RESEARCH COMMUNICATION

Changes in Biological and Virulent Characteristics of *Helicobacter pylori* Exposed to High Salt

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Abstract

The effect of high salt environments on biological characteristics of *Helicobacter pylori* is still unclear. In the present study, we therefore investigated biological characteristics of the bacterium exposed to high salt concentrations. *H. pylori* strain, L301, was cultured in media supplemented with different concentrations (3%, 15% and 30%) of sodium chloride (NaCl) under microaerophilic conditions for 48 h. Morphology was assessed by light microscopy, the ATP content was quantitated by single-tube fluorescent light-emission and the levels of CagA and UreB proteins were determined by Western blotting. After exposure to NaCl, *H. pylori* transformed from common spiral shape to U or even coccoid shapes. The ATP content was significantly higher in 30% NaCl group than in 15% and 3% NaCl group and the level of CagA protein increased with the salt concentration. The urease reaction was all strongly positive in *H. pylori* exposed to different salt concentrations. The level of 8-OHdG expression was significantly increased in GES-1 cells co-cultured with *H. pylori* exposed to high salt, compared with the level in uninfected cells. *H. pylori* survives under exposure to high salt concentrations up to 30%, exhibiting changes in mobility, morphology and CagA expression, associated with increased 8-OHdG in the gastric epithelial cells, indicative of DNA damage.

Keywords: *H. pylori* - gastric epithelial cells - high salt - DNA damage

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Introduction

It is acknowledged that *H. pylori* infection is closely associated with diseases such as chronic gastritis, peptic ulcer, gastric cancer, and B-cell lymphoma (Nilsson et al., 2002; Sachs, Wen, and Scott, 2009). Chaput et al found that changes in the biological characteristics of *H. pylori* were accompanied by changes in polysaccharide in the cell wall, which may help the bacterium escape host immune response (Chaput et al., 2006). These findings indicate that *H. pylori* can adapt to the changing environment by self-regulation in order to adhere to and colonize the gastric mucosa, and thus cause consequent gastroduodenal diseases.

H. pylori infection is thought to be an initial etiological factor for the development of gastric cancer, which is a multifactorial and multi-step process (Szabo and Ohshima, 1997). It has been suggested that sodium chloride (NaCl) concentration is an environmental factor closely related to *H. pylori* infection and gastric diseases (Ishii et al., 2006; Yermilov et al., 1996). Epidemiological studies have shown that development of gastric atrophy in the presence of *H. pylori* infection is closely associated with high dietary salt intake (Szabo and Ohshima, 1997).

In human epidemiologic studies and also in animal models (Kuroiwa et al., 2007; Naito and Yoshikawa, 2002; Nozaki et al., 2002), a link has been shown between high salt intake and the development of gastric cancer. In the studies to detect the transmission routes of *H. pylori*, researchers observed that contaminated food or water, some of which with very high NaCl concentration, may serve as reservoir in the transmission of *H. pylori* (Carbone et al., 2005; Vale and Vitor, 2010). However, due to the strain-specific difference in salt resistance or the limitation of experimental methods, the studies evaluating the effect of high salt environments on biological characteristics of *H. pylori* have produced inconsistent results (Loh, Torres, and Cover, 2007; Yan et al., 2008). In addition, the interaction between *H. pylori* and very high salt environments (e.g. 30% NaCl) has never been reported.

Therefore, the present study was conducted to investigate the changes in biological characteristics on exposure to high salt concentrations (including 30% NaCl). In addition, the effect of high-salt exposed bacteria on 8-OHdG expression in gastric epithelial cells was assessed as an index of DNA damage.

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Materials and Methods

Culture and treatment of H. pylori with high-salt

H. pylori strain, L301, which is positive for CagA and ureB, was kindly provided by the Third Laboratory, Cancer Institute, China Medical University, Shenyang, China. The bacteria were grown on brain heart infusion agar containing 7% sheep blood, 0.4% BBLTM IsoVitaleXTM Enrichment (Becton, Dickinson and Company, Franklin Lakes, NJ USA), 0.08% amphotericin B (Sigma), 0.2% vancomycin (Eli Lilly, Indianapolis, USA), and 0.5% trimethoprim (Sigma), and incubated under microaerophilic conditions (5% O2, 10% CO_2, and 85% N2) at 37˚C and 95% humidity. In the present study, the above media supplemented with different concentrations of NaCl (3%, 15%, and 30%, respectively) were prepared; 3% NaCl group was used as a control.

