

Research Article

CagA-Positive *Helicobacter pylori* Infection and Reduced Sperm Motility, Vitality, and Normal Morphology

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Helicobacter pylori (HP) infection, particularly when caused by strains expressing CagA, may be considered a concomitant cause of male and female reduced fertility. This study explored, in 87 HP-infected males, the relationship between infection by CagA-positive HP strains and sperm parameters. HP infection and CagA status were determined by ELISA and Western blotting; semen analysis was performed following WHO guidelines. The amino acid sequence of human enzymes involved in glycolysis and oxidative metabolism were “blasted” with peptides expressed by HP J99. Thirty-seven patients (42.5%) were seropositive for CagA. Sperm motility (18% versus 32%; $P < 0.01$), sperm vitality (35% versus 48%; $P < 0.01$) and the percentage of sperm with normal forms (18% versus 22%; $P < 0.05$) in the CagA-positive group were significantly reduced versus those in the CagA-negative group. All the considered enzymes showed partial linear homology with HP peptides, but four enzymes aligned with four different segments of the same *cag* island protein. We hypothesize a relationship between infection by strains expressing CagA and decreased sperm quality. Potentially increased systemic levels of inflammatory cytokines that occur in infection by CagA-positive strains and autoimmune phenomena that involve molecular mimicry could explain the pathogenetic mechanism of alterations observed.

1. Introduction

Helicobacter pylori (HP), a Gram-negative bacterium that colonizes the stomach, is the main etiologic factor in the development of gastritis, peptic ulcer disease, and malignant gastric lesions [1, 2]. The clinical consequences of HP infection are not limited to the gastroduodenal tract since many studies reported that it could contribute to cause extradiigestive disorders [3] and to play a role in the development of autoimmune diseases [4]. Recently, rising evidence has indicated a relationship between HP infection and reduced fertility, in both men and women. The first observation on this topic was made by some of our group [5] who reported that the infection is significantly more common in both men and women with fertility problems; in semen, follicular fluid, and vaginal secretions of infected individuals, we detected specific antibodies, which cross-reacted *in vitro* with spermatozoa, suggesting the existence of an autoimmune phenomenon.

The amino acid alignment of the human tubulin, a constituent of sperm axoneme, with HP peptides indicated a possible antigenic mimicry with the cytotoxin-associated gene A protein (CagA) and other HP antigens. CagA is expressed by HP strains endowed with enhanced inflammatory potential and the infection by CagA-positive strains is significantly associated with gastric and extragastric diseases such as cardiovascular disorders and autoimmune thyroid diseases [6]. CagA is expressed by one of the 30 genes enclosed in the pathogenicity island *cag* and it is a marker of the presence of such an insertion in the bacterial chromosome. Most strains harboring *cag* also express the vacuolating toxin VacA, which contributes to the aggressiveness of the strains.

Later on, in a Japanese study [7], HP infection was reported to be twice as prevalent in women with idiopathic procreation problems, compared to women with known causes of fertility disorders. Lastly, an Italian team have recently found that *in vitro* sperm motility through cervical

mucus of infected women was significantly reduced, concluding that anti-HP IgG antibodies could possibly hamper the meeting of sperm with the oocyte [8].

The previous observation, made in a small number of patients, that HP infection was associated with reduced sperm motility [9, 10] prompted us to carry out the present study, in which we explored the role of CagA in decreasing sperm motility in a large male population.

To substantiate whether antigenic mimicry mechanisms may influence the sperm motility, we aligned the amino acid sequence of some enzymes involved in energy production, with peptides expressed by *Helicobacter pylori* J99 (taxId: 85963) strain possessing the pathogenicity island *cag*. Should a homology exist, the immune response against HP infection could also react with human cells with possible harmful consequences.

