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Helicobacter pylori

Effect of NaCl and *Helicobacter pylori* vacuolating cytotoxin on cytokine expression and viability

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Abstract

AIM: To determine whether *Helicobacter pylori* (*H pylori*) vacuolating cytotoxin (VacA) regulates release of pro-inflammatory cytokines (IL-1β, IL-8, TNF-α, and IL-6) or alters gastric epithelial cell viability and to determine whether NaCl affects these VacA-induced changes.

METHODS: Vacuolating activity was determined by measuring the uptake of neutral red into vacuoles of VacA-treated human gastric epithelial (AGS) cells. AGS cell viability was assessed by direct cell counting. Specific enzyme-linked immunosorbent assays (ELISA) and reverse transcriptase-polymerase chain reaction(RT-PCR) were performed to examine the effects of *H pylori* VacA and NaCl on cell pro-inflammatory cytokine production in AGS cells. Immunohistochemical staining of gastric tissue from Mongolian gerbils was used to confirm VacA-induced pro-inflammatory cytokine production and the effects of NaCl on this VacA-induced response.

RESULTS: Addition of VacA alone reduced AGS cell viability ($P < 0.05$), and this reduction was enhanced by high doses of NaCl ($P < 0.05$). VacA alone induced expression of TNF-α, IL-8 and IL-1β, while NaCl alone induced expression of TNF-α and IL-1β. Changes in mRNA levels in the presence of both VacA and NaCl were more complicated. For the case of TNF-α, expression was dose-dependent on NaCl. IL-6 mRNA was not detected. However, low levels of IL-6 were detected by ELISA. Positive immunohistochemical staining of IL-1, IL-6, and TNF-α was found in gastric tissue of *H pylori*-infected gerbils fed with either a normal diet or a high salt diet. However, the staining of these three cytokines was stronger in *H pylori*-infected animals fed with a 5g/kg NaCl diet.

CONCLUSION: VacA decreases the viability of AGS cells, and this effect can be enhanced by NaCl. NaCl also affects the production of pro-inflammatory cytokines induced by VacA, suggesting that NaCl plays an important role in *H pylori*-induced gastric epithelial cell cytotoxicity.

Key words: *Helicobacter pylori*; Vacuolating cytotoxin; Cytokine; Gerbil; AGS cell

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death[1, 2]. Epidemiological studies in humans and rodents have demonstrated that chronic gastritis caused by *Helicobacter pylori* (*H pylori*) is a strong risk factor for this malignancy[3]. Current models suggest that *H pylori* is not directly carcinogenic but rather acts indirectly on inducing cancer. For example, it may act through inflammatory mediators or by decreasing gastric acid secretion, thus favoring the formation of mutagenic adducts[4]. *H pylori* expresses several major proteins that are critical to the pathogenicity of the bacterium[5]. The vacuolating cytotoxin, VacA, is an *H pylori*-secreted virulence factor that induces the formation of large cytoplasmic vacuoles in epithelial cells, which precedes cultured cell death both *in vitro* and *in vivo*[6-9]. In the past decade, a considerable number of studies have contributed to our understanding of the mechanism of VacA-induced intracellular vacuolation[6].

Salt consumption is considered a risk factor for gastric cancer[10]. *H pylori*-associated mucosal atrophy is partially associated with a high salt diet[11]. Excessive NaCl intake enhances *H pylori* colonization in mice and humans, while chronic salt intake may exacerbate gastritis by increasing *H pylori* colonization[12]. Furthermore, salt intake may interfere with *H pylori* infection and modify the cancer risk[13]. The high prevalence of *H pylori* infection and high salt diets may significantly affect the rate of occurrence of gas-

tric carcinogenesis[14-17].