Observation of motility and morphology and measurement of ATP content of H. pylori

The morphology of *H. pylori* was identified by Warthin-Starry silver staining (Jhala et al., 2003). Moreover, ultrastructure was also observed under transmission and scanning electron microscope, respectively. For scanning electron microscopy (H-600, Olympus, Japan), fixation, dehydration, and coating were performed as previously described (Sato et al., 2003).

For the measurement of ATP content, *H. pylori* were harvested and then washed with phosphate-buffered saline (PBS, pH7.3-7.4) centrifugation 10 min, 5000×g, 3 times, and then detected with the Bactiter-Glo Microbial Cell Viability Assay (Zhou et al., 2008).

Urease activity

H. pylori cultured on the agar plates with different concentrations of NaCl was collected 24, 48, 72, 96, and 120 hours after culture, and then incubated on the urease medium. A positive result was recorded when the color of the medium changed from yellow to pink.

Expression of CagA and UreB proteins in H. pylori as detected by Western blotting

Bacteriawere lysed in radio-immunoprecipitation assay (RIPA) buffer (Beyotime, China). The protein levels were determined using a mouse anti-CagA monoclonal antibody (Santana, Bethel, USA) and a rabbit anti-ureB polyclonal antibody (Santana). A rabbit anti- GAPDH (Santana, Bethel, USA) was used to detect GAPDH as a control.

Determination of 8-hydroxy-2'-deoxyguanosine in GES-1 cells after infection with H. pylori by immunofluorescence

GES-1 cells were co-cultured with *H. pylori* as described above, and then washed twice with PBS and fixed on slides with acetone at 4˚C. The slides were incubated in bovine serum albumin for 30 min and with mouse anti-human 8-hydroxy-2'-deoxyguanosine (anti-8-OHdG) antibody (1:20, JaICA, Shizuoka, Japan) overnight at 4oC. The slides were washed twice with PBS, and then incubated with goat anti mouse IgG (Santana) at 37˚C for 1 h. The cells were observed under upright fluorescence microscope (Ke, Ning, and Wang, 1994). The optical density of 10 visions was detected by image analysis system and calculated the average optical density value.

Statistical analysis

Statistical analysis was performed by using the SPSS® version 11.5 (SPSS, Chicago, IL). The results were expressed as the mean ± standard deviation (SD) and comparisons between groups were performed by using the Student-t test. A P value of less than 0.05 (two-sided) was considered as statistically significant.

Results

Effect of high salt concentrations on mobility and ATP content of H. pylori

H. pylori were collected from the agar plate into PBS, and the dynamic changes observed under the inverted microscope. In cultures with different concentrations of NaCl for 48 h, *H. pylori* represented weak drill-like movement.

The ATP contents in *H. pylori* exposed to 30% NaCl were significantly higher than those in *H. pylori* exposed to 3% or 15% NaCl at 24, 72, 96, and 120 h (P<0.05). Compared with 3% NaCl, the ATP contents at 15% NaCl were significantly higher at 24, 72, 96, and 120 h (P<0.05) (Table 1).

Effect of high salt concentrations on morphology of H. pylori

H. pylori exposed to different concentrations of salt was positive for Warthin-Starry silver staining, and exhibited following morphological changes: the bacterial cells were rod-shaped, short rod-shaped, or even coccoid-shaped and in various sizes (Figure 1).

Transmission electron microscopy showed that the majority of *H. pylori* cells exposed to 30% NaCl were in U form (Figure 2A), chain-shaped (Figure 2B), or coccoid (Figure 2C) with or without homogenous cytoplasm. Under the scanning electron microscope, *H. pylori* cells exposed to 30% NaCl lost the spiral shape, and a substantial proportion of the cells were coccoid-shaped, and had a rough and irregular surface with single polar

Table 1. ATP Concentration in *H. Pylori* Exposed to Different Concentrations of NaCl at Different Time Points (10^4 relative light units)

Group	0 h	24 h	48 h	72 h	96 h	120 h
3% NaCl	1.0±0.10	0.05±0.01	0.3±0.07	0.3±0.01	1.1±0.40	0.7±0.46
15% NaCl	1.0±0.10	0.9±0.5*	0.5±0.03	0.9±0.25*	2.0±0.14*	0.07±0.04*
30% NaCl	1.0±0.10	20.2±4.00**	9.6±0.15**	5.7±1.87**	4.5±0.57**	3.8±0.42**

*P<0.05 vs. 3% NaCl; **P<0.05 vs. 15% NaCl.

