Sperm Parameters and Semen Levels of Inflammatory Cytokines in *Helicobacter pylori*—infected Men

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OBJECTIVE To explore the relationships between seminal interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) with semen parameters, sperm apoptosis, and necrosis in subjects infected by *Helicobacter pylori* (HP) expressing and not expressing CagA.

METHODS In 109 selected patients, HP infection and seminal levels of IL-6 and TNF-α were determined using enzyme-linked immunosorbent assays. Western blotting was used to detect antibodies to CagA. Semen parameters were determined following World Health Organization guidelines and sperm apoptosis and necrosis by annexin V and propidium iodide assay.

RESULTS Twenty-eight subjects were infected by HP (HP+); among them, 12 were CagA seropositive (CagA+) and 16 were negative (CagA−). Eighty-one men were HP seronegative (HP−). Semen TNF-α and IL-6 concentrations were increased in HP+ vs HP− groups (TNF-α: 41 pg/mL vs 27 pg/mL; IL-6: 11 pg/mL vs 5 pg/mL; $P < .01$). In comparison to the HP− group, CagA+ group showed reduced sperm motility (24% vs 32% motile sperm; $P < .05$), enhanced necrosis (33.5% vs 21% necrotic sperm; $P < .05$), and increased cytokines levels (TNF-α: 46 pg/mL vs 27 pg/mL; $P < .01$; IL-6: 17.5 pg/mL vs 5 pg/mL; $P < .01$). Sperm motility of CagA+ group was lower vs CagA− group (24% vs 36.5% motile sperm; $P < .05$). Both IL-6 and TNF-α levels positively correlated with the percentage of necrotic sperm ($P < .001$).

CONCLUSION CagA+ HP infection increases semen levels of inflammatory cytokines, which may reduce sperm motility and determine sperm damage and contribute to reduce the reproductive potential in men. UROLOGY 86: 41−47, 2015. © 2015 Elsevier Inc.

The bacterium *Helicobacter pylori*, a microaerophilic, gram-negative, and spiral-shaped organism, colonizes the stomach and stimulates an intense cellular and immune response (chronic gastritis), which concurs, together with bacterial factors, to cause peptic ulceration in 15%-20% and gastric carcinoma in 1%-3% of infected individuals according to the virulence of the infecting organisms and the geographic areas.[1,2]

The prevalence of *H pylori* infection in dyspeptic patients in Italy is about 40%[3]; outside Italy, in adult population, it ranges from approximately 15%-20% in industrialized countries to >80% in developing areas. The infection is asymptomatic in approximately 50% of cases.[4]

Not all strains show the same virulence; those harboring the chromosomal insertion named *cag* pathogenicity island (*cag*PAI) are endowed with an enhanced inflammatory and carcinogenetic potential.[5] A *cag*PAI gene, *cag*A, encodes for a protein named CagA, which is considered a marker for the presence of *cag* in the bacterial chromosome and which stimulates the production of serum antibodies detectable by serologic tests. In addition to CagA, other bacterial constituents, such as peptidoglycans, are translated into mucocytes where they react with constituents of the natural immunity. An important effect of CagA inside the cells concerns its reaction with the products of tumor suppressor genes such as p53; CagA subverts p53 tumor suppressor pathway and induces a substantial antiapoptotic effect.[6] The clinical importance of infection by strains harboring *cag*PAI, in addition to the production of the oncoprotein CagA, resides in the stimulation of an elevated systemic inflammatory status, which may have consequences on organs other than the stomach.

Numerous studies have shown that the outcome of *H pylori* infection could be putatively associated with extradigestive disorders.[7] Recently, increasing evidences support that *H pylori* infection seems to negatively influence even the reproductive sphere both in women[8,9] and men.[10-12] As regards specifically men, *H pylori*

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infection, particularly when caused by strains expressing CagA, has been proposed as a possible concomitant cause of reduced fertility and altered semen quality, consisting in reduced motility and increased percentage of unviable sperm.[10-12]

The exact mechanism by which *H pylori* may influence the sperm quality is still unknown although some hypotheses involving antigenic mimicry have been proposed.[10,11] To explain the reduced sperm quality in individuals infected by CagA-positive *H pylori* strains, we supposed a concomitant involvement of inflammatory response to the infection. *H pylori* strains bearing *cag*A were found to induce increased local and systemic levels of interleukin 8 (IL-8), IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and a cell inflammatory response in the gastric mucosa compared to the levels of inflammatory mediators generated by infection by CagA-negative strains[13]; at this purpose, we demonstrated that *H pylori*—infected men, especially those with serum antibodies to the CagA protein, had increased systemic levels of TNF-α.[11]

The aim of this study was to verify whether *H pylori* infection could influence the levels of proinflammatory cytokines, such as IL-6 and TNF-α, directly in the seminal plasma. In addition, sperm parameters and sperm apoptosis and necrosis were evaluated in the same individuals.

