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# DNA Damage and oxidative stress induced by *Helicobacter pylori* in gastric epithelial cells: protection by vitamin C and sodium selenite

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The direct effect of intact *Helicobacter pylori* on gastric epithelial cells SGC-7901 and the protection given by the antioxidants vitamin C and sodium selenite were studied. Incubation of SGC-7901 cells with *H. pylori* simultaneously caused a significant increase of DNA damage (DNA strand breakage and DNA fragmentation) and ROS formation, as well as a significant decrease of intracellular GSH content in a *H. pylori* multiplicity of infection (MOI) dependent manner in gastric cells. ROS formation was strongly positively correlated while GSH content was negatively correlated with DNA strand breakage and fragmentation, indicating that DNA damage may be mainly caused by *H. pylori*-induced oxidative stress in gastric cells. The antioxidants, vitamin C and sodium selenite, directly increased GSH content while diminishing ROS formation and DNA damage in *H. pylori*-infected SGC-7901 cells, indicating that vitamin C and sodium selenite can protect gastric cells against *H. pylori* damage. The protections by vitamin C and sodium selenite further demonstrated that DNA damage may be derived from oxidative stress in *H. pylori*-infected gastric cells. The results suggested that DNA damage caused by *H. pylori*-induced oxidative stress may be one important factor in the pathogenesis of *H. pylori*, and that vitamin C and sodium selenite may have a preventive or therapeutic role against *H. pylori*-associated gastric diseases.

## 1. Introduction

*Helicobacter pylori* infects the stomachs of more than 50% of the human population worldwide and is the principal cause of gastritis and peptic ulcer (Dunn et al. 1997). Epidemiological studies have suggested an association between *H. pylori* and gastric cancer, and the World Health Organization has defined *H. pylori* as a type I carcinogen (IARC 1994). The mechanism of *H. pylori*-mediated pathogenesis has been extensively studied, but the underlying pathogenic mechanism, by which *H. pylori* infection results in gastroduodenal pathology, has not, so far, been entirely understood.

DNA damage plays an important role in many severe diseases including carcinogenesis (Olinski et al. 2002). A high degree of DNA damage of tumor-associated lymphocytes and a low total antioxidant level in cancer patients with gastric cancer, gastritis, lung adeno-carcinoma, small cell lung cancer, lung squamous cell carcinoma, breast cancer and thyroid cancer have been found by our laboratory (Su 2001; Liu et al. 2003). Metastasis of tumors is a major cause of unsuccessful therapy in cancer patients and DNA damage has been implicated as an important factor in metastasis. DNA damage was 2-fold higher in metastatic breast cancer than in nonmetastatic (Malins et al. 1996). DNA damage increased in *H. pylori*-infected human gastric mucosa, and *H. pylori* eradication attenuated this damage (Farinati et al. 1998; Hahm et al. 1997; Baik et al. 1996; Pignateli et al. 2001), but there are also conflicting reports that DNA damage, detected by comet assay, was significantly lower in *H. pylori* gastritis than in normal gastric mucosa (Everett et al. 2002). Obst et al. (2000) reported that *H. pylori* extract caused DNA synthesis inhibition, apoptosis (DNA fragmentation) and poly(ADP-ribose) formation *in vitro*, indicating *H. pylori* induced DNA damage. Reactive oxygen species (ROS) are supposed to be involved in tumour intiation and promotion (Halliwell and Aruoma 1991; Burdon 1995). The ROS level in *H. pylori*-positive gastric mucosa is higher than in *H. pylori*-negative mucosa (Davies et al. 1994; Zhang et al. 1997; Drake et al. 1998). Bagchi et al. showed that *H. pylori* directly stimulated production of ROS in cultured gastric cells (Bagchi et al. 1996, 2002). Here, we hypothesised that DNA damage derived from *H. pylori*-induced oxidative stress was the underlying pathogenic mechanism of *H. pylori* infection. To address this, we studied the effects of intact *H. pylori* on DNA damage and ROS formation in gastric epithelial cells SGC-7901.