It has been reported that cytokines play an important role in gastric cancer. Proinflammatory cytokines such as interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) may up regulate cyclooxygenase-2 (COX-2) during gastric carcinogenesis[18]. Genotypes of IL-1β (-511 T/T) and TNF-α (-308 A/A) are associated with the risk of non-cardia gastric cancer. The presence of TNF-α SNPs -308 and -1031 might favor *H pylori* infection and promote an inflammatory response in infected gastric mucosa[19]. IL-1β genotype has been found to increase the risk of distal gastric cancer[20]. IL-1β is one of the potent proinflammatory cytokines elicited by *H pylori* infection[21, 22]. In human gastric cancer cells, IL-1β induces vascular endothelial growth factor (VEGF), a dominant angiogenic factor in gastric cancer[23]. Moreover, levels of IL-1β and IL-8 have been found to be significantly higher in *H pylori*-positive gastric cancers compared with controls, and eradication of *H pylori* can significantly reduce the levels of these cytokines[24]. Genetic polymorphisms identified in *IL-6* can be attributed to ethnicity and appear to be independent of the clinical outcome of *H pylori* infections[25].

The present study was to examine the combined effects of VacA and NaCl on cell viability and pro-inflammatory cytokine expression both in AGS cells and in Mongolian gerbils.

MATERIALS AND METHODS

Preparation of VacA

The toxin-producing strain, *H pylori* ATCC49503, was used as the source of VacA. *H pylori* was grown under microaerophilic conditions on Columbia blood agar plates with vigorous shaking in a controlled microaerophilic atmosphere of 50 mL/L O_2 and 100 mL/L CO_2 at 37℃ for 3-4 d, harvested and re-suspended in brucella broth (Difco) supplemented with 100 mL/L fetal calf serum. *H pylori* cells were grown for 48 h at 37℃ in an Erlenmeyer flask with shaking at 60 r/min. Cells were then pelleted by centrifugation at 500 r/min for 20 min. The culture supernatants were collected and pooled. Solid ammonium sulfate was added to the pooled material at 4℃ to 50% saturation. The precipitate was collected by centrifugation at 12000 r/min for 20 min, dissolved in 60 mmol/L Tris-HCl (pH 7.7) containing 0.1 mol/L NaCl and subjected to liquid chromatography on a Superose 6 HR 10/30 column (1cm × 30 cm) equilibrated with 60 mmol/L Tris-HCl (pH 7.7) containing 0.1 mol/L NaCl. The material was eluted with the same buffer at a flow rate of 0.5 mL/min. The eluted protein was monitored by Western blotting using polyclonal anti-VacA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The fractions containing VacA were collected, pooled, diluted with deionized water to the intended concentration and maintained at -20℃[26]. VacA concentration was determined by Micro-BCA assay (Bio-Rad, Hercules, CA).

Vacuolating activity assay

The human gastric epithelial cell line AGS (ATCC CRL 1739) was maintained in Ham's F-12 medium supplemented with 100 mL/L fetal calf serum and 50 mg/L penicillin-streptomycin (Life Technologies, Inc.) under 50mL/L CO_2 at 37℃. AGS cells were seeded in 96-well culture plates (5×10^3 cells/well in 90 μL) and cultured as monolayers for 20 h in a 50 mL/L CO_2 atmosphere at 37℃. Samples (10 μL) of VacA were added to achieve the indicated final concentrations and cells were incubated for an additional 2-8 h at 37℃[27]. Vacuolating activity was determined by measuring the uptake of neutral red into vacuoles in VacA-treated cells. Cells were incubated for 5 min at room temperature with 50 μL of freshly prepared 0.5 g/L neutral red in PBS containing 3 g/L BSA and washed three times with 0.1 mL of PBS containing 3 g/L BSA. After addition of 0.1 mL 700 mL/L ethanol in water containing 0.4 mL/L HCl, absorbance at 540 nm (A_{540}) was measured. Vacuolating activity was determined by subtracting the A_{540} of cells incubated without VacA from the A_{540} of VacA-treated cells. To evaluate the effect of NaCl on vacuolating activity, AGS cells were incubated with 40 mg/L VacA and 0-10 mmol/L NaCl for the indicated periods.

Assessment of AGS cell viability

AGS cells were seeded to a subconfluent density of 5×10^4 cells/well in 24-well plates and incubated at 37℃ overnight. The supernatant was discarded before co-incubation. AGS cells were grown with NaCl alone or in the presence of VacA and NaCl in F-12 medium supplemented with 100 mL/L FBS. Control cells were inoculated into F-12 and incubated for up to 72 h in triplicate. At the end of each time of incubation, cell viability was determined in a hemacytometer by trypan blue exclusion.

