Zinc chloride inhibits *Helicobacter pylori* growth and reduces expression of interleukin-1β by gastric epithelial cells

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Helicobacter pylori is a Gram-negative bacterium that colonises the gastric mucosa in half of the world's human population.[1] It is associated with gastritis, peptic ulcer disease and development of gastric carcinoma and primary B-cell gastric lymphoma.[2] *H. pylori* infection almost always causes inflammation of the gastric mucosa, the distribution and severity of which varies widely and affects the clinical outcome. Host genetic factors (in conjunction with bacterial and/or environmental factors) determine the immune and inflammatory responses to *H. pylori* infection.

Interleukin (IL)-1β is upregulated in the presence of *H. pylori* and is important in initiating and amplifying the inflammatory response to *H. pylori* infection.[3] A pluripotent pro-inflammatory cytokine, IL-1β has a central role in the pathogenesis of *H. pylori*-induced mucosal inflammation, and IL-1β gene expression and protein production are increased in *H. pylori* infection and reduce following successful eradication.[4–6]

H. pylori infection-associated gastric mucosal damage is mainly caused by increased reactive oxygen species (ROS) and consequent oxidative stress.[7–9] *H. pylori*-induced gastric mucosal inflammation is characterised by reduction of copper/zinc superoxide dismutase (SOD).[10] It has been shown that after *H. pylori* eradication, the gastric mucosal content of copper/zinc SOD returns to normal levels.[10,11]

Zinc is an essential trace element for the immune system. It is an abundant intracellular micronutrient that participates in diverse cellular processes.[12] Zinc deficiency is accompanied by an immunodeficiency, resulting in a predisposition to infection. However, the immune function is delicately regulated by zinc, as both increased and decreased zinc levels result in a disturbed immune function.

In vitro cell culture systems have been used extensively to study the interactions between *H pylori* and the human gastric epithelium. The co-culture of the human gastric cancer cell line AGS and different strains of *H. pylori* has been used as a model for bacterial-epithelial interactions.

The aim of this study is to determine the effect of ZnCl on *in vitro* culture of *H. pylori* and expression of IL-1β by gastric epithelial cell lines (AGS, ATCC CRL-1739) in response to *H. pylori* following treatment with ZnCl.

Twenty-four clinical isolates of *H. pylori* were used for this study, obtained between January and May 2012. The *H. pylori* reference strains ATCC 49503 was used as a control. Columbia blood agar (Oxoid) medium supplemented with 10% defibrinated sheep blood and Dents supplement (containing vancomycin, trimethoprim and polymyxin) was incubated at 37°C under microaerophilic conditions produced by strips in an anaerobic jar (Oxoid, UK) for three to five days.

Plates were examined for bacterial growth and typical colonies were selected for identification. The identity of *H. pylori* was confirmed by Gram stain and also by the production of urease and catalase. *H. pylori* isolates were defined as Gram-negative spiral-shaped bacilli that were catalase-positive and rapidly (<30 min) urease-positive. *H. pylori* ATCC 43504 and ATCC 43526 were used as *cagA*-positive controls, and ATCC 51932 as the *cagA*-negative control. Clinical isolates positive for cytotoxin-associated gene A and E (*cagA* and *cagE*) (GenBank accession numbers JN232379, JN232378 and JN232377) were used in the study.

Zinc chloride salt solution was prepared immediately before use and filtered prior to addition to the culture medium. In order to determination minimum inhibitory concentration (MIC) of ZnCl, a dilution range of 5, 10, 20 and 40 μg/mL was used. The inoculum used contained 10^9 colony-forming units (cfu) adjusted to 0.5 McFarland and the plate was incubated at 37°C in a microaerophilic environment for five to seven days. Susceptibility to ZnCl was determined by a viable colony count and bismuth subsalicylate (BSS) was used as a control. Plates were examined for *H. pylori*

