OPEN

Influence of *H. pylori* CagA Coupled with Alcohol Consumption on Cytokine Profiles in Men

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Abstract: The aim of this study was to evaluate the effect of *Helicobacter pylori* (*H pylori*) cytotoxin-associated gene A (CagA) coupled with chronic alcohol ingestion on cytokine profiles.

A total of 215 male subjects were divided into the following 4 groups: 130 alcohol *H pylori* CagA-negative consumers (CagA−) (group A), 50 alcohol *H pylori* CagA-positive consumers (CagA+) (group B), 24 nonalcohol *H pylori* CagA-negative consumers (group C), and 11 non-alcohol *H pylori* CagA-positive consumers (group D). The serum CagA, C-reactive protein (CRP), interleukin (IL)-6, IL-10, E-selectin, adiponectin (ADP), and tumor necrosis factor-α (TNF-α) levels were measured through enzyme-linked immunosorbent assays (ELISAs).

After adjusting for age and mean alcohol drinking history, a multivariable linear regression analysis revealed that the mean daily alcohol consumption, IL-6, TNF-α, and ADP levels were significantly increased with increases in the serum CagA concentrations ($P = 0.008$, $P = 0.000$, $P = 0.000$, and $P = 0.006$, respectively). The serum IL-6 and IL-10 levels of group A were significantly lower than those of group B (all $P = 0.000$). Furthermore, the serum IL-6 and IL-10 levels of groups A and C were significantly lower than those of group D (all $P = 0.000$), and the serum IL-6 and IL-10 levels of group C were significantly lower than those of group B (all $P = 0.000$). The serum ADP and E-selectin levels of groups B and D were significantly higher than those of group A ($P = 0.000$). The serum ADP levels of group B were significantly higher than those of group C ($P = 0.000$), and the serum ADP and E-selectin levels of group C were significantly lower than those of group D ($P = 0.000$ and $P = 0.005$, respectively). Finally, the serum TNF-α levels of groups B, C, and D were significantly higher than those of group A (all $P = 0.000$), and the serum TNF-α levels of group C were significantly higher than those of group D ($P = 0.005$).

In conclusion, *H pylori* CagA may result in significantly higher levels of several inflammatory markers in both alcohol consumers and non-alcohol consumers. However, chronic alcohol ingestion coupled with *H pylori* CagA positivity does not result in significant changes in cytokine profiles.

Abbreviations: ADP = adiponectin, ANOVA = one-way analysis of variance, CagA− = CagA-negative, CagA = cytotoxin-associated gene A, CagA+ = CagA-positive, CIMT = carotid intima-media thickness, CRP = C-reactive protein, CSX = cardiac syndrome X, DU = duodenal ulcer, ELISAs = enzyme-linked immunosorbent assays, ERK1/2 = extracellular signal-regulated kinase, GC = gastric cancer, GERD = gastroesophageal reflux disease, *H pylori* = *Helicobacter pylori*, IL-10 = interleukin-10, IL-17 = interleukin-17, IL-6 = interleukin-6, IL-8 = interleukin-8, LPS = lipopolysaccharide, NF-κb = nuclear factor-κB, PHG = portal hypertensive gastropathy, STAT3 = signal transducer and activator of transcription 3, TLRs = toll-like receptors, TNF-α = tumor necrosis factor-α, VacA = vacuolating cytotoxin.

INTRODUCTION

*H*elicobacter pylori (*H pylori*) is a human pathogen that colonizes the stomach epithelium.[1] Under normal circumstances, the host is unable to clear the infection, which may lead to life-long chronic inflammation.[2] According to previous studies, *H pylori* is associated with gastric diseases such as peptic ulceration and gastric adenocarcinoma and with exogastric diseases, such as cardiovascular and cerebrovascular diseases and type 2 diabetes mellitus. Cytotoxin-associated gene A (CagA) seropositivity in particular is associated with increased risks of certain diseases involving the lower stomach and duodenum and decreased risks of gastroesophageal reflux disease (GERD) and its sequelae.[3] According to 1 study, gastric cancer (GC) patients have higher *H pylori* and CagA seroprevalence than the matched controls, confirming that CagA-positive (CagA+) *H pylori* infection is associated with a higher risk of GC.[4] Additionally, CagA+ *H pylori* strains may contribute to the pathogenesis of early atherosclerosis by aggravating immunoinflammatory reactions.[5] Coronary artery disease is also significantly associated with *H pylori* infection, particularly with strains expressing CagA proteins.[6] In addition, CagA+ *H pylori* strains may act as a trigger and play a role in the pathogenesis of cardiac syndrome X (CSX) via chronic inflammation.[7] Conversely, colonization with CagA+ *H pylori* is not an independent risk factor for severe coronary heart disease.[8] Therefore, the relationships of CagA+ and CagA− strains with their hosts and with diseases remain unknown. Generally, chronic alcohol consumption is associated with enhanced susceptibility to both systemic and mucosal infections. Different quantities of alcohol consumption have different effects on

