotene, ascorbic acid, or both agents independently resulted in significant regression of lesions. These results support the hypothesis that oxidative stress may represent the final common path of HP carcinogenesis (2).

In our previous retrospective study, we investigated the activity of SOD in gastric mucosal biopsies in HP-positive specimens compared with HP-negative ones. In patients with HP infection, mucosal SOD activity was significantly increased in the antrum, but not in the corpus. This might be a response of the host to protect mucosa against possible tissue injury caused by ROMs. The SOD activity was significantly higher in HP-positive chronic antral-gastritis (CAG) than in HP-negative cases. The severity and activity of CAG had a positive relationship with SOD activity (4).

The aim of the present prospective study was to investigate the changes in the activity of SOD in gastric mucosal biopsy specimens before and after eradication of HP.

Patients and Methods

Patients and biopsies. Thirteen never-treated *Helicobacter*-positive patients (7 male, 6 female, mean age 56.7 years; range 26-79) were studied. After informed consent was obtained, biopsy specimens were taken during diagnostic gastroscopy. One patient (a 26-year-old man) was diagnosed by histological examination as having *Helicobacter heilmannii* (HH) in the stomach. The other 12 subjects were infected with HP. Patients were treated with a triple combination of omeprazole (20 mg once daily), clarithromycin (500 mg twice daily) and amoxycillin (1000 mg twice daily) for 7 days. This was followed by H2 receptor blocker treatment. After 88.3±12.6 days from the termination of the eradication treatment, the patients underwent repeated gastroscopy. Two antral and 2 corpus biopsy specimens were obtained both before and after treatment to be used for histology and for determination of mucosal SOD activity. (SODa=SOD activity in the antrum; SODc=SOD activity in the corpus; U/mg protein). Histology was performed by routine pathology.

Tissue extraction. Each biopsy specimen was homogenized manually in 300 μl phosphate buffer (0.01 mol/l, pH 7.0) in a mortar using a pestle. The homogenates were centrifuged (3000/min, for 10 min).

Determination of SOD activity. SOD activity in the supernatant was determined using a RANSOD (RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, UK, Cat. No. SD 125) kit. The principle of this technique is based on the xanthine/xanthine-oxidase (XOD) system. In the presence of xanthine, XOD produces superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. This reaction is inhibited by SOD, through competition with INT for the flux of superoxide. The degree of inhibition of this reaction correlates with the SOD activity. SOD activity was determined in a Cobas Mira Plus (Roche Diagnostics) clinical chemistry analyzer.

Briefly, 5 μl homogenate was added to 170 μl XOD (80U/l). The reaction started as described above. The amount of red formazan was detected by spectrophotometry at 500 nm and at 37˚C. A calibration curve based upon 6 standards between 0.36 and 5.7 μg/ml SOD was used. SOD activity was expressed in U/mg protein.

Determination of protein concentration in the supernatant. Protein was determined as described by Bradford (5). Measurements were done in a microplate reader (Multiskan Plus MK II, Helsinki, Finland) at 585 nm using bovine albumin as standard.

Statistical analysis. All data were reported as mean±SD. The significance of the difference between the mean SOD activities before and after eradication treatment was determined by the Mann-Whitney rank test (*U*-test). A *p*-value less than 0.05 was used as the level of significance.

Results

Eradication was successful in 9 patients on the basis of HP negativity at the control gastroscopy. Three patients remained HP-positive. Eradication of HH was unsuccessful.

Four subjects had a small amount of HP (HP+) in their biopsy specimens during the first gastroscopy, in one case with mild CAG and in 3 cases with moderate CAG in the antrum. Low SOD activities were found before eradication (SODa=2.32±1.04; SODc=1.42±1.20 U/mg prot; n=4). SODc activities showed a great variation. Only the patient who was diagnosed with mild CAG in the antrum showed complete healing after successful eradication. In this patient SOD activities were low at entry and were under the detection limit after eradication.

Three patients showed moderate CAG associated with HP+, 1 subject with ulcus bulbi. In 2 of these patients SODa activities and severity of CAG were diminished following successful eradication, and the ulcus bulbi healed. One subject had focal intestinal metaplasia (FIM) with moderate CAG at entry. In this patient, after unsuccessful treatment, the levels of HP and SODa activity were higher than before treatment and IM was detected in the antrum. Finally, in patients with HP+ (n=4) eradication was successful in 3 cases. One patient remained HP-positive and had a large HP count in the stomach following treatment.