2. Materials and Methods

2.1. Subjects. From January 2010 to May 2013, we enrolled 97 males (aged 18–45 years) with HP infection attending the Department of Medical and Surgical Sciences and Neurosciences for dyspepsia. Patients did not take antibiotics potentially active against HP, including proton pump inhibitors, in the past three months. Their HP infectious status was previously unknown. The inclusion criteria encompassed also BMI <25 kg/m², no history of diabetes, radiotherapy, chemotherapy, chronic illnesses, medication, or autoimmune disorders. The subjects showed normal follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) evaluated in serum by chemiluminescence using commercial kit (Beckman Coulter Access for FSH, LH, and Testosterone, Beckman Coulter S.p.A., Milano, Italy). Normal ranges were 0.7–11.00 mUI/mL for FSH (sensitivity 0.2 mUI/mL, intra- and interassay coefficient of variation <10%), 0.8–8.0 mUI/L for LH (sensitivity 0.2 mUI/mL, intra- and interassay coefficient of variation <10% and 2.7–10.9 ng/mL for T (sensitivity 0.1 ng/mL, intra- and inter-assay coefficient of variation <10%).

Clinical and physical examinations and scrotal Eco-color Doppler were performed on all patients to exclude the possible presence of varicocele. Testis size appeared in the normal range in all patients.

All subjects gave their permission to collect blood samples in order to investigate their HP infectious status and semen samples for determination of semen parameters and for microbiological investigations; they signed a written informed consent for this research, and the local Ethical Committee has approved the study.

After semen analysis, 10 individuals were excluded from the study: 3 subjects (2 CagA negative and 1 CagA positive) resulted azoospermic; 6 other patients (2 CagA negative and 4 CagA positive) had semen culture positive for *Escherichia coli* (3 men), *Enterococcus faecalis* (2 men), and *Staphylococcus epidermidis* (1 man); in addition, one CagA-negative patient showed the sperm defect of possible genetic origin named “dysplasia of the fibrous sheath,” characterized by short, thick tails with a peculiar dysplastic fibrous sheath, as detected

by transmission electron microscopy, which severely compromises sperm motility [11]. The azoospermic subjects and the patient with the sperm showing “dysplasia of the fibrous sheath” were excluded from the study after three different semen analyses which confirmed the first diagnosis. The study was then performed on a group of 87 subjects.

2.2. Determination of *H. pylori* Infection. HP infectious status was determined in serum using a commercially available enzyme-linked immunosorbent assay with a sensitivity and specificity of around 96% (*Helicobacter pylori* IgG, HpG screen ELISA kit, Genesis Diagnostics Ltd, Littleport, UK). Infection was confirmed by Western blotting (WB). WB 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). Strips were then cut and immunoblotted with serum samples diluted at 1:100 in PMT for immunoglobulin G (IgG). 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 addition of the substrate (H₂O₂ in a solution of 4-chloro-1-naphthol in 0.05 M pH 6.8 Tris buffer). The reaction was stopped with water. Anti-HP whole cell suspension and anti-CagA rabbit polyclonal antibodies (kindly donated by R. Rappuoli, Novartis, Siena) were used as positive controls.

2.3. 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, and sperm concentration and motility were evaluated according to WHO guidelines [12]. Sperm morphology was assessed by the Papanicolaou (PAP) staining modified for spermatozoa following the WHO guidelines [12]. Sperm vitality was assessed in semen samples showing a progressive motility <40%: 37 specimens of CagA-positive group, 41 of CagA-negative one. The specimens were stained with 0.5% eosin Y (CI 45380) in a 0.9% aqueous sodium chloride solution. A few minutes after staining, the samples were observed by light microscope, and the stained (dead) cells and unstained (living) cells were scored.

2.4. Alignment. The amino acid sequence of some of human enzymes involved in the energy production was “blasted” in the protein databases at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov/>) [13], with proteins of *Helicobacter pylori* J99 (taxId: 85963). Sequences longer than five amino acids were also included, even if the alignment was interrupted by

one or two nonmatching amino acids. Five amino acids in a row is the minimum number to form an epitope, also called antigenic determinant, that is, the specific region on an antigen that an antibody recognizes and binds to. We consider also sequences longer than five amino acids because the longer the amino acid sequence, the higher the probability that two proteins are similar and antibodies may also be able to bind the native protein.