We also explored the potential therapeutic effects of vitamin C and sodium selenite on *H. pylori*-associated gastric diseases. Vitamin C and sodium selenite are important antioxidants associated with cancer prevention. Our research group has found that vitamin C and sodium selenite inhibited cell growth and stimulated redifferentiation in human hepatoma cells (Kang et al. 2000; Zheng et al. 2002a, 2002b). Epidemiological evidence showed that dietary vitamin C and sodium selenite reduce gastric can-

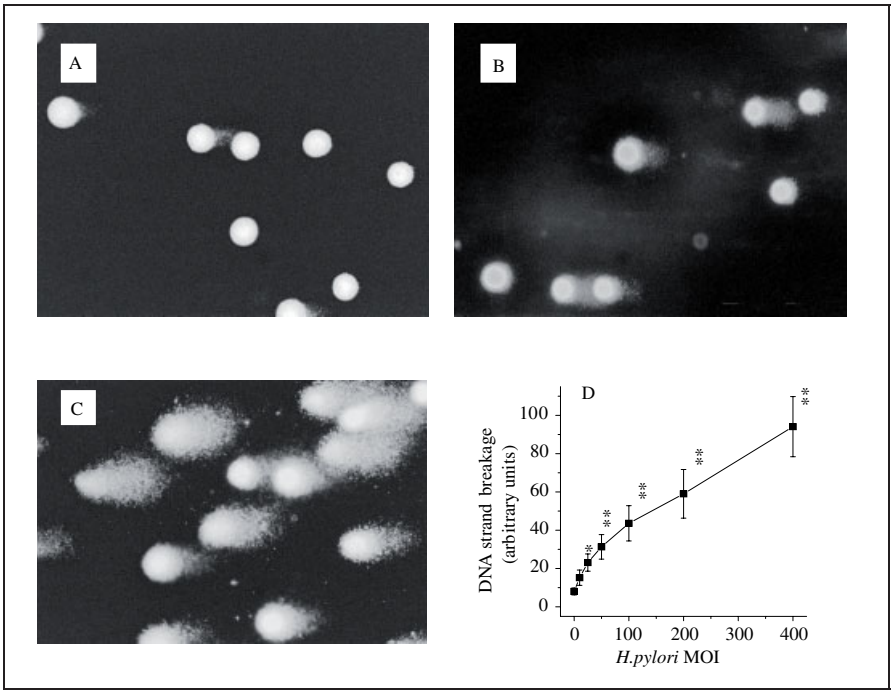



Fig. 1:
Increase of DNA strand breakage in SGC-7901 cells by *H. pylori*. Cells were incubated for 24 h in RPMI 1640 medium alone or with *H. pylori* at indicated multiplicity of infection (MOI). DNA strand breakage were determined by comet assay (A: control, B: with *H. pylori* at MOI of 50, and C: with *H. pylori* at MOI of 400) and scored by arbitrary units (D). Data represent means ± SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs control.

cer risk (Correa et al. 1998; Yang 2000; Mark et al. 2000; Blot et al. 1993; Drake et al. 1998). The vitamin C content in gastric juice infected by *H. pylori* declined and returned to a normal level after eradication of the bacterium (Correa et al. 1998; Drake et al. 1998; Fraser and Woollard 1999; Zhang et al. 1998), but few reports have been concerned with the effect of vitamin C on gastric cells infected by *H. pylori*. The effect of sodium selenite on *H. pylori*-related gastric cell damage has not been reported.

## 2. Investigations, results and discussion

### *2.1. H. pylori induced DNA damage in gastric epithelial cells*

In this experiment, DNA damage was determined by DNA strand breakage (comet assay) and fragmentation (ELISA assay). Within the multiplicity of infection (MOI, i.e. the ratio of the number of bacteria to the number of host cells) range $10 \sim 400$, *H. pylori* increased DNA strand breakage (Fig. 1) and fragmentation (Fig. 2) in gastric epithelial cells SGC-7901 in an MOI dependent manner. The significant increase of both DNA strand breakage and fragmentation began at MOI more than 25.