Higher levels of SODa and SODc were seen in 5 patients with moderate amounts HP (HP++) in the stomach during the first gastroscopy (SODa=5.69±1.73; SODc=5.58±2.21 U/mg prot; n=5). They all were diagnosed as having mild CAG in the antrum. In 1 patient, the histology revealed mild atrophy and FIM. In 3 cases, following successful eradication, SODa and SODc activities decreased markedly (SODa=1.02±1.07; SODc=1.45±1.55 U/mg prot; n=3), and the moderate CAG became mild in response to treatment. In 2 subjects eradication was unsuccessful, the initial SODa activity did not decrease and the mild CAG further persisted. SODc activities tended to increase.

Considerably higher SODa activities were measured in subjects with a large number of HP (HP+++) (SODa=10.29±2.34 U/mg prot; n=3) at entry compared to patients with HP+ or HP++. SODc activities were practically zero. All these patients had severe CAG, 1 with moderate atrophy and FIM. One subject was diagnosed as having erosive inflammation in the antrum. In spite of the succesful eradication of a large number of bacteria in all cases, SODa activities showed marked decrease. SODc activities did not change. In 1 case severe CAG showed complete healing and 2 patients had mild CAG in their second biopsy specimens.

There was an association between severity of HP infection and SODa activities. Patients with HP+ had lower SODa activity (2.32±1.04 U/mg prot; n=4) than those with HP++ (5.94±1.86 U/mg prot; n=7). Subjects with HP+++ had the highest SODa activity (10.29±2.34 U/mg prot; n=3), whereas no such association was seen between HP infection and SODc activities.

Samples were also graded by the severity of CAG. Patients with mild CAG exhibited low SODa activities (1.14±0.90 U/mg prot; n=8). In subjects with moderate CAG, SODa activities (5.11±2.06 U/mg prot; n=11) were similar to those found earlier in healthy subjects (5.17±1.16 U/mg prot; n=11). Strongly elevated SODa activities were found in patients with severe CAG (10.29±2.34 U/mg prot; n=3).

The presence of FIM and IM did not show any association with SODa activity. In one patient diagnosed with erosive CAG, the SODa activity was elevated (8.83 U/mg prot), probably due to the large amount of HP.

A small amount of HH was present in one patient accompanied by moderate CAG before and after treatment as well. SOD activities at entry were extremely high (SODa=15.31 U/mg prot; SODc=13.68 U/mg prot). Despite unsuccessful eradication, no SOD activities were detected in the biopsy specimens following treatment.

Table I. *Group of patients with successful eradication of Helicobacter pylori (n=9, 5M/4F, age range: 45-79 years).*

	SOD activity (antrum) mean± SD (U/mg protein)	SOD activity (corpus) mean± SD (U/mg protein)	*H. pylori*	Histology
Before treatment	6.08± 1.31 (n=9)	2.99± 1.03 (n=9)	+ (n=3) ++ (n=3) +++ (n=3)	mild CAG (n=2) moderate CAG (n=4) severe CAG (n=3) UB (n=1) atrophy (n=2) FIM (n=2)
After treatment	0.48± 0.73* (n=9)	0.63± 1.08** (n=9)	neg. (n=9)	mild CAG (n=2) moderate CAG (n=7)

+: small; ++: moderate; +++: large amount of *H. pylori*
CAG: chronic antral gastritis
UB: ulcus bulbi
FIM: focal intestinal metaplasia
*$p<0.001$ SOD activity in the antrum before *vs.* after eradication treatment
**$p>0.05$ SOD activity in the corpus before *vs.* after eradication treatment

Discussion

The major finding of our study was that the SOD activity of gastric mucosa in the antrum declined significantly ($p<0.001$), and the severity of CAG in the antrum diminished substantially following successful eradication of HP (Table I).

Out of the 4 patients with HP+ at entry, eradication was successful in 3 cases and SODa activities were reduced in response to treatment. In 1 subject, eradication was unsuccessful, the HP count and SODa activity increasing markedly after treatment.

Patients with HP++ and moderate CAG at entry (n=5) had higher SODa activities than those measured in HP+ subjects. In 3 cases eradication was successful, SODa and SODc activities decreased and CAG became less apparent. After treatment, 2 subjects remained HP++ with moderate CAG and with unaltered SOD activities.

In patients with HP+++ and severe CAG at entry, much higher SODa activities were measured than in subjects with HP+ and HP++.