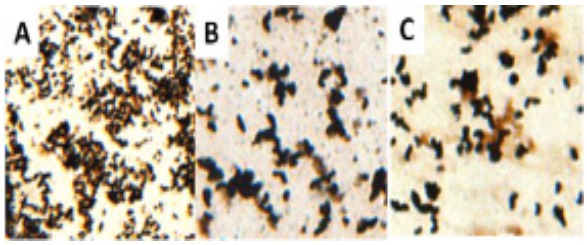

Figure 1. Warthin-Starry Silver Staining Illustrating the Morphology of *H. pylori* exposed to 3% (A, ×1000), 15% (B, ×1000) and 30% (C, ×1000) NaCl concentrations

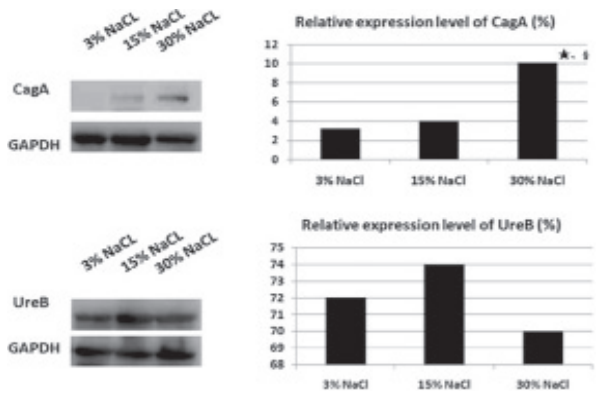

Figure 3. Western Blotting Illustrating the Expressions of CagA (A) and UreB (B) Proteins in *H. pylori* exposed to 3% (lane 1), 15% (lane 2) and 30% (lane 3) of NaCl.

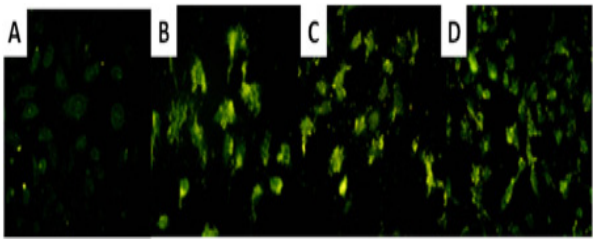

Figure 4. . Immunofluorescence Illustrating the Expression of 8-OHdG in Uninfected GES-1 Cells (A) and GES-1 cells co-cultured with *H. pylori* exposed to 3% (B), 15% (C) and 30% (D) (all ×400). In uninfected GES-1 cells, the cell membrane is distinct and slightly stained, but the nucleus is not stained. In GES-1 cells co-cultured with *H. pylori* exposed to 3%, 15%, or 30% NaCl, both the cell membrane and nucleus are stained.

flagella (Figure 2D). The number of coccoid cells of *H. pylori* appeared to increase with the concentrations of NaCl and culture time.

Effect of high salt concentrations on urease activity and the expression of CagA and UreB of H. pylori

The urease reaction was strongly positive (change within 5 min) for the bacteria exposed to different concentrations of NaCl for 48 h.

In *H. pylori* exposed to different concentrations of salt, CagA and UreB proteins were still expressed.. The expression of CagA protein increased with salt concentration, and the difference was significant among *H. pylori* exposed to3%, 15%, and 30% NaCl (P<0.05). There was no significant difference in the level of UreB protein at 3%, 15%, and 30% NaCl (P>0.05).