Cytokine and cytokine mRNA measurement

After AGS cells were incubated for 24 h. *H pylori* VacA (40 mg/L) and 10 mmol/L NaCl were added. The cells were incubated for an additional 4 h. The supernatants were then collected and stored at -20℃ until assay. The levels of IL-6, IL-8, IL-1β and TNF-α in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Immunotech, France) according to the manufacturer's instructions. In these assays, the lower limits of detection were 3 ng/L for IL-6, 8 ng/L for IL-8, 1.5 ng/L for IL-1β and 5 ng/L for TNF-α.

Total RNA was extracted from AGS cells (incubated as indicated above, under cytokine measurement) using Isogen (Nippon Gene, Tokyo, Japan). Aliquots (2.5 μg) of total RNA were incubated at 70℃ for 5 min, chilled on ice and reverse transcribed in a final volume of 10 μL of a solution containing 200 MU/L Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen Life Technologies), first-strand buffer (250 mmol/L Tris-HCl, pH 8.3 at room temperature, 375 mmol/L KCl, 15 mmol/L $MgCl_2$) containing 0.1 mol/L DTT and 2.5 mmol/L dNTPs plus random primer (6 mer) pd(N)6 (TaKaRa, Japan). Reactions were incubated at 22℃ for 10 min, at 37℃ for 60 min, heated to 80℃ for 5 min, and stored at -20℃ until use.

The resultant cDNA from above (1 μL) was added to a 29-μL reaction containing PCR reaction buffer, 1 μL of 4 nmol of each primer (IL-1 β, IL-6, IL-8, TNF-α),

Table 1 Viability of AGS cells grown in the presence of NaCl or VacA and NaCl (*P* value, *t*-test)

VacA (mg/L)	NaCl (mmol/L)	24 h	48 h	72 h
(-)				
	0	-	-	-
	2.5	0.12	0.07	0.22
	5	0.04	0.09	0.18
	10	0.06	0.11	0.15
50				
	0	0.03	0	0.01
	2.5	0.02	0	0.01
	5	0.01	0.02	0
	10	0	0	0
50				
	0	-	-	-
	2.5	0.45	0.36	0.13
	5	0.27	0.08	0.01
	10	0.12	0.01	0.04

0.1 μL of 5 MU/L of Taq DNA polymerase and water. Oligonucleotide primers were designed based on previous reports[28]. PCR was performed with an automatic thermal cycler using an initial denaturation step at 95 ℃ for 5 min followed by 36 cycles of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and an extension at 72 ℃ for 1 min. The final cycle included an extension for 7 min at 72 ℃ to ensure full extension of the products. Aliquots (5 μL) of each PCR product were analyzed by electrophoresis on 1.5 g/L agarose S gels (Wako Chemical Co., Ltd., Osaka, Japan) containing ethidium bromide, and the bands were examined under UV light to detect amplified DNAs.

Animal experiment

H pylori-infected and non-infected 7-wk-old male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan) were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12 h light-12 h dark cycle. The animals were classified into four groups according to *H pylori* infection and NaCl diet ($n = 3$). *H pylori*-infected and non-infected gerbils were given autoclaved distilled water *ad libitum* and CE-2 irrigated with 50 g/kg NaCl (Kyudo Co. LTD, Japan) or CE-2 only (normal diet). After 4 mo, the gerbils were sacrificed and stomach tissue was obtained and frozen.

The frozen tissue blocks were sectioned (6 μm thick) as previously described[28] and the sections were fixed with 4 g/L paraformaldehyde and acetone for 10 min each. The sections were treated with 30 mL/L H_2O_2 (Wako, Japan) in methanol for 5 min followed by blocking with 10 ml/L normal rabbit serum (Vector Laboratories, Inc., Burlingame, CA) for 10 min. We used goat polyclonal antibodies as primary antibodies directed against the following mouse proteins: TNF-α, IL-1β, and IL-6 (R & D Systems, Inc.). Tissue sections were incubated for 60 min at room temperature in a moist chamber with the desired primary antibody diluted according to the manufacturer's instructions. Biotinylated rabbit anti-goat immunoglobulin G (Vector Laboratories) was then applied for 30 min at room temperature. After three times of 5-min rinse with PBS, the signals from the antibodies were amplified using an ABC kit (Vector Laboratories) for 30 min and visualized with DAB reagent (Vector Laboratories) for 5 min. The sections were counterstained with Mayer hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), dehydrated and mounted using histological mounting medium (Fisher Scientific). Negative control reactions contained purified goat IgG (Vector Laboratories) instead of specific primary antibodies.