Our results are in line with those published by Noguchi *et al*. (3). These authors also found that the mean SOD activity was significantly higher in HP-positive than in -negative mucosa, and decreased markedly following HP eradication.

Iacopini *et al*. (6) investigated oxidative damage by nitrotyrosine immunohistochemistry in the whole gastric mucosa in HP-positive gastritis at baseline and at 12 months after eradication. After one year, total nitrotyrosine levels tended to decrease, but remained unchanged in IM. HP eradication resulted in a complete healing of inflammation, but not of IM. These results appear to indicate that eradication of HP decreases oxidative stress in gastric mucosa, contributing to the protection against HP-associated carcinogenesis in the stomach.

In our earlier study, HP-negative patients with normal endoscopy and histology constituted the healthy control group. In these subjects, the mean SODa (5.17±1.16 U/mg prot; n=11) and SODc (5.28±2.36 U/mg prot; n=12) did not differ. In contrast, in the present study, markedly lower SOD activities were measured in HP-negative biopsy specimens following successful eradication (SODa=0.48±0.73 U/mg prot; SODc=0.63±1.08 U/mg prot; n=9). No unequivocal

Table II. *Group of patients with unsuccessful eradication of Helicobacter pylori.*

Patient and sample	SOD activity (antrum) (U/mg protein)	SOD activity (corpus) (U/mg protein)	*H. pylori*	Histology
A (F, 43y)				
Before treament	3.23	n.d.	+	moderate CAG,
After treatment	6.18	n.d.	+++	moderate CAG,
B (M, 64y)				
Before treatment	6.75	2.25	++	moderate CAG
After treatment	8.51	7.20	++	moderate CAG
C (F, 58y)				
Before treatment	3.93	5.13	++	moderate CAG
After treatment	4.58	7.14	++	moderate CAG

+: small; ++: moderate; +++: large amount of *H. pylori*
CAG: chronic antral gastritis
FIM: focal intestinal metaplasia
IM: intestinal metaplasia
n.d.: not detected

explanation can be offered as yet for the low mucosal SOD activities 88.3±12.6 days after successful eradication of HP. It is feasible that the re-establishment of baseline SOD activity requires a longer period after eradication treatment.

In subjects with unsuccessful eradication and consistent severity of CAG, SODa activity did not significantly change from the initial value. In 1 patient's HP count, the severity of CAG and SODa activity even increased following treatment (Table II).

These results appear to support our earlier finding observed in a retrospective study, namely that SODa activities are correlated with bacterial count and severity of CAG. SODc activities exhibited a large inter-individual variation. This might be a consequence of the fact that HP-associated gastritis is predominant in the antrum.

Although HP infection is closely associated with increased gastric cancer risk, Skopeliton *et al*. (1) suggested that it alone is unlikely to be capable of inducing gastric cancer. One reason is that infection rates exceed cancer rates by 190 times. On the other hand, some populations that experience almost universal infection with HP have very low rates of gastric cancer or its precursors.

HP strains show a high grade of genetic diversity. Two bacterial genes, *cagA* and *vacA,* are determinants of virulence. The presence of the *cagA* gene is associated with more severe pro-inflammatory response and with increased oxidative stress. It appears that HP strains which possess the *cagA* gene elicit more pronounced inflammatory responses than other strains. This gene is associated with increased risk of gastric carcinoma when compared to uninfected controls and persons with *cagA*-negative infections. Genotypic variations in the *vacA* gene are also related to higher cancer risk (2).

HP gastritis is not a sufficient factor in itself to induce gastric carcinogenesis. Other factors, such as geographical and dietary variabilities, are needed for the progression of the disease. Subsequent additional genetic changes involving tumor suppressor genes or oncogenes may also be responsible for tumor progression (1).

It is not clear whether oxidative stress is also present in HP asymptomatic humans. Felley *et al*. (7) investigated the expression in the antrum and corpus of inducible nitric oxide synthase (iNOS), SOD, CAT and interleukin-8 (IL-8) production in HP-infected asymptomatic humans, and the effect of eradication of HP. Activities of iNOS, SOD, CAT and the concentration of IL-8 were significantly associated with HP infection. A decrease in the activities of iNOS and CAT was observed after HP eradication. The authors concluded that oxidative stress also occurs in asymptomatic HP-positive patients and can be modulated by HP eradication.