The aligned enzymes were as follows.

Glycolysis. Hexokinase, phosphoglucose isomerase, phosphofruktokinase, aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

Pyruvate Dehydrogenase

Krebs Cycle. Citrate synthase, aconitase, isocitrate dehydrogenase, α -ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase.

Oxidative Phosphorylation. NADH-coenzyme Q oxidoreductase, succinate-Q oxidoreductase, electron transfer flavoprotein-Q oxidoreductase, Q-cytochrome c oxidoreductase, cytochrome c oxidase, and ATP synthase.

2.5. Statistical Analysis. All groups of quantitative variables were checked for normal distribution by the Kolmogorov-Smirnov test, and the *F* test was used to compare variances between the groups.

Since the conditions of normality of distribution and of homogeneity of variance were not satisfied, comparisons between the variables from HP CagA-positive and HP CagA-negative groups were performed using the Mann-Whitney *U* test. The variables were expressed as median and minimum and maximum values. Statistical analysis was performed using the StatGraphicsPlus (version 5.0) statistical package. $P < 0.05$ was considered significant.

3. Results and Discussion

To investigate the role of CagA in reduced semen quality, we have analyzed semen samples from a group of men with HP-infection, establishing their CagA status. The characteristics of the 87 HP infected subjects, grouped following CagA status, are shown in Table I. Thirty-seven HP-infected patients (42.53%) were positive for CagA.

Medians of semen volume and sperm concentrations were comprised between 25th centile and 50th centile in both groups, the median of progressive sperm motility percentage was under 2.5th centile in the group of CagA-positive patients and at 5th centile in those negative for CagA, the median of normal forms was between 50th centile and 75th centile in both groups, and finally the medians of vitality were <2.5th centile [12].

Applying statistics, we obtained that sperm motility and vitality in the CagA-positive group were significantly lower than those observed in the CagA-negative group (motility:

18% versus 32%; vitality: 35% versus 48%; $P < 0.01$). Using such a numerous group of infected patients, we confirmed that the infection by HP strains expressing CagA may exert a negative effect on human sperm motility and vitality. These results are concordant with previous observations that in infertile male seropositive for CagA, the frequency of sperm death, expressed as apoptosis and necrosis, was increased with respect to that detected in infected men seronegative for CagA or uninfected [9]. Another interesting result emerged from this analysis: the percentage of sperm with normal forms appeared to be significantly lower in the CagA-positive group with respect to that observed in CagA-negative one (18% versus 22%; $P < 0.05$). This result can be explained by the increase of unviable sperm with broken plasma membrane (Eosin-stained) in CagA-positive group: the acrosome, the most fragile organelle of the spermatozoon, is reacted or absent in necrotic sperm [14]. In addition, the manchette, the structure able to model the sperm shape during spermatogenesis, is constituted by microtubules, and it is known that human tubulin, the major constituent of this structure, shows homologies with some HP proteins, suggesting the phenomenon of antigenic mimicry [5].

The other analyzed variables such as pH, volume, and sperm concentration were similar in both groups (pH: $P = 0.875$, volume $P = 0.594$ and sperm concentration $P = 0.718$). Also similar, between the two groups, were the levels of analyzed hormones, such as FSH (CagA positive: 4.30 mUI/mL (1.80 mUI/mL–10.10 mUI/mL); CagA negative: 3.80 mUI/mL (1.70 mUI/mL–11 mUI/mL)), LH (CagA positive: 2.50 mUI/mL (1.20 mUI/mL–6.50 mUI/mL); CagA negative: 2.60 mUI/mL (1 mUI/mL–6.70 mUI/mL)) and T (CagA positive: 5.60 ng/mL (2.93 ng/mL–9.86 ng/mL); CagA negative: 5.47 ng/mL (2.97 ng/mL–9.98 ng/mL)). To the best of our knowledge no clues on this topic are present in the literature; a recent research reported that infection may regulate the seminal levels of ghrelin, a hormone endowed with anti-inflammatory properties [15] and involved in reproduction [16], suggesting that the infection may influence the semen concentration of peptides involved in the regulation of testicular function [17].