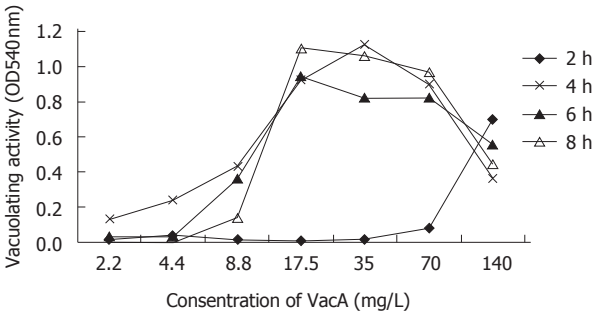

Figure 1 Activity of VacA-induced vacuolization.

Statistical analysis

All data in each experiment were expressed as the mean ± SE. The statistical significance of the response of cytokine production to VacA was evaluated using the Student's *t*-test. Differences between cytokine levels were considered significant at $P < 0.05$.

RESULTS

VacA visualization

VacA, partially-purified from a toxin-producing *H pylori* strain, was visualized on a Western blot with a VacA-specific antibody (Table 1). An antibody-reactive protein at 87 ku, the molecular weight expected for the mature toxin[26] was found.

VacA-induced vacuolation in AGS cells

AGS cells showed dose-dependent vacuolation induced by VacA (Figure 1). Different VacA concentrations were shown for the indicated incubation times. Vacuolization was quantified by measuring the uptake of neutral red into vacuoles. The experiments were carried out in triplicate. When VacA was incubated with AGS cells at 4, 6 and 8 h, its activity was stable in the range of 17-70 mg/L. Vacuolation was not seen in AGS cells in response to VacA after incubation for 2 h, except at the maximum concentration.

VacA-induced vacuolating activity was not influenced by NaCl

The time course for vacuolation within cultured cells in the presence of VacA and NaCl showed the effect of NaCl on the vacuolating activity (Figure 2). The vacuolating activity was shown as a function of incubation time at different NaCl concentrations. Cells were induced with 40 mg/L VacA. The experiments were carried out in triplicate.

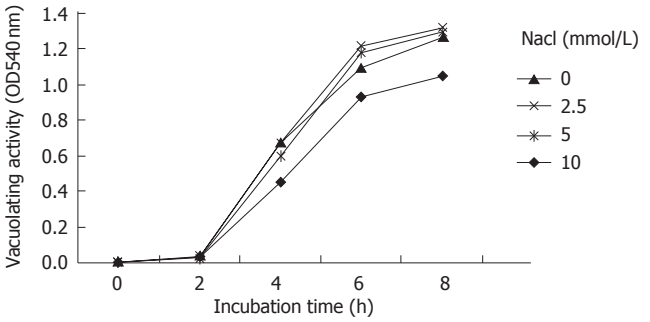

Figure 2 Time course of VacA-induced vacuolization of AGS cells at different NaCl concentrations.

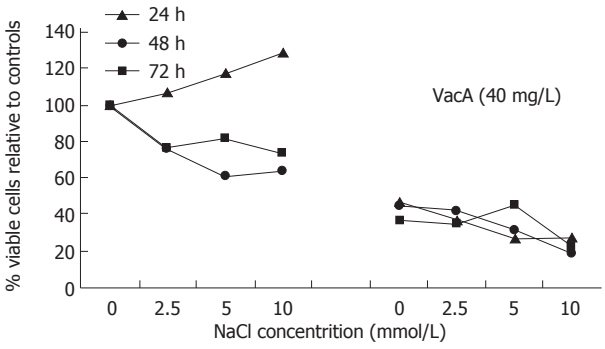

Figure 3 Viability of AGS cells grown in the presence of NaCl alone or VacA and NaCl.