HP possesses self-protecting mechanisms against oxidative stress. The HP protein NapA (neutrophil-activating protein) has been shown to contribute to the ability of the bacterium to survive under oxidative stress conditions. NapA is an iron-binding protein. It co-localizes with the nuclear material, suggesting that it can interact with DNA *in vivo*. Cooksley *et al*. (8) demonstrated that mutation of NapA resulted in increased DNA damage in HP. This antioxidant protein can protect HP against oxidative stress-induced lethality.

In the patient infected with HH, moderate CAG was shown both before and after treatment. SOD activities were markedly elevated at entry. Eradication was not successful. Following treatment, SOD activities were under the detection limit.

HH is a corkscrew-like spiral bacterium. (9) HH colonizes the antrum of rats in large numbers and induces no significant inflammatory response. (10) In humans, HH gastritis has an endoscopic appearance that resembles HP gastritis. Fathalla *et al*. (9) reported 2 cases of HH gastritis with endoscopic features in children who presented with a history of severe abdominal pain. They found that HH gastritis responds well to treatment with H2 blockers, clarithromycin and amoxicillin. In contrast, in the study of Danon *et al*. (10), eradication of HH with a triple combination was not successful in rats.

The results of this study confirmed that SOD plays an important role as an antioxidant against ROMs generated in HP-infected gastric mucosa and, subsequently, in the

maintenance of mucosal cell turnover. Our data also support the potential etiological role of HP in the development of gastric cancer, through the increased inflammatory response and oxidative stress caused by the bacterium.

In conclusion, the current study showed that successful eradication of HP is effective in lowering the severity of CAG in association with a reduction of oxidative stress in gastric mucosa. These results also have implications for research regarding the need for eradication therapy in asymptomatic HP-infected subjects.

It remains to be elucidated whether the pronounced decrease in SOD activity in response to successful eradication is only transient or if initial activity is re-established in the gastric mucosa overtime. Endoscopic follow-up examinations may answer this question.

References

1 Skopelitou AS, Mitselou A, Katsanos KH, Alexopoulou V and Tsianos EV: Immunohistochemical expression of Fhit protein in *Helicobacter pylori* related chronic gastritis, gastric precancerous lesions and gastric carcinoma: correlation with conventional clinicopathologic parameters. Eur J Gastroenterol Hepatol *15*: 515-523, 2003.

2 Correa P: Chemoprevention of gastric cancer: has the time come? J Clin Oncol *21(23s)*: 270s-271s, 2003.

3 Noguchi K, Kato K, Moriya T, Suzuki T, Saito M, Kikuchi T, Yang J, Imatani A, Sekine H, Ohara S, Toyota T, Shimosegawa T and Sasano H: Analysis of cell damage in *Helicobacter pylori*-associated gastritis. Path Intern *52*: 110-118, 2002.

4 Farkas R, Selmeci L, Tulassay Z and Pronai L: Superoxide-dismutase activity of the gastric mucosa in patients with *Helicobacter pylori* infection. Anticancer Res *23*: 4309-4312, 2003.

5 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*: 248-254, 1976.

6 Iacopini F, Consolazio A, Bosco D, Marcheggiano A, Bella A, Pica R, Paoluzi OA, Crispino P, Rivera M, Mottolese M, Nardi F and Paoluzi P: Oxidative damage of the gastric mucosa in *Helicobacter pylori*-positive chronic atrophic and nonatrophic gastritis, before and after eradication. Helicobacter *8*: 503-512, 2003.

7 Felley CP, Pignatelli B, Van Melle GD, Crabtree JE, Stolte M, Diezi J, Corthesy-Theulaz I, Michetti P, Bancel B, Patricot LM, Ohshima H and Felley-Bosco E: Oxidative stress in gastric mucosa of asymptomatic humans infected with *Helicobacter pylori*: effect of bacterial eradication. Helicobacter *7*: 342-348, 2002.

8 Cooksley C, Jenks PJ, Green A, Cockayne A, Logan R and Hardie KR: NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator. J Med Microbiol *52*: 461-469, 2003.

9 Fathalla B, Chan M and Ayas M: Two cases report of *Helicobacter heilmannii* gastritis with endoscopic features resembling *Helicobacter pylori* gastritis. J Pediatr Gastroenterol Nutr *25*: 468, 1997.

10 Danon SJ, Moss ND, Larsson H, Arvidsson S, Ottosson S, Dixon MF and Lee A: Gastrin release and gastric acid secretion in the rat infected with either *Helicobacter felis* or *Helicobacter heilmannii*. J Gastroenterol Hepatol *13*: 95-103, 1998.

Received May 13, 2005
Accepted July 21, 2005