Western blotting illustrating CagA (A) and UreB (B)

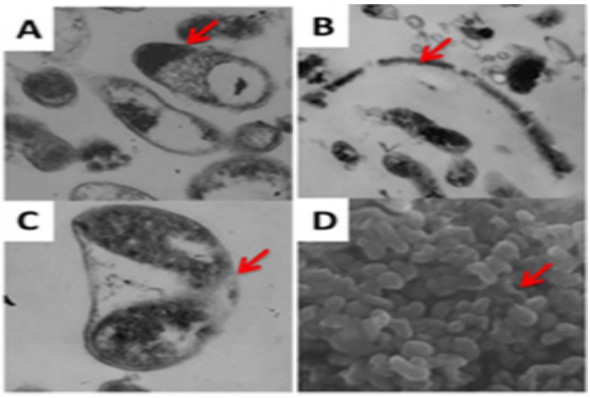

Figure 2. Representative Images of Transmission and Scanning Electron Microscope Illustrating the Morphology of *H. pylori* Exposed to 30% NaCl. A & C, TEM, ×20000; B, TEM, ×10000; and D, SEM, ×2500

Table 2. Western Blotting Illustrating the Expression of CagA and UreB Proteins in *H. pylori* Exposed to 3%, 15% or 30% of NaCl

Group	Relative expression level of CagA (%)	Relative expression level of UreB (%)
3% NaCl	3.21	72.73
15% NaCl	3.99	74.81
30% NaCl	10.09*§	70.20

*P<0.05 vs. *H. pylori* exposed to 3% NaCl; §, P<0.05 vs. *H. pylori* exposed to 15% NaCl.

Table 3. Immunofluorescence Illustrating the Expression of 8-OHdG in GES-1 Cells with or Without Co-culture with *H. pylori* Exposed to 3%, 15% or 30% of NaCl

Group	Total gray value (average)
GES-1	1099.03±448.677
3% NaCl L301+GES-1	31969.40±18707.6*
15% NaCl L301+GES-1	20643.06±6219.87*
30% NaCl L301+GES-1	16844.10±4482.67*§

*P<0.05, compared with GES-1 cells without *H. pylori* infection; *P<0.05 vs. 3% NaCl L301+GES-1

proteins were expressed at lower levels in *H. pylori* cells exposed to different concentrations of NaCl (Figure 3). Moreover, CagA expression was NaCl concentration dependent (P<0.05), the level of CagA increased with NaCl concentration elevation. There was no significant difference in UreB expression levels among *H. pylori* control group and after exposure to 3%, 15%, and 30% NaCl (Figure 3, Table 2).

8-OHdG expression in GES-1 cells infected with H. pylori cultured with high salt

Overall, the expression levels of 8-OHdG was significantly greater in GES-1 cells infected with *H. pylori* exposed to different concentrations of NaCl than in those without *H. pylori* infection. (Figure 4, Table 3). However, the 8-OHdG level was significantly decreased in cells infected with *H. pylori* exposed to 30% NaCl, compared with the level in cells infected with *H. pylori* exposed to 3% NaCl (P=0.035<0.05). The 8-OHdG level was lower, albeit not statistically significant, in cells infected exposed

to 15% NaCl than in those infected with *H. pylori* exposed to 3% NaCl (P=0.06).

Discussion

The present study demonstrated that *H. pylori* survived in the present of salt at concentration of up to 30%. Bacteria exposed to high salt led to changes in the morphology, ATP contents, CagA expression. In addition, the expression of 8-OHdG was decreased in gastric epithelial cells co-cultured with *H. pylori* exposed to high salt.

The reported epidemiological studies have provided evidence for a combined effect of salt or salted food consumption and *H. pylori* infection in gastric carcinogenesis (Lee et al., 2003; Wang, Terry, and Yan, 2009). In our preliminary study, we found that individuals who liked high salt diet such as bacon were more likely to have *H. pylori* infection than those who did not in a high risk population in Zhuanghe region, an area with high incidence of gastric cancer (Yuan, 2005). Several studies have been conducted to identify the underlying effects of high salt on the biological behaviors of *H. pylori* (Gancz, Jones, and Merrell, 2008; Loh, Torres, and Cover, 2007; Wang, 2007). However, those studies are all conducted under relative lower NaCl concentrations (<30%), and thus the adaptation to the very high salt environment is still unclear. Therefore, the present study investigated the tolerance ability of a local *H. pylori* strain, L301, in very high salt environment (i.e. 30%), and subsequently the changes of the biological characteristics.