At all NaCl concentrations, the vacuolating activity was minimal after incubation for 2 h. Time-dependent vacuolating activity increased linearly, doubling every two hours between 2 and 6 h of incubation. Because the curves at all NaCl concentrations were similar, NaCl did not appear to significantly affect VacA-induced vacuolation. As a control, the same concentrations of NaCl were added to AGS cells without VacA and no vacuolating activity was observed (data not shown).

Viability of AGS cells was decreased after coculture with VacA and NaCl

The effect of NaCl on AGS viability was assessed with and without VacA at the indicated incubation times. Cell viability was determined by trypan blue exclusion. The experiments were carried out in triplicate. Upon incubation with increasing levels of NaCl for 72 h, there was an apparent slight increase in the viability of AGS cells. However, culture for 24 or 48 h apparently decreased the viability of AGS cells (Figure 3). These results, however, were not statistically significant ($P > 0.05$) compared with control AGS cells at all incubation time (Table 1). Furthermore, the viability of AGS cells cultured with VacA and 10 mmol/L NaCl was significantly reduced ($P < 0.05$) compared with VacA alone after incubation for 48 and 72 h. Although the average viability values after incubation for 24 h were similar to those at 48 and 72 h, the viability at 10 mmol/L and 0 mmol/L NaCl was not significantly different ($P > 0.05$) because large variances were obtained for the 24-h samples. The reduced viability in the presence of VacA was similar at all three incubation times.

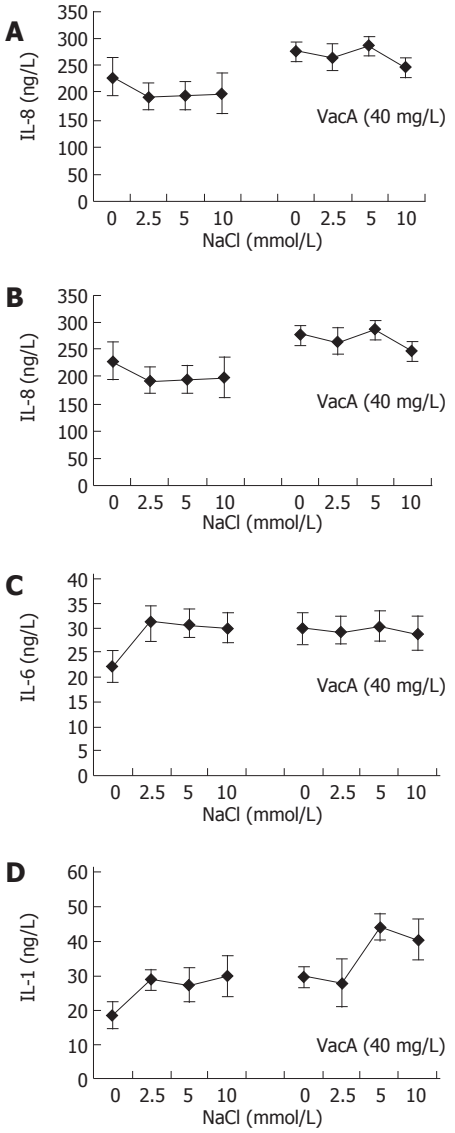

Figure 4 Production of cytokines induced by NaCl alone or VacA and NaCl.

VacA-induced cytokine production in AGS cells

To examine cytokine production after cell culture with VacA combined with NaCl, AGS cells were incubated with VacA or VacA and NaCl. The secretion of TNF-α, IL-8, IL-6 and IL-1β assessed by ELISA, is shown in Figure 4. The experiments were carried out in triplicate. In addition, we assessed the cytokine response of cultured human AGS cells to NaCl alone. The secretion of TNF-α increased in cells exposed to VacA, but this increase was inhibited in the presence of both VacA and NaCl. However, the secretion of TNF-α seemed to increase slightly in culture with NaCl alone ($P > 0.05$, Figure 4A). The secretion of IL-8 increased in cells exposed to VacA compared with cell-only control cultures or those exposed to NaCl alone. The secretion increased when VacA was combined with NaCl. However, NaCl did not appear to increase IL-8 secretion in the presence of VacA. NaCl did not induce IL-8 secretion from AGS cells at any concentration tested (Figure 4B). IL-6 secretion increased in cultures incubated with VacA or NaCl or both VacA and NaCl (Figure 4C). IL-1β secretion increased after culture with VacA or NaCl

