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

INTRODUCTION

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 50% of cases *ca.*[4]

Not all strains show the same virulence; those harbouring the chromosomal insertion named *cag* pathogenicity island (*cag*PAI) are endowed with an enhanced inflammatory and carcinogenetic potential[5]. A *cag*PAI gene, *cagA*, 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 serological tests. In addition to CagA, other bacterial constituents, such as peptidoglican, 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 tumour suppressor genes such as p53; CagA subverts p53 tumour suppressor pathway and induces a substantial antiapoptotic effect[6]. The clinical importance of infection by strains harbouring *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 upon organs other than the stomach.

Numerous studies have shown that the outcome of *H. pylori* infection could be putatively associated with extra-digestive 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* 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 *cagA* 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.

MATERIALS AND 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/exclusion criteria for this study consisted in non azoospermic men with a normal 46, XY karyotype evaluated by conventional cytogenetic analysis, BMI<25 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 (FSH: 0.7-11.00 mU/ml), luteinizing hormone (LH: 0.8-8.0 mU/L) and testosterone (T: 2.7-10.9 mg/ml) evaluated in serum by chemiluminescence using commercial kit (Beckman Coulter Access for FSH, LH and Testosterone, Beckman Coulter S.p.A., Milano, Italy).

A full history was recorded, then clinical and physical examinations (Prof. NF, MD, PhD) 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 three months, including proton pump inhibitors. Their *H. pylori* infection status was previously unknown.

Based on clinical history, physical examination and routine laboratory analysis, we identified the following possible confounding variables: smoking habit (≥ 10 cigarettes/day), the presence of varicocele, corrected varicocele (*i.e.* patients that experienced varicocele in the past; all patients had correction of varicocele and at the 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 socio economical 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 96% *ca.* (*Helicobacter pylori* IgG, HpG screen ELISA kit, Genesis Diagnostics Ltd, Littleport, UK.) has 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's buffer at 100 °C for 5 min 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 three times with PMT, and a peroxidase labeled antibody to human IgG, diluted in PMT 1:2000 (Sigma Che. Co., Milan) was added and incubated at room temperature for 90 min. Strips were washed three 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 min at 37 °C. Volume, pH, sperm concentration and motility were evaluated

according to WHO guidelines[14]. Sperm morphology was assessed by the Papanicolaou (PAP) staining modified for spermatozoa following the WHO guidelines[14].

Leukocytes were identified by peroxidase stain; leukocytospermia has been defined as a concentration of $>1x10^6$ cells/ ml in semen.[14]

Detection of membrane phosphatidylserine (PS) externalization and membrane integrity using the Annexin V/Propidium iodide assay.

The detection of PS externalization and membrane integrity was performed by Vybrant apoptosis assay (Invitrogen Ltd, Paisley, United Kingdom) based on fluorescein isothiocyanate (FITC)–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, recognised by annexin V protein, from the inner to the outer plasma membrane layer, is a critical step in apoptosis; PI enters into necrotic cells with broken membranes.

Aliquots of 109 semen samples were washed with PBS, centrifuged, suspended in annexin-binding buffer obtaining a cell density of approximately $1x10^6$ sperm/ml, and finally treated following the manufacturer's instructions. A drop of sperm suspension was smeared on glass slide and then mounted in glycerol containing 5% n-propylgallate. Observations and photographs were made with a Leitz Aristoplan light microscope (Leica, Wetzlar, Germany) equipped with a fluorescence apparatus. A minimum of 300 sperm was scored for each sample, identifying intact cells (unstained: AnV negative, PI negative), apoptotic cells (green: AnV positive, PI negative) and necrotic sperm (red: AnV negative, PI positive). Sperm with both green and red signals were considered necrotic since they had a broken plasma membrane.

IL-6 and TNF-α level measurements

For each semen sample, 500 µl of whole semen were recovered 1h after collection and fractioned by centrifugation (1500 rpm for 15 min). The supernatant, composed of seminal plasma without spermatozoa, was immediately stored at -80 °C until analyses were performed.

IL-6 and TNF-α levels were determined by ELISA (Human IL-6 BMS213/2CE BMS213/2TENCE, and TNF-α BMS223/4CE BMS223/4TENCE, Bender MedSystems GmbH, Vienna, Austria) and the results were expressed in pg/ml as reported in the manufacturer's instructions.

Statistical analysis

The data were expressed as median and interquartile range (IQR: 25°-75° centile) or as absolute frequency and percentage (%). The possible associations between confounding variables and/or the presence of *H. pylori* infection and CagA seropositivity were analysed by the chi-square or Fisher's exact test. Comparisons between the *H. pylori* positive, *H. pylori* negative, CagA positive and CagA negative groups were performed using the Mann-Whitney U test.