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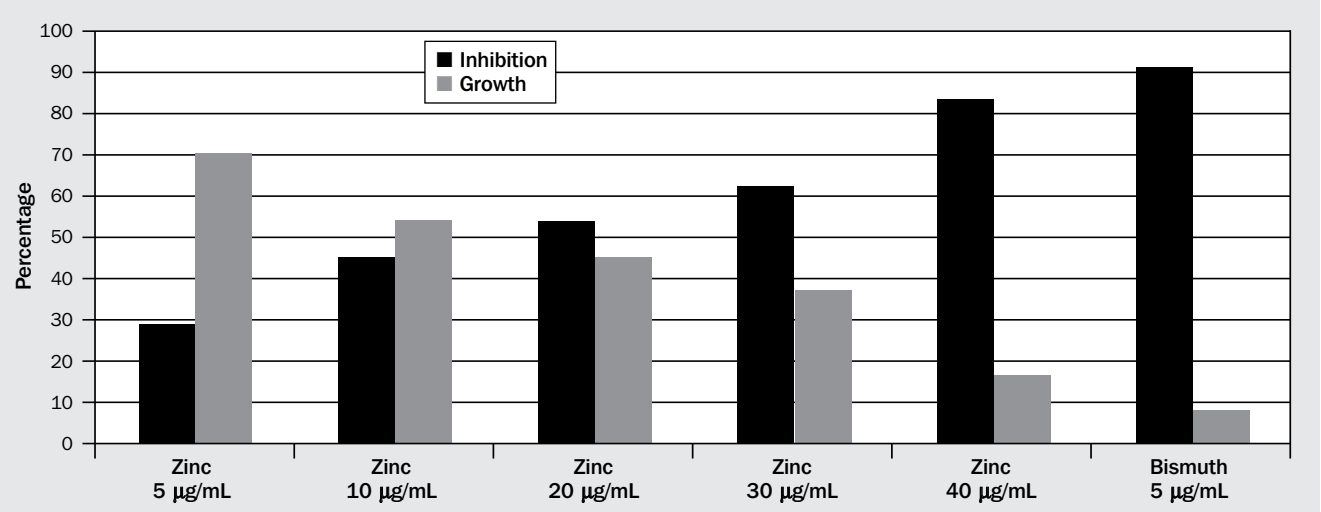

Fig. 1. *H. pylori* growth in response to exposure to zinc chloride and bismuth subsalicylate concentration.

Table 1. Interleukin-1β induction in AGS cells by clinical isolates of *H. pylori*.

H. pylori strain	Diagnosis	CagA	CagE	IL-1β (pg/mL)					
				AGS	AGS + *H. pylori*	*H. pylori* + Zn (5 μg/mL)	*H. pylori* + Zn (40 μg/mL)	AGS + bismuth	AGS + bismuth + *H. pylori*
PK2164593	Duodenal ulcer	+	+	2.74	2.94	3.06	2.94	2.73	2.98
PK2134140	Duodenal ulcer	+	+	2.73	2.98	3.02	1.18	2.74	2.62
PK2134141	Duodenal ulcer	+	+	2.73	3.14	3.14	1.85	2.74	2.98
PK70212	Duodenal ulcer	+	+	2.72	2.78	2.94	2.94	2.73	2.94
PK120312	Duodenal ulcer	+	+	2.71	2.90	3.49	1.18	2.71	3.06
PK160312	Duodenal ulcer	+	+	2.73	1.06	3.06	1.85	2.72	2.98
PK270312	Gastritis	+	+	2.74	2.94	3.06	2.94	2.73	2.98
PK240412	Gastritis	+	+	2.73	2.98	3.02	1.18	2.74	2.62
PK210412	Gastritis	+	+	2.73	3.14	3.14	1.85	2.74	2.98
PK120412	Gastritis	+	+	2.72	2.78	2.94	2.94	2.73	2.94
PK130412	Gastritis	+	+	2.71	2.90	1.49	1.18	2.71	3.06
PK170312	Gastritis	+	+	2.73	3.06	3.06	1.85	2.72	2.98
PK180312	Gastritis	+	+	2.74	2.94	3.06	2.94	2.73	2.98
PK10212	Gastritis	+	+	2.73	2.98	3.02	1.18	2.74	2.62
PK110212	Gastritis	+	+	2.73	3.14	3.14	1.85	2.74	2.98
PK120212	Gastritis	+	+	2.72	2.78	2.94	2.94	2.73	2.94
PK160212	Gastritis	+	+	2.71	2.90	3.49	1.18	2.71	3.06
PK90212	Gastritis	+	+	2.73	1.06	3.06	1.85	2.72	2.98
PK80412	Gastritis	+	+	2.74	2.94	3.06	2.94	2.73	2.98
PK110412	Gastritis	+	+	2.73	2.98	3.02	1.18	2.74	2.62
PK10412	Gastritis	+	+	2.73	3.14	3.14	1.85	2.73	2.98
PK90412	Gastritis	+	+	2.72	2.78	2.94	.94	2.74	2.94
PK160412	Gastritis	+	+	2.71	2.90	3.49	1.18	2.74	3.06
PK170412	Gastritis	+	+	2.73	3.06	3.06	1.85	2.73	2.98