the human body. A previous study showed that modest alcohol intake, from 10 to 20 g of ethanol per day, may have decreased cardiovascular mortality.[9] Another study revealed that a significant decrease in carotid intima-media thickness (CIMT) occurred with increased alcohol consumption of daily intake levels of up to 80 g in men.[10] A recent study revealed that heavy and binge alcohol consumption is an important risk factor related to an increasing incidence of gastric cancer in a population not infected with *H pylori*.[11] Additionally, as shown in several studies, chronic inflammation-associated cytokine profiles are present during the development of certain diseases. However, the associations between CagA+ *H pylori* coupled with alcohol intake and inflammation remain controversial. Therefore, the objective of the present study was to explore the effect of *H pylori* CagA coupled with chronic alcohol ingestion on cytokine profiles by measuring the serum levels of inflammatory and anti-inflammatory cytokines.

MATERIALS AND METHODS

Ethics Statement

This protocol was approved by the Clinical Research Ethics Committee of Taishan Hospital of Shandong Province, and written informed consent was obtained from all of the enrollees.

Subject Selection

A total of 215 male subjects undergoing health examination were enrolled between January 2012 and May 2015. All of the subjects were evaluated based on whether they chronically ingested alcohol by a questionnaire on alcohol consumption. *H pylori* CagA positivity was diagnosed based on the serum *H pylori* CagA levels at Taishan Hospital, Shandong Province. The subjects enrolled in this study included 180 subjects that chronically ingested alcohol and 35 control subjects. The diagnosis of chronic alcohol ingestion was defined as a daily ethanol intake >40 g in men for a period >5 years.[12] Additionally, a serum *H pylori* CagA level of at least 80 pg/mL was considered positive (CagA+), whereas a *H pylori* CagA level >80 pg/mL was considered negative (CagA−). The subjects were divided into the 4 following groups according to whether they exhibited *H pylori* CagA positivity: 130 alcohol *H pylori* CagA-negative consumers (group A), mean age of 46.84 ± 7.08 years, mean alcohol drinking history of 5.77 ± 1.81 years, and mean daily alcohol consumption of 66.23 ± 21.68 g; 50 alcohol *H pylori* CagA-positive consumers (group B), mean age of 46.23 ± 7.04 years, mean alcohol drinking history of 5.63 ± 1.59 years, and mean daily alcohol consumption of 67.38 ± 19.58 g; 24 nonalcohol *H pylori* CagA-negative consumers (group C), mean age of 48.04 ± 6.53 years; and 11 nonalcohol *H pylori* CagA-positive consumers (group D), mean age of 46.00 ± 5.64 years. A cross-sectional study was conducted to evaluate the correlations between *H pylori* CagA and the profiles of cytokines associated with alcohol consumption based on the serum levels of *H pylori* CagA, C-reactive protein (CRP), interleukin (IL)-6, IL-10, adiponectin (ADP), E-selectin, and tumor necrosis factor-α (TNF-α) measured through enzyme-linked immunosorbent assays (ELISAs).

The exclusion criteria included the following: smoking, fever, infectious diseases, anti-inflammatory drugs, antibiotics, medication for killing *H pylori,* and suffering from other diseases.

Experimental Setup and Reagents

The experiment equipment included an Enzyme Standard Instrument (Type ANTHOS 2010, Austria), and the reagents used in this study included *H pylori* CagA, CRP, IL-6, IL-10, ADP, E-selectin, and TNF-α ELISA kits (Shanghai Enzyme-Linked Immune Co. Ltd, produced by R&D Systems).

Blood Collection and Handling

On the same day as the general health examination, peripheral venous blood samples were collected after overnight fasting for at least 10 hours.[13] For measurements of serum *H pylori* CagA, CRP, IL-6, IL-10, ADP, E-selectin, and TNF-α, the samples were collected into ice-cold tubes containing EDTA (1 mg/mL) and centrifuged at 3000 rpm/minute for 10 minutes. The plasma was then stored at −70°C until being assayed. To ensure the reliability of the experimental results, all of the serum samples were carefully preserved and not subjected to repeated freeze-thaw cycles. Finally, all of the parameters were measured by ELISA according to the manufacturer's instructions.