The Pearson correlation coefficient was calculated for bivariate correlation analysis between values of IL-6 and TNF-α with sperm necrosis. $P<0.05$ (two-tailed) was considered statistically significant. All analyses were performed using the SPSS 16 statistical package for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The demographic and clinical characteristics of studied individuals are reported in Table 1.

Among 109 analyzed cases, 28 men (25.7%) were seropositive for *H. pylori* infection (HP+) while 81 individuals (74.3%) were seronegative and therefore were considered uninfected (HP-). Among the infected subjects, 12 men (42.8%) were seropositive for CagA (CagA+), indicating that they were colonized by strains expressing this marker of increased pathogenicity; 16 infected patients (57.1%) did not show anti-CagA antibodies and they were considered infected by "less virulent" *H. pylori* strains (CagA-).

No associations were found between the confounding variables and *H. pylori* infection or CagA status (respectively: smoking habit P=0.993 and P=0.570; varicocele P=0.196 and P=0.673; corrected varicocele: P=0.450 and P=0.670, leukocytospermia P=0.965 and P=0.718). Therefore, these variables were not considered for statistical calculation.

Median and interquartile ranges of considered variables of the 109 subjects are reported in table 2. Medians of sperm concentration resulted between the 25th and 50th centile[14] in all analyzed groups, whereas the medians of progressive motility percentages were in the 5th centile[14] in HP- and HP+ groups, between the 5th and 10th centile[14] in CagA- group and <2.5th centile in the CagA+ group. Normal sperm morphology percentage was in the 75th centile[14] in HP-, HP+ and CagA- groups and between 50th and 75th[14] in CagA+ group.

In the HP+ group, the semen concentrations of TNF-α and IL-6 were significantly increased respect to HP- group (TNF-α: 41 pg/ml *vs*. 27 pg/ml; IL-6: 11 pg/ml *vs*. 5 pg/ml, P<0.01, Table 2). No differences were observed as far as the other parameters were considered. Respect to the HP- group, the CagA + group showed enhanced levels of inflammatory cytokines (TNF-α : 46 pg/ml *vs*. 27 pg/ml, P<0.01; IL-6: 17.5 pg/ml *vs*. 5 pg/ml, P<0.01, Table 2) concomitant with reduced sperm motility (24% *vs*. 32%, P<0.05) and increased percentage of necrotic sperm (33.5% *vs*. 21%, P<0.05).

In the CagA+ group, sperm motility was significantly reduced with respect to that observed in the CagA- group (24% *vs*. 36.5%, P<0.05, Table 1). No difference was detected in the other considered parameters.

Semen parameters and levels of inflammatory cytokines in the CagA- group were not significantly different from those observed in the HP- group (Table 2).

In order to assess the relationship between cytokine levels and sperm necrosis, correlations have been performed between these values. IL-6 (r=0.52, P<0.001; Figure 1a) and TNF-α (r=0.55, P<0.001; Figure 1b) levels were both positively correlated with the percentage of necrotic sperm.

DISCUSSION

Recent surveys have highlighted the observation that *H. pylori* infection may adversely affect the human reproductive potential in both men and women and that it may exert an unfavorable outcome on sperm quality.[8-12] For many years, our research group has focused on the study of the possible influence of *H. pylori* infection on sperm quality, particularly in relation to the presence of strains expressing CagA. We demonstrated in different researches a relationship between the infection by strains expressing CagA and decreased sperm quality, mainly concerning motility, morphology and viability.[11,12,15] Even in the present research we have confirmed that sperm motility was significantly reduced in individuals infected by CagA positive strains. Concomitantly, the percentage of necrotic sperm, which is closely related to the motility decrement, was increased in this group of patients compared to sperm from uninfected individuals. In our opinion, this observation could have a clinical value since similar results were obtained by our group evaluating sperm necrosis by transmission electron microscopy[11] in the same type of patients.

It is noteworthy that in a very recent paper, El-Garem et al.[16] reported that *H. pylori* treatment significantly improved sperm motility in infertile men with asthenozoospermia and elevated anti-*H. pylori* seminal IgA, supporting our observations.