H. pylori switched from the typical spiral form to long rod, U, or even coccoid form in the high salt environment in the present study, which indicates that the bacterium may switch from active proliferative phase into "non-culturable" phase, during which the bacteria are more likely to escape host immunity and to infect the new host (She et al., 2003; Wang et al., 1997). We further measured the ATP contents exposed to different concentrations of NaCl. It was found that the ATP contents were significantly higher in *H. pylori* exposed to 30% NaCl than those exposed to 3% and 15% NaCl, suggesting that the ATP contents in *H. pylori* increases with the concentrations of NaCl, and switches from active form to dormant form under high salt. In addition, although *H. pylori* cultured in high salt environment (30%) did not grow to form colonies in the present study, previous studied have demonstrated that some *H. pylori* bacterial cells that become coccoid form in a hostile environment are still viable and re-cultivable (Bumann et al., 2004; She et al., 2003; Sorberg et al., 1996; Wang et al., 1997).

Urease activity plays an essential role in *H. pylori* colonization of the gastric mucosa (Sachs et al., 2005; van Vliet et al., 2002; Zaidi et al., 2009). In this study, high salt did not affect the urease activity and the expression of the urease subunit, UreB, of *H. pylori*. These findings indicate that urease activity is not abolished by the high concentration of salt, and thus may still facilitate the development of gastric cancer; however, the underlying mechanism is required to be further investigated. CagA is accepted as the most important virulence biomarker that is associated with vacuolating cytotoxin A (VacA) of *H. pylori* (Tuo et al., 2004). In the present study, the CagA expression levels of the *H. pylori* strain, L301, were increased proportionally with NaCl concentrations. Thus, *H. pylori* not only tolerates to very high salt conditions but also up-regulates CagA protein in response to high salt conditions accordingly.

Furthermore, we found that the expression level of 8-OHdG was significantly increased in GES-1 cells that were co-cultured with *H. pylori* exposed to different concentrations of NaCl, compared with the level in cells without *H. pylori* infection (Cooke et al., 2003). Therefore, a high salt environment may lead to gastric carcinogenesis through up-regulating 8-OHdG expression in individuals infected with *H. pylori*.

In conclusion, *H. pylori* survives in the exposure of high salt at concentration of up to 30%. Exposure to high salt results in changes in mobility, morphology and CagA expression. *H. pylori* exposed to a very high salt increases the expression of 8-OHdG of gastric epithelial cells, indicating DNA damage of the cells. These findings indicate that *H. pylori* can adapt to the very high salt environment, such as high-salt food or water, and importantly, high dietary salt and *H. pylori* infection are synergistic in the development of gastric cancer. Further investigation is required to explore the underlying mechanisms on how high salt affects the biological changes of *H. pylori*, and how these two "carcinogens" interact in gastric carcinogenesis.

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References

Bumann D, Habibi H, Kan B, et al (2004). Lack of stage-specific proteins in coccoid *Helicobacter pylori* cells. *Infect Immun*, **72**, 6738-42.

Carbone M, Maugeri TL, Gugliandolo C, et al (2005). Occurrence of *Helicobacter pylori* DNA in the coastal environment of southern Italy (Straits of Messina). *J Appl Microbiol*, **98**, 768-74.

Chaput C, Ecobichon C, Cayet N, et al (2006). Role of AmiA in the morphological transition of *Helicobacter pylori* and in immune escape. *PLoS Pathog*, **2**, e97.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J*, **17**, 1195-214.

Gancz H, Jones KR, Merrell DS (2008). Sodium chloride affects *Helicobacter pylori* growth and gene expression. *J Bacteriol*, **190**, 4100-5.

Ishii Y, Umemura T, Kanki K, et al (2006). Possible involvement of NO-mediated oxidative stress in induction of rat forestomach damage and cell proliferation by combined treatment with catechol and sodium nitrite. *Arch Biochem Biophys*, **447**, 127-35.