Statistical Analysis

The SPSS statistical package (version 19.0 for Windows; SPSS Inc., Chicago, IL) was used for all of the statistical analyses. For the 4 groups, the normality of the distribution and the homogeneity of variances were assessed using the Kolmogorov test before the statistical analysis. To allow for covariance and confounders, multivariable linear regression analysis was performed to investigate the associations of the serum CagA concentrations with cytokine profiles after adjusting for age, mean alcohol drinking history, and mean daily alcohol consumption. If the variables followed a normal distribution, the variables are expressed using the means ± standard deviations. One-way analysis of variance (ANOVA) was used to analyze multiple sample means, and the Bonferroni post-hoc test was used to analyze differences between 2 groups. However, if variables without a normal distribution were expressed as medians and ranges, Kruskal–Wallis ANOVA was used for comparisons among the 4 groups, and the Mann–Whitney U test was used for comparisons between 2 groups. Differences with $P < 0.05$ were considered statistically significant. However, when the Bonferroni post-hoc test was used, statistical significance was accepted at $P \leq 0.008$.

RESULTS

No differences in age were found among the 4 groups or between 2 groups, and no differences in the mean alcohol drinking history and mean daily alcohol consumption were found between group A and group B (all $P > 0.05$). After adjusting for age and the mean alcohol drinking history, a multivariable linear regression analysis showed that the mean daily alcohol consumption and the IL-6, TNF-α, and ADP levels were significantly increased with increases in the serum CagA concentration ($P = 0.008$, $P = 0.000$, $P = 0.000$, and $P = 0.006$, respectively), but the CRP, IL-10, and E-selectin levels did not significantly change with changes in the serum CagA concentration (all $P > 0.05$).

The serum CRP, IL-6, and IL-10 concentrations in the 4 groups are listed in Table 1. The differences in the serum IL-6 and IL-10 levels were significant (H = 69.53, $P = 0.000$, and H = 84.560, $P = 0.000$, respectively). The serum IL-6 and IL-10 levels in group A were significantly lower than those in group B (all $P = 0.000$). Furthermore, the serum IL-6 and IL-10 levels in

TABLE 1. Comparison of Serum CRP, IL-6, and IL-10 Levels Among the 3 Groups (ng/L, Medians With Ranges)

Groups	N	CRP	IL-6	IL-10
A	130	3.16 (2.13−8.22)	12.40 (7.00−91.00)[*,†]	319.55 (14.00−1112.00)[§,‖]
B	50	3.26 (2.23−29.94)	31.50 (9.00−109.00)[‡]	699.05 (10.00−2921.00)[¶]
C	24	2.87 (2.18−7.55)	14.00 (7.00−47.00)[†]	224.10 (126.00−825.00)[‖]
D	11	2.28 (2.18−9.63)	51.10 (21.00−100.00)	642.90 (45.00−1773.00)
H		7.247	69.53	84.560
P		0.064	0.000	0.000

CRP = C-reactive protein, IL-6 = interleukin-6, IL-10 = interleukin-10.
[*] $P = 0.000$ (IL-6: group A vs group B).
[†] $P = 0.000$ (IL-6: groups A and C vs group D).
[‡] $P = 0.000$ (IL-6: group B vs group C).
[§] $P = 0.000$ (IL-10: group A vs group B).
[‖] $P = 0.000$ (IL-10: group A and C vs group D).
[¶] $P = 0.000$ (IL-10: group B vs group C).

groups A and C were significantly lower than those in group D (all $P = 0.000$), and the serum IL-6 and IL-10 levels in group C were significantly lower than those in group B (all $P = 0.000$). However, no differences in the serum CRP levels were found among the 4 groups or between 2 groups (all $P > 0.008$).

The serum ADP, E-selectin, and TNF-α concentrations in the 4 groups are listed in Table 2. The differences in the serum ADP, E-selectin, and TNF-α levels were significant (H = 74.50, $P = 0.000$; H = 44.81, $P = 0.000$; and H = 57.22, $P = 0.000$, respectively). The serum ADP and E-selectin levels in groups B and D were significantly higher than those in group A ($P = 0.000$). Additionally, the serum ADP level in group B was significantly higher than that in group C ($P = 0.000$), and the serum ADP and E-selectin levels in group C were significantly lower than those in group D ($P = 0.000$ and $P = 0.005$, respectively). Finally, the serum TNF-α levels in groups B, C, and D were significantly higher than those in group A (all $P = 0.000$), and the serum TNF-α level in group C was significantly higher than that in group D ($P = 0.005$).