To explain the relationship between *H. pylori* infection and semen quality, two possible hypotheses have been proposed. First of all, the existence of antigenic mimicry phenomena between CagA and other bacterial antigens and human peptides has been supposed. For instance, the infection may induce an immune response against bacterial cell constituents, which may also react with the host's tissues. At this purpose we demonstrated a close structural homology between *H. pylori* antigens and human beta-tubulin[10] and some enzymes of glycolysis and oxidative metabolism processes that provide sperm with the energy used for motility.[12]

The other plausible mechanism that may explain the association of *H. pylori* infection with extra-digestive diseases, including the reduced sperm quality, involves the inflammatory response to the infection. It is known that HP strains expressing CagA stimulate a local and systemic overproduction of IL-8, IL-1β, IL-6 and TNF-α, respect to the levels of inflammatory mediators stimulated by CagA-negative strain infection.[11,13,17] The main goal of this study was the evaluation of some inflammatory cytokines directly in human semen samples of infected and uninfected individuals in order to verify the illustrated hypothesis. Levels of both cytokines IL-6 and TNF-α were increased in *H. pylori* positive respect to *H. pylori* negative groups, especially if infected men had anti-CagA serum antibodies. These results most probably indicate that the sperm damage observed in *H. pylori* positive individuals, in particular if infected by strains expressing CagA, could be induced by an enhanced systemic inflammatory status, which may also involve the testicular environment and damage spermatozoa. Numerous studies have proved that high cytokine levels may determine an alteration of sperm characteristics.[18-21] In our case, for the first time, we have detected increased amounts of proinflammatory cytokines directly in semen samples during infections by CagA positive *H. pylori* strains, which could realistically be responsible for the reduced sperm quality observed in infected individuals. The proposed mechanism is supported by the observation that both cytokines showed significant a positive correlation with the percentage of necrotic sperm.

Eradication of subjects, especially those infected by *H. pylori* expressing CagA, could be followed by a reduction of necrotic sperm and an increase of motility[16]; since other studies have shown a strong decline of inflammatory cytokine levels in the gastric mucosa samples, as well as systemically[22], there is the possibility that the same event would take place in the seminal fluid.

At this point, we wondered whether cytokines have reached the seminal fluid from the systemic circulation or have been produced in the male genital tracts. The germinal epithelium of the adult testis is excluded, in normal conditions, from the systemic circulation by a protective structure, the blood-testis barrier (BTB), whose primary function is to create the appropriate microenvironment where germ cells can develop, while haploid germ cells are protected from potentially dangerous

cytotoxic molecules and the interaction of autoantigens with interstitial immune cells is prevented. The impermeability of BTB is guaranteed by a high number of tight junctions composed by occludin and other proteins that enable cells to bind to the same molecules on adjacent cells.[23,24] Pérez et al.[25] showed, either in vivo and in vitro experiments, that rat Sertoli cells, in the presence of IL-6, reveal a downregulation of occludin expression; consequently, Sertoli tight junctions may undergo a redistribution of occludin and other structural proteins and a reduced transepithelial electrical resistance. According to our hypothesis, high concentrations of systemic cytokines, as occurs in CagA positive *H. pylori* infection, may damage the Sertoli tight junctions. It is plausible that this damage could even occur in the epididymis, where the tight junctions are much less effective than in the testicular BTB. Once BTB impermeability is interrupted, the circulatory cytokines could spillover into the semen and damage spermatozoa.

There might be another source of cytokines in the testes. It has been shown that in infected patients *H. pylori* antigens diffuse through the inflamed gastric mucosa.[26] After disruption of epithelial cell junctions, the bacteria can pass through the gastric wall facing direct immune response from lymphocytes, mast cells, neutrophils, and dendritic cells.[27] Tests carried out in animals experimentally infected have confirmed that *H. pylori* constituents and substances secreted by the microorganisms may penetrate into the blood vessels and sensitize macrophages and other white blood cells.[28,29] Once sensitized, immune cells may gain access to the portal circulation and the lymphatic system and could eventually reach the gonad district and go over the damaged tight junctions. IL-6, in fact, besides modulating the occludin expression, may increase endothelial permeability through modifications in the distribution of endothelial tight junction proteins, supporting inflammatory cell extravasation.[30] The inflammatory cells might therefore infiltrate the interstitium, where they keep on secreting chemokines, as they have been stimulated along the transit through the stomach.

These hypotheses could possibly explain the observation, made for the first time, that increased levels of some inflammatory cytokines detected in the semen of *H. pylori* infected individuals might be

responsible for the reduced sperm quality commonly observed in these individuals.

In conclusion, the results of the present study may help explain the reason why men infected by CagA positive *H. pylori* organisms present sperm alterations: because they have increased amounts of potentially harmful proinflammatory cytokines in their semen. Should the reported results be confirmed by conclusions of other investigations, we believe that physicians should consider the eventuality of routinely testing men, who suffer from idiopathic fertility disorders, for *H. pylori* infection and the CagA status. This is important not only for the fertility problem, but also because *H. pylori* should be eradicated since it can cause different pathologies

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Figure captions

Figure 1. Scatter plots of sperm necrosis percentages and IL-6 (a) or TNF-α (b) levels determined in the semen of 109 subjects.