The exact mechanism by which HP may influence sperm quality is still unknown, although some hypotheses involving antigenic mimicry have been proposed [5, 9]. For instance, infected individuals may mount a humoral and cellular immune response to bacterial constituents and products that might cross-react with epithelial cells of various organs. (For a comprehensive review on HP infection and autoimmunity, consult [18].) In a previous study, we showed that antibodies raised in rabbit against HP, as well as serum samples from HP-infected patients, recognized immunologically human sperm, suggesting the existence of an antigenic mimicry between HP antigens and sperm peptides [5]. As far as the autoimmune cell response is concerned, there are lines of evidences of a cross-reactivity of CD4+ Th1 cells, infiltrating the gastric epithelium in response to the infection, with epitopes of the gastric K+/H+ ATPase, the proton pump, with deleterious consequences for the gastric mucosa [19]. These observations prompted us to align aminoacidic sequence of HP with some enzymes involved in glycolysis and oxidative

TABLE 1: Medians (minimum–maximum) of pH, volume, sperm concentration, percentages of motility, normal morphology, and vitality in patients infected by HP strains expressing CagA and in patients infected by CagA-negative HP strains.

HP patients	pH	Volume (mL)	Sperm/mL × 10 ⁶	Progressive sperm motility %	Normal forms %	[§] Vitality %
CagA positive <i>n</i> = 37	7.3 (7.2–7.6)	3 (1–8)	58 (1–287.50)	18** (2–30)	18* (7–34)	35** (10–59)
CagA negative <i>n</i> = 50	7.3 (7.2–7.6)	3 (1–7)	63 (1–232)	32 (15–64)	22 (8–36)	48 (32–65)

***P* < 0.01; **P* < 0.05; [§]sperm vitality was calculated in samples with progressive sperm motility <40%; number of CagA positive: 37; number of CagA negative: 41.

Aldolase

Query 7 LTPEQKKEL 15
LTPE KK L
Sbjct 637 LTPEAKKKL 645

Phosphoglycerate mutase

Query 132 YNYSISKERRYAGLKPGE LPTCESLKD TIARALPFWNEEIVPQIKAGKRV LIAAHGNSLR 191
Y + +S+ R E CE L AR F ++ + KA K L A N
Sbjct 1244 YLDCVSRARN-----EKEKQECEKLLTPEAR--KFLEKQRQKDKAIKDCLKNADPNDR A 1296
Query 192 GIVKHLEGMSDQ 203
I+K L+G+SD+
Sbjct 1297 AIMKCLDGLSDE 1308

Pyruvate kinase

Query 117 YRPVAIALDTKGPEIR TGILQGGPESEVELVKGSQVLVTVDPAFRTRGNANTVWVDYPNI 176
Y P+ I L +K TGI+ G +V + G+ +L +D + GN +V P +
Sbjct 1587 YTPIEITLTSKVDATLTGIVSGVVAKDVWNMNGTMIL--LDKGTKVYGNYSVKGKGTPI M 1644
Query 177 VRVVPVGGRIYIDDGLI 193
R++ V + DG+I
Sbjct 1645 TRLMIVFTKAITPDGVI 1661

Succinate dehydrogenase

Query 539 ISKLYGDLKHLKTFD 553
+ ++Y DL+ KTFD
Sbjct 1411 VDRIYSDLR SRKTFD 1425

FIGURE 1: Alignment of four human enzymes with different segments of the same *cag* protein of *Helicobacter pylori* J99 (taxId: 85963) (sequence ID: ref. NP_223194.1). Explanatory information—“Query”: amino acid sequence of human peptides. “Subject”: amino acid sequence of the bacterial proteins. Numbers in the sequence represent the initial and final positions of the amino acid. “+” indicates that the aligned amino acids are different, although, from an antigenic point of view, they are equivalent.

metabolism processes, since both pathways are involved in providing energy for sperm movement [20, 21].