DISCUSSION

H pylori may induce strong mucosal inflammation and local and systemic immune responses, which manifest as significant changes in both inflammatory cytokines, including TNF-α,[14] E-selectin,[14] IL-8,[14] and IL-6,[14−16] and anti-inflammatory cytokines, such as IL-10,[17,18] as well as IL-17 and IL-23.[19] For example, infection of gastric epithelial cells by *H pylori* stimulates the activation of nuclear factor-κB (NF-κB) and the upregulation of interleukin-8 (IL-8) expression.[20] Moreover, both IL-6 and IL-11 are strongly upregulated in gastric cancer biopsies, and it has been shown that IL-6 may increase STAT3 and ERK1/2 activation in *H pylori*-dependent gastritis. Moreover, IL-11 expression is associated with adenocarcinoma development.[15] These lines of evidence suggest that IL-6 I and L-11 may serve as potent inducers of human gastric cancer. Additionally, *H pylori* CagA may activate the STAT3 signaling pathway in vitro and in vivo,[21] suggesting that chronic *H pylori* infection may promote the development of gastric cancer. However, in a contradictory study, *H pylori* did not cause changes in the systemic secretion of certain cytokines.[18,22] Therefore, the relationship between *H pylori* and inflammation remains unclear. Nevertheless, *H pylori* CagA is known to play a critical role in various diseases via changes in inflammatory cytokines. According to a previous study, the *H pylori* CagA+ strain may induce severe gastritis,[23] and the CagA pathogenicity island provides a growth advantage to *H pylori* strains and is associated with an increased inflammatory response at the gastric mucosal level.[24] In duodenal ulcer (DU) patients, the serum IL-12 but not IL-13 concentrations were influenced by

TABLE 2. Comparison of Serum ADP, E-Selectin, and TNF-α Levels Among the 3 Groups (ng/L, Medians With Ranges)

Groups	N	ADP	E-Selectin	TNF-α
A	130	1192.00 (96.00−7489.00)	25.40 (4.00−194.00)	212.20 (29.00−1518.00)
B	50	2491.00 (860.00−10412.00)[*,†]	72.70 (6.00−265.00)[§]	601.80 (1.00−2078.00)[¶]
C	24	1107.00 (110.00−4398.00)[‡]	75.00 (15.00−265.00)[‖]	350.60 (169.00−1284.00)[¶,#]
D	11	3032.00 (1582.00−6354.00)[*]	108.95 (35.00−213.00)[§]	973.50 (427.00−1676.00)[¶]
H		74.50	44.81	57.22
P		0.000	0.000	0.000

ADP = adiponectin, TNF-α = tumor necrosis factor-α.
[*] $P = 0.000$ (ADP: groups B and D vs group A).
[†] $P = 0.000$ (ADP: group B vs group C).
[‡] $P = 0.000$ (ADP: group C vs group D).
[§] $P = 0.000$ (E-selectin: groups B and D vs group A).
[‖] $P = 0.005$ (E-selectin: group C vs group D).
[¶] $P = 0.000$ (TNF-α: groups B, C, and D vs group A).
[#] $P = 0.005$ (TNF-α: group C vs group D).

bacterial CagA, independent of the vacuolating cytotoxin (VacA) status, suggesting that high IL-12 levels may contribute to the susceptibility to DU in CagA+ individuals.[25] Furthermore, high IL-18 levels in CagA+ subjects predispose these individuals to susceptibility to digestive ulcers.[26] And, serum IL-8[27,28] and nitric oxide (NO)[29] levels are significantly correlated with CagA+ *H pylori* strain infection. A recent study indicated that the upregulation of miR-155 and miR-146b decreases. In addition, *H pylori* CagA+ (HpcagA+) results in IL-6 overexpression, which may weaken the eradication of HpcagA+ and contribute to ulcer development.[4] Moreover, the significantly increased activation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK1/2) in *H pylori*-dependent gastritis was found to be further enhanced in the presence of CagA+ *H pylori* strains.[15] As a result, the combined detection of the serum IL-8, NO, and *H pylori* CagA levels will contribute to the early diagnosis of precancerous lesions in the stomach.[30] In the present study, after adjusting for age and the mean alcohol drinking history, a multivariable linear regression analysis showed that the serum CagA concentrations are associated with the mean daily alcohol consumption and the levels of certain inflammatory cytokines, such as IL-6, TNF-α, and ADP, supporting the viewpoint that CagA is involved in the inflammatory response. Simultaneously, our present subgroups study have determined that in *H pylori* CagA+ subjects without chronic alcohol ingestion, the levels of several inflammatory markers such as IL-6, ADP, E-selectin and TNF-α, and anti-inflammatory markers such as IL-10 were significantly increased compared with their levels in patients of the control group, who were neither chronic alcohol consumers nor *H pylori* CagA+. Thus, *H pylori* CagA may enhance the levels of inflammatory and anti-inflammatory markers, consequently causing an inflammatory response. However, contradictory results suggest that CagA is not required for the *H pylori*-induced activation of NF-κB and upregulation of IL-8 expression in gastric epithelial cells[21] and that host inflammatory responses in the gastric mucosa are not correlated with the expression of the babA2, cagA, and vacAs1 genes.[31] To date, the conclusions reached by studies on the relationship between *H pylori* CagA and the profile of inflammatory cytokines are incongruous and thus require further investigations.