METHODS

Patients

From January 2013 through September 2013, we selected 109 semen samples from male subjects (aged 25-46 years) attending the Department of Molecular and Developmental Medicine, University of Siena. The inclusion and exclusion criteria for this study consisted in non azoospermic men with a normal 46, XY karyotype evaluated by conventional cytogenetic analysis, body mass index <25 kg/m^2, and no history of diabetes, radiotherapy, chemotherapy, chronic diseases, medication, or autoimmune disorders. All subjects were negative to bacteriological analysis of semen samples and showed normal follicle-stimulating hormone (0.7-11.00 mU/mL), luteinizing hormone (0.8-8.0 mU/L), and testosterone (2.7-10.9 mg/mL) evaluated in serum by chemiluminescence using commercial kit (Beckman Coulter Access for follicle-stimulating hormone, luteinizing hormone, and testosterone; Beckman Coulter S.p.A., Milano, Italy).

A full history was recorded, then clinical and physical examinations (N.F.) and scrotal eco-color Doppler were performed in all patients to detect the possible presence of varicocele. Patients did not suffer from dyspeptic symptoms nor had they taken antibiotics potentially active against *H pylori* in the past 3 months, including proton pump inhibitors. Their *H pylori* infection status was previously unknown.

On the basis of clinical history, physical examination, and routine laboratory analysis, we identified the following possible confounding variables: smoking habit ($\geq$10 cigarettes/day), the presence of varicocele, corrected varicocele (ie, patients that experienced varicocele in the past; all patients had correction of varicocele, and at enrolment, they showed a negative result of eco-color Doppler), and the presence of leukocytospermia ($>10^6$ leukocytes/mL of semen).[14]

Semen specimens were used for the determination of sperm parameters (including apoptosis and necrosis) and for evaluation of IL-6 and TNF-α levels. All enrolled individuals were Italian men and came from similar socioeconomic environment. The individuals provided a written informed consent before the inclusion in this study that was approved by the Ethics Committee of Azienda Ospedaliera Universitaria Senese, CEAOUS.

Determination of *H pylori* Infection

A commercially available enzyme-linked immunosorbent assay with a sensitivity and specificity of approximately 96% (*H pylori* IgG, HpG screen ELISA kit; Genesis Diagnostics Ltd, Littleport, UK) had been used to determine *H pylori* infection. Infection was confirmed by Western blotting, which was also used to detect antibodies to *H pylori* CagA. Briefly, a whole-cell suspension of *H pylori* CCUG 17874 (a CagA-positive and cytotoxic strain) was denatured in Laemmli buffer at 100°C for 5 minutes and electrophoresed in 10% polyacrylamide gel with sodium dodecylsulphate. Resolved proteins were transferred electrophoretically onto nitrocellulose membranes, and free sites were saturated with 3% skim milk in phosphate-buffered saline (PBS) pH 7.4 containing 0.1% Triton X (PMT). Nitrocellulose membranes were cut in strips that were used to perform the test. Antigens immobilized on each strip were immunoblotted with serum samples diluted at 1:100 in PMT. After overnight incubation at room temperature, strips were washed 3 times with PMT, and a peroxidase-labeled antibody to human IgG, diluted in PMT 1:2000 (Sigma Che. Co., Milan, Italy) was added and incubated at room temperature for 90 minutes. Strips were washed 3 times with PMT, once with PBS-Triton X, and twice with 0.05 mol/L pH 6.8 Tris buffer. The reaction was visualized by the addition of the substrate (H_2O_2 in a solution of 4-chloro-1-naphthol in 0.05 M pH 6.8 Tris buffer). The reaction was stopped with water. Anti—*H pylori* whole-cell suspension and anti-CagA rabbit polyclonal antibodies (kindly donated by R. Rappuoli, Novartis, Siena) were used as positive controls.

Semen Analysis

Semen samples were collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 minutes at 37°C. Volume, pH, sperm concentration, and motility were evaluated according to World Health Organization guidelines.[14] Sperm morphology was assessed by the Papanicolaou staining modified for spermatozoa following the World Health Organization guidelines.[14]

Leukocytes were identified by peroxidase stain; leukocytospermia has been defined as a concentration of $>1 \times 10^6$ cells/mL in semen.[14]

Detection of Membrane Phosphatidylserine Externalization and Membrane Integrity Using the Annexin V and Propidium Iodide Assay

The detection of phosphatidylserine (PS) externalization and membrane integrity was performed by Vybrant apoptosis assay (Invitrogen Ltd, Paisley, United Kingdom) based on fluorescein isothiocyanate—annexin V (AnV; green fluorescence) and propidium iodide (PI; red fluorescence). These compounds are able to label dead cells, differentiating apoptosis and necrosis. The translocation of PS, recognized by AnV protein, from the inner to the outer plasma membrane layer, is a critical step in apoptosis; PI enters into necrotic cells with broken membranes.