All the considered enzymes showed partial linear homology with HP peptides; four enzymes aligned with four different segments of the same *cag* protein (Table 2, Figure 1).

In addition, NADH dehydrogenase (ubiquinone) Fe-S protein 1 showed partial linear homology with a different *cag* island protein, sequence ID: ref. NP_223213.1. Finally, aldolase partially aligned with vacuolating cytotoxin (VacA), sequence ID: ref. NP_222995.1.

The observation that four different enzymes (Table 1) showed partial structural homology with different segments of the same *cag* island protein could suggest the existence of sperm damage mediated by antigenic mimicry mechanisms. Of course, the considered enzymes are part of ubiquitous processes shared by all kinds of cells, so the damage caused by autoimmune reaction cannot be severe. On the other hand we have to consider that the HP infection lasts for the patient's entire life (if not treated) and that the immune reactions occurring in patients infected by CagA positive HP strains,

TABLE 2: Linear homologies of different human enzymes with *cag* island protein of *Helicobacter pylori* J99 (taxId: 85963) (sequence ID: ref. NP_223194.1).

Enzyme	Expected value	Identity (%)	Positivity (%)	N. gaps (%)
Aldolase	0.4	78	77	0
Phosphoglycerate mutase	2.4	31	44	9
Pyruvate kinase	0.061	29	48	2
Succinate dehydrogenase	2.1	26	47	12

Expected value: significance of aligned amino acid sequences; identity %: percentage of identical amino acids in the two compared sequences; positivity %: percentage of amino acids that share the same behaviour from a chemical or an antigenic point of view; N. gaps %: number of intervals lacking linear homology between two homologue sequences.

which are endowed with enhanced inflammatory potential, could concur to determine a more severe damage.

Another plausible concomitant explanation for the observed reduced sperm quality, in terms of vitality, motility, and normal morphology, could consist in the inflammatory response to the infection. HP strains bearing *cagA* were found to induce increased local and systemic levels of interleukin-8 (IL-8), IL-1 β , IL-6, tumor necrosis factor- α (TNF- α), and inflammation in the gastric mucosa, compared to the levels of inflammatory mediators generated by infection by CagA-negative strains [22–24]; to this purpose we demonstrated that HP infected men, especially those with serum antibodies to the CagA protein, have increased systemic levels of TNF- α , a proinflammatory cytokine that may cause sperm damage [9]. We are working on measuring cytokines levels directly in seminal plasma of men infected by HP expressing/nonexpressing CagA.

Since we hypothesized mechanisms such as antigenic mimicry and inflammation, it is important to discuss how and where the sperm damage may occur. The blood-testis barrier (BTB) protects haploid germ cells against immune attack; however, it is possible that in case of increased level of interleukins (as in case of infection by CagA-positive HP) the BTB might be damaged; it has been recently reported that IL-6 might be involved in the downregulation of expression of occludin, a protein found in the tight junctions constituting the BTB, and in the modulation of BTB permeability that occurs in rats undergoing autoimmune orchitis [25]. Indeed, it is not excluded that the damage could occur in the epididymis, where the tight junctions are much less effective than in the testis [26], or directly in the semen.

4. Conclusions

We definitely demonstrated, in a quite large population of HP-infected males, a relationship between the presence of strains expressing CagA and decreased sperm quality, especially concerning motility and viability. We propose two possible explanations, not mutually exclusive, for the obtained results:

- (1) the existence of an autoimmune phenomenon that involves molecular mimicry,
- (2) an enhanced inflammatory response causing an increased cytokine production.

In our opinion, there are enough indications that the chronic infection by HP expressing CagA could contribute to the damage of organs other than the gastroduodenal tract and to negatively influence many functions [3], including reproduction. If other studies will confirm our findings, treatment of *H. pylori* infection is advisable. We tend, however, to prescribe the appropriate eradication therapy independently of the putative impact of infection upon the reproductive sphere; our opinion is that there is no advantage for infected people to harbour an organism that causes chronic gastritis in all, peptic ulceration in 20% ca. and gastric cancer in 3% ca. of them.

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