and the procedure was repeated for cultures defined as resistant. The control consisted of *H. pylori* ATCC 49503 incubated without ZnCl and BSS for five to seven days.

The human gastric adenocarcinoma epithelial cell line (AGS; ATCC CRL 1739) was obtained from American Type Cell Culture (Philadelphia, USA). Cells were grown in monolayer culture in RPMI 1640 (Sigma) medium supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 10% fetal bovine serum (Invitrogen). Cells were grown in 75 cm^2 tissue culture flasks at 37°C in an atmosphere of 5% CO_2 and 95% air and were passaged every three to five days. Cell culture supernatants were harvested and analysed for cytokines by an enzyme-linked immunosorbent assay (ELISA) technique using a commercially available kit (human IL-1β, BD OptEIA). The lower limit of detection for the assay was 2 pg/mL

Cells were pretreated with ZnCl in two different concentrations and then infected with clinical isolates of *H. pylori* for six hours. The supernatant was collected and stored at –80°C before analysis. The level of IL-1β in the supernatant from the AGS cells was determined using an ELISA kit (BD), following the manufacturer's instructions.

The Statistical Package for Social Science (SPSS, release 19) was used for data analysis. Results were presented as mean±standard deviation (SD) for quantitative variables and number (%) for qualitative variables. $P<0.05$ was considered statistically significant (all values two-sided). Non-parametric, Mann Whitney and Kruskal-Wallis tests were used to compare the effect of different concentrations of ZnCl and BSS on the expression of IL-1β by AGS cell.

Twenty (83%) *H. pylori* strains were susceptible to ZnCl (40 μg/mL) and four (17%) were resistant, while 22 (92%) were susceptible to BSS (20 μg/mL) and two (4%) were resistant. In comparison, 95% (19/20) isolates were susceptible to ZnCl (40 μg/mL) while 92% (22/24) were susceptible to BSS ($P=0.321$) and did not exhibit any growth in culture (Fig. 1).

IL-1β secretion by the AGS cell varied from 2.71–2.74 pg/mL, with a mean of 2.72 pg/mL (Fig. 1, Table 1). *H. pylori* strains increased secretion of IL-1β by the AGS cell line ($P<0.0001$; Fig. 2). The expression of IL-1β from AGS cells was not inhibited by pretreatment with ZnCl (5 μg/mL). *In vitro* interaction of AGS cells and ZnCl in the presence of *H. pylori* led to significant secretion of IL-1β ($P= 0.012$; Fig. 3). When ZnCl concentration was increased to 40 μg/mL it increased secretion of IL-1β ($P=0.0003$; Fig. 3). Comparison of AGS cell with *H. pylori* and exposure to 5 μg/mL and 40 μg/mL ZnCl demonstrated a progressive increase in IL-1β ($P=0.0004$; Fig. 3).