Changes in the cytokine balance are responsible for several of the systemic and hepatic manifestations of alcoholism. Ethyl alcohol consumption in humans and experimental animals is also associated with an increased incidence and severity of infections, which is attributed to the immunosuppression that is associated with ethyl alcohol consumption.[29] Several studies have demonstrated that alcohol consumption may result in certain cytokine alterations such as altered IL-6, IL-8, IL-10, IL-12, and TNF-α levels[32,33] as well as ICAM-1 and E-selectin levels.[34] BMI is the only predictor of high-sensitivity CRP levels and TNF-α levels because obesity induces increases in certain serum inflammatory markers.[35] Additionally, alcohol consumption increases insulin sensitivity, and obesity-related alterations in insulin sensitivity are coupled with alterations in inflammatory genes. Alcohol may specifically improve insulin sensitivity by increasing the expression of anti-inflammatory genes.[36] Studies have demonstrated that the sources and functions of cytokines, including cytokines abnormally secreted by natural killer cells from patients with chronic alcoholism, depend on both the existence of liver disease and the status of ethyl alcohol intake.[37] Chronic ethanol exposure can improve the responsiveness of lipopolysaccharide (LPS)-stimulated macrophage IL-6 and TNF-α production, indicating that this effect may result from ethanol-induced alterations in intracellular signaling through nuclear factor (NF)-κB.[38] Toll-like receptors (TLRs) also play an important role in the innate immune response and link the innate and adaptive responses. Thus, the elimination of TLR4 abolishes the effects of ethanol on the innate and adaptive inflammatory responses induced by ethanol treatment in macrophages.[39] Although a few studies have examined the interaction between *H pylori* infection and chronic alcohol ingestion and found that alcohol consumption appears to be associated with *H pylori* infection,[40] alcohol consumption, particularly wine consumption, may reduce the risk[41,42] and facilitate the elimination of *H pylori* infection,[43] suggesting a protective mechanism of adequate alcohol consumption that is mediated by ''adaptive cytoprotection.'' However, studies on the association between ethanol ingestion and both *H pylori* CagA and cytokine profiles are lacking. According to our previous study, *H pylori* infection may result in significant changes in the cytokine profiles of the inflammatory marker E-selectin and the anti-inflammatory marker IL-10 in chronic alcohol consumers because of the interaction between *H pylori* infection and chronically ingested alcohol.[13] Our present study demonstrates that *H pylori* CagA is correlated with IL-6, IL-10, ADP, E-selectin, and TNF-α levels in chronic alcohol consumers. Furthermore, regardless of chronic alcohol ingestion, the levels of inflammatory markers such as CRP, IL-6, E-selectin, ADP, E-selectin and TNF-α and the anti-inflammatory marker IL-10 were significantly higher in *H pylori* CagA+ subjects than in *H pylori* CagA-subjects. However, a comparison of *H pylori* CagA+ subjects with and without chronic alcohol ingestion and *H pylori* CagA-subjects with and without chronic alcohol ingestion demonstrated that their cytokine profiles did not differ. Therefore, chronic alcohol ingestion does not result in significant changes in the cytokine profiles of subjects who are *H pylori* CagA+. The combination of the results described in the literature and the findings of the present study may lead to the conclusion that although the consumption of alcohol may appear to reduce *H pylori* infection, it does not significantly reduce the inflammatory response.

In conclusion, *H pylori* CagA may result in significantly higher levels of several inflammatory markers in subjects with and without chronic alcohol consumption. However, chronic alcohol consumption does not result in significant changes in the cytokine profiles of subjects who are *H pylori* CagA+. The effect of chronic alcohol consumption on serum cytokine levels requires additional studies with larger sample sizes.

Our study has several limitations. First, the sample size used in this study was small, and the sample was biased in that it only included male subjects. Second, the potential impact of other factors, such as overweight, other drugs, and potentially influential variables, was not controlled. Third, our conclusions are only based on a prospective observational study, which has certain limitations, rather than on a completely randomized controlled study. Finally, the effects of different types and amounts of alcohol in *H pylori* CagA+ subjects were not examined in our study.

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