Jhala NC, Siegal GP, Klemm K, Atkinson BF, Jhala DN (2003). Infiltration of *Helicobacter pylori* in the gastric mucosa. *Am*

J Clin Pathol, **119**, 101-7.
Ke Y, Ning T, Wang, B (1994). Establishment and characterization of a SV40 transformed human fetal gastric epithelial cell line-GES-1. *Zhonghua Zhong Liu Za Zhi*, **16**, 7-10.
Kuroiwa Y, Ishii Y, Umemura T, et al (2007). Combined treatment with green tea catechins and sodium nitrite selectively promotes rat forestomach carcinogenesis after initiation with N-methyl-N'-nitro-N-nitrosoguanidine. *Cancer Sci*, **98**, 949-57.
Lee SA, Kang D, Shim KN, et al (2003). Effect of diet and *Helicobacter pylori* infection to the risk of early gastric cancer. *J Epidemiol*, **13**, 162-8.
Loh JT, Torres VJ, Cover TL (2007). Regulation of *Helicobacter pylori* cagA expression in response to salt. *Cancer Res*, **67**, 4709-15.
Naito Y, Yoshikawa T (2002). Molecular and cellular mechanisms involved in *Helicobacter pylori*-induced inflammation and oxidative stress. *Free Radic Biol Med*, **33**, 323-36.
Nilsson HO, Blom J, Abu-Al-Soud W, et al (2002). Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. *Appl Environ Microbiol*, **68**, 11-9.
Nozaki K, Shimizu N, Inada K, et al (2002). Synergistic promoting effects of *Helicobacter pylori* infection and high-salt diet on gastric carcinogenesis in Mongolian gerbils. *Jpn J Cancer Res*, **93**, 1083-9.
Sachs G, Weeks DL, Wen Y, et al (2005). Acid acclimation by *Helicobacter pylori*. *Physiology*, **20**, 429-38.
Sachs G, Wen Y, Scott DR (2009). Gastric infection by *Helicobacter pylori*. *Curr Gastroenterol Rep*, **11**, 455-61.
Sato F, Saito N, Konishi K, et al (2003). Ultrastructural observation of *Helicobacter pylori* in glucose-supplemented culture media. *J Med Microbiol*, **52**, 675-9.
She FF, Lin JY, Liu JY, Huang C, Su DH (2003). Virulence of water-induced coccoid *Helicobacter pylori* and its experimental infection in mice. *World J Gastroenterol*, **9**, 516-20.
Sorberg M, Nilsson M, Hanberger H, Nilsson LE (1996). Morphologic conversion of *Helicobacter pylori* from bacillary to coccoid form. *Eur J Clin Microbiol Infect Dis*, **15**, 216-9.
Szabo C, and Ohshima, H (1997). DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide*, **1**, 373-85.
Tuo BG, Sellers ZM, Smith AJ, et al (2004). A role for CagA/VacA in *Helicobacter pylori* inhibition of murine duodenal mucosal bicarbonate secretion. *Dig Dis Sci*, **49**, 1845-52.
Vale FF, Vitor JM (2010). Transmission pathway of *Helicobacter pylori*: does food play a role in rural and urban areas? *Int J Food Microbiol,* **138**, 1-12.
van Vliet AH, Poppelaars SW, Davies BJ, et al (2002). NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. *Infect Immun*, **70**, 2846-52.
Wang X, Sturegard E, Rupar R, et al (1997). Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*. *J Med Microbiol*, **46**, 657-63.
Wang XQ, Terry, PD, and Yan, H (2009). Review of salt consumption and stomach cancer risk: epidemiological and biological evidence. *World J Gastroenterol*, **15**, 2204-13.
Yan YG, Zhao G, Ma JP, Cai SR, Zhan WH (2008). Effects of different Helicobacter pylori culture filtrates on growth of gastric epithelial cells. *World J Gastroenterol*, **14**, 3745-9.
Yermilov V, Yoshie Y, Rubio J, Ohshima H (1996). Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett*, **399**, 67-70.
Yuan Y (2005). Comprehensive prevention and treatment for high risk population in high risk area with gastric cancer in Zhuanghe region, Liaoning province. *Bull Chinese Cancer*, **5**, 55-9.
Zaidi SF, Yamamoto T, Refaat A, et al (2009). Modulation of activation-induced cytidine deaminase by curcumin in *Helicobacter pylori*-infected gastric epithelial cells. *Helicobacter*, **14**, 588-95.
Zhou YN, Coleman WG Jr, Yang Z, et al (2008). Regulation of cell growth during serum starvation and bacterial survival in macrophages by the bifunctional enzyme SpoT in *Helicobacter pylori*. *J Bacteriol*, **190**, 8025-32.