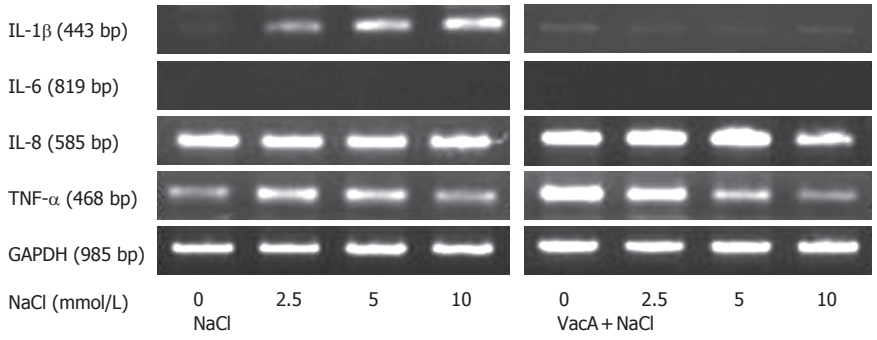

Figure 5 Expression of cytokines induced by NaCl or VacA with NaCl (RT-PCR) in gastric tissue of *H pylori*-infected gerbils (×200) fed with a normal diet (top) and a 50g/kg NaCl diet (bottom).

alone. The addition of 5 or 10 mmol/L NaCl enhanced VacA-induced IL-1β secretion, although it was not dose-dependent (Figure 4D).

Cytokine mRNA expression induced by VacA in AGS cells

To examine the ability of VacA combined with NaCl to induce the production of cytokines, cytokine-specific mRNA expression in AGS cells was analyzed by RT-PCR at 4 h post-induction. The expression of TNF-α, IL-8, IL-6 and IL-1β mRNAs is shown in Figure 5. TNF-α mRNA expression increased slightly after culture with NaCl, and there was a significant increase in TNF-α expression after culture with VacA. TNF-α mRNA expression decreased after culture with VacA combined with NaCl and displayed a significant NaCl dose-dependence. IL-8 mRNA expression was not stimulated by NaCl alone. However, the IL-8 mRNA PCR product increased after culture with VacA especially with VacA combined and NaCl, although a decrease was observed at the highest NaCl concentration. IL-6 mRNA was not detected in the control cells or upon exposure to any of the above conditions. IL-1β mRNA expression was induced in response to NaCl in a dose-dependent manner. Expression was also induced upon exposure to VacA, but this effect was not stimulated by NaCl.

Proinflammatory cytokine production in gastric tissue

Positive staining of IL-1β, IL-6, and TNF-α in gastric tissue was found in all but one of the *H pylori*-infected subjects ($n=6$) regardless of diet. However, we observed more staining of all three cytokines in *H pylori*-infected gerbils on a 50 g/kg NaCl diet than in infected gerbils on a normal diet, suggesting a potential role of NaCl in the up-regulation of VacA-induced pro-inflammatory cytokine production in gastric epithelium. IL-6 displayed less increase in staining than the other two cytokines, consistent with the *in vitro* ELISA results. There was no significant difference between the specific primary antibody staining and the IgG negative control staining in tissues from uninfected animals fed with 50 g/kg NaCl and positive staining was not observed in the untreated gerbils (data not shown).

DISCUSSION

The neutral red uptake assay is often used for quantitatively determining *H pylori* cytotoxicity *in vitro*[9]. Because the shortest time point at which vacuolation was induced in this study was 4 h, 4 h incubation was chosen for further experiments. The appropriate dose of VacA to induce vacuolation was determined to be 17.5 mg/L, and 40 mg/L VacA decreased AGS cell viability to 40% after 24 h. Therefore, 40 mg/L VacA was used in experiments to assess VacA-induced cytokine production.

Significant vacuolating activity is stimulated by acidic ($pH<5.5$) or alkaline ($pH>9.5$) conditions[27]. We evaluated the effect of high Na^+ and Cl^- concentrations on vacuolization induced by VacA. AGS cells exposed *in vitro* to VacA at various concentrations of NaCl had vacuolization rates similar to those exposed to VacA alone, suggesting that VacA may be activated by exposure to acidic or alkaline conditions but not under the osmotic conditions.