Bismuth subsalicylate had no effect on IL-1β secretion by AGS cells compared to *H. pylori* strains ($P=0.494$). Secretion

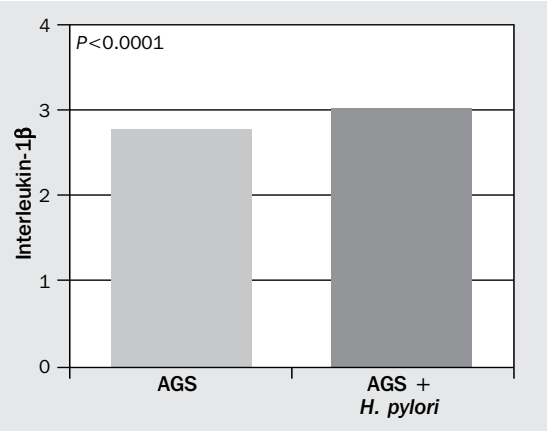

Fig. 2. AGS cell secretion of IL-1β in the presence or absence of *H. pylori*.

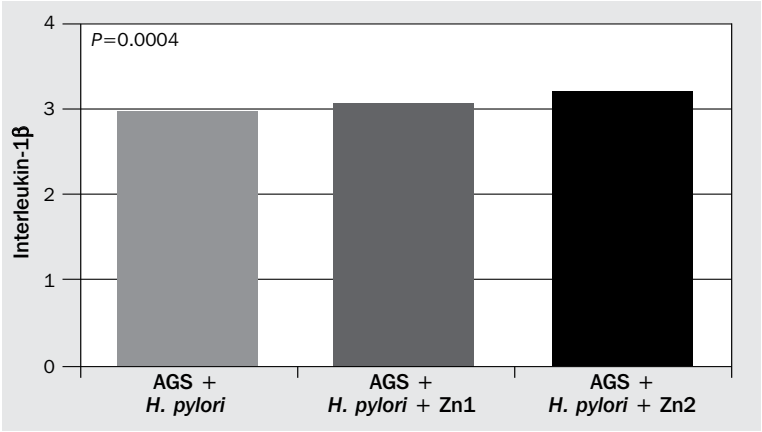

Fig. 3. AGS cell secretion of IL-1β in the presence of *H. pylori* and zinc at 5 μg/mL (Zn1) or 40 μg/mL (Zn2).

of IL-1β from AGS cells in response to *H. pylori* was not inhibited by BSS at 5 μg/mL (P=0.874).

This study has shown that *H. pylori* clinical isolates are comparable in their susceptibility to ZnCl and BSS. The majority of *H. pylori* isolates cultured from patients with non-ulcer dyspepsia and peptic ulcer were found to be susceptible to ZnCl, as were those associated with chronic gastritis and chronic active gastritis.

The secretion of IL-1β by AGS cells was increased by the presence of *H. pylori* strains (Fig. 2). As ZnCl concentration increased, it augmented the IL-1β secretion by the AGS cells (Fig. 3). In comparison, BSS demonstrated no effect on IL-1β secretion by the AGS cells and also had no effect on *H. pylori*-stimulated secretion of IL-1β.

The present study suggests that ZnCl augmented the pro-inflammatory reaction by AGS cells, as demonstrated by the increasing level of IL-1β against *H. pylori* infection *in vitro*. However, the work did not include study of IL-1β messenger RNA expression or other cytokines (e.g., IL-8) that have been reported to be increased with IL-1β in previous studies.[13] Several have shown the benefits of zinc supplementation in infectious diseases.[14,15]

It is known that cellular homeostasis tightly controls zinc levels to modulate the immune response.[16] It is possible that IL-1β and other cytokines produced by gastric epithelial cells in response to *H. pylori* or other inflammatory agents play a role as autocrine or paracrine mediators of growth.

While this study suggests that ZnCl promotes a T helper 1 (Th1) reaction by stimulating IL-1β secretion by gastric epithelial cells in response to *H. pylori* infection, further work is in progress to study the expression of other cytokines in response to ZnCl.

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