H pylori has been shown to either reduce or enhance gastric epithelial cell viability *in vitro*[29]. All strains of *H pylori* decrease cell viability of microvascular endothelial cells after 72 h[30]. Furthermore, co-culture of *H pylori* with AGS cells significantly decreases cell viability[29]. Consistent with these studies, our results demonstrated that VacA alone could reduce AGS cell viability ($P<0.05$), which was enhanced by high doses of NaCl ($P<0.05$).

Increased levels of proinflammatory cytokines induced by VacA in AGS cells and gerbils can be considered a cytotoxic effect. Consistent with our findings, others have noted that the damage caused by *H pylori* is partially attributed to the enhanced secretion of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, particularly in response to VacA-expressing *H pylori* strains[31,32]. It has been reported that gastric *H pylori* infections induce mucosal production of various cytokines, including IL-1β, IL-6, IL-8 and TNF-α[33]. Our study further confirmed the cytokine response to the combined effect of NaCl and VacA *in vitro* and *in vivo*. First, we examined the expression profile of proinflammatory cytokines in response to stimulation by NaCl alone. The addition of NaCl alone to AGS cells resulted in a dose-dependent increase in IL-1β mRNA level, whereas expression of the other three cytokine mRNAs did not change significantly both *in vitro* and *in vivo*. It has been reported that normal human dermal fibroblasts ele-vate IL-1β mRNA levels upon exposure to 0.5 mmoL/L NaCl and that normal human epidermal keratinocytes increase levels of IL-6 and IL-8 mRNA in response to NaCl[34]. These reported differences in IL-6 and IL-8 expression in response to NaCl exposure may be attributable to differences in cell types or the higher NaCl concentration used in the other studies, which was fivefold higher than that in our study. We found that NaCl could

not alter VacA-induced IL-8 expression. However, the induction of IL-8 by VacA alone is consistent with previous studies that *H pylori* infection increases mucosal production of IL-8 and interaction between viable *H pylori* and AGS cells increases levels of IL-8 mRNA expression and protein secretion[33, 35].

It has also been reported that polymorphisms within the IL-1β and TNF-α genes are associated with a risk for gastric carcinoma in individuals infected with *H pylori*[36]. Our study demonstrated that VacA-induced expression of IL-1β and TNF-α mRNAs could be up-regulated by NaCl in gerbils, whereas NaCl increased IL-1β expression and decreased VacA-induced TNF-α expression in AGS cells.

IL-6 is a multifunctional cytokine that plays a central role in host defense mechanisms[37-40]. IL-6 production upon *H pylori* infection could be induced in response to urease in human AGS cells[41]. IL-6 can also be induced by VacA in bone marrow-derived mast cells[42]. These findings are consistent with our ELISA results, but we were unable to detect IL-6 mRNA with RT-PCR under any of the conditions we tested.

VacA directly activates AGS cells to produce proinflammatory cytokines, which might be a host early innate immune response, suggesting that VacA plays a role in the pathogenesis of *H pylori*-infected gastritis although the cag pathogenicity island is closely related to induction of proinflammatory cytoikine[43]

High-salt diet (NaCl 75g/kg versus 2.5g/kg) intake enhances *H pylori* colonization in mice and humans, while chronic salt intake might exacerbate gastritis by increasing *H pylori* colonization[12]. Our study indicated that excessive dietary NaCl (50 g/kg) influenced cytokine production in the Mongolian gerbil model of *H pylori* infection. VacA may be regulated by NaCl to influence cytokine production through a variety of mechanisms. One mechanism might be through an effect on VacA channels, which are likely to be an important component in the mode of action of this toxin[36].

In conclusion, the virulent *H pylori* cytotoxin, VacA, decreases the viability of AGS cells, which can be enhanced by NaCl. NaCl also affects the production of proinflammatory cytokines induced by VacA. Our results suggest that NaCl plays an important role in *H pylori*-induced AGS cell cytotoxicity. However, the interactions between *H pylori*, NaCl and gastric cancer are complex, more studies are required to understand the mechanism by which NaCl affects the progression of *H pylori*-related gastric cancer.

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