Our results showed that intact *H. pylori* caused DNA damage, which is consistent with previous studies indicating that DNA damage increased in *H. pylori*-positive gastric mucosa and that *H. pylori* eradication attenuated this damage (Farinati et al. 1998; Hahm et al. 1997; Baik et al. 1996; Pignatelli et al. 2001). *H. pylori* also induced DNA damage in cultured gastric cells (Obst et al. 2000; Bagchi et al. 2002). Taking all this together, we proposed that DNA damage induced by *H. pylori* might be one of the most important pathogenetic factors leading finally also to gastric cancer.

Single cell gel electrophoresis (or comet assay) is a highly sensitive technique that detects DNA damage (strand breaks and alkali labile sites) within a single cell. It was first established by Singh et al. (1988) and has been used extensively to detect DNA damage *in vitro* (McKelvey-Martin et al. 1993). During electrophoresis, DNA with strand breakage and alkali labile sites migrates towards the anode, and the cell appears as a "comet", with a fluorescent head and a tail extending towards the anode. Normal cells retain a circular appearance. In our experiment, DNA strand breakage, detected by comet assay, was determined by scoring 5 "comet" grades and assigning arbitrary units (Collins 2004). Increased DNA strand breakage was found in *H. pylori*-infected gastric cells, which was different from a report by Everett et al. (2002) in which DNA damage was lower in *H. pylori* gastritis than in normal gastric mucosa. The different results might derive from the method of scoring "comets", by which Everett scored DNA damage as comet % (percentage of cells with comet

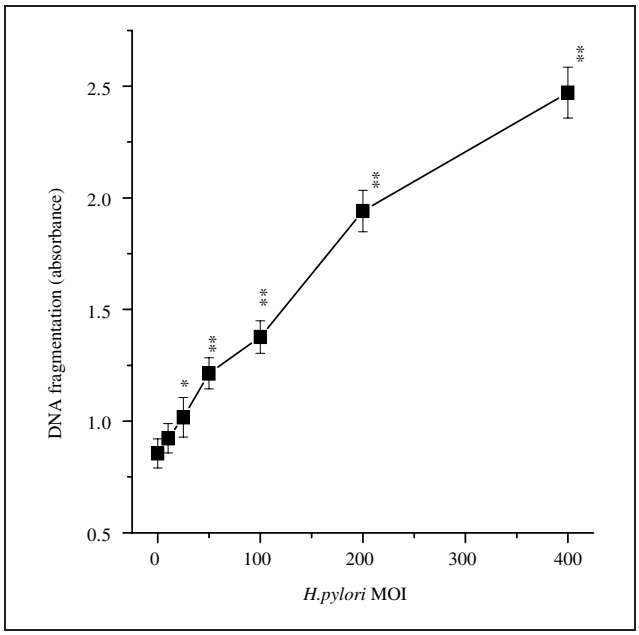



Fig. 2: Increase of DNA fragmentation in SGC-7901 cells by *H. pylori*. Cells were incubated for 24 h in RPMI 1640 medium alone or with *H. pylori* at indicated multiplicity of infection (MOI). DNA fragmentation was determined by quantifying the amount of oligonucleosome-bound DNA in the supernatant of cell lysate and was expressed as absorbance. Data represent means ± SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs control.

in total). We found that the comet % declined but that arbitrary units actually increased in cells which were severely damaged by *H. pylori* (more than MOI 400) (data not shown). For example, when the MOI increased from 400 to 800, the comet % decreased from 62.1 to 37.3, while the arbitrary units increased from 94.1 to 135.7. Therefore we propose that arbitrary units rather than comet % give a superior representation of DNA damage in the comet assay. Collins (2004) states in a review that relative tail intensity bears a linear relationship with DNA break frequency in the comet assay.

### *2.2. H. pylori enhanced ROS formation and decreased intracellular GSH content in gastric epithelial cells*

After exposure to *H. pylori* for 1 h, ROS formation was enhanced in a MOI dependent manner. Significant enhancement of ROS formation began at *H. pylori* MOI over 10 (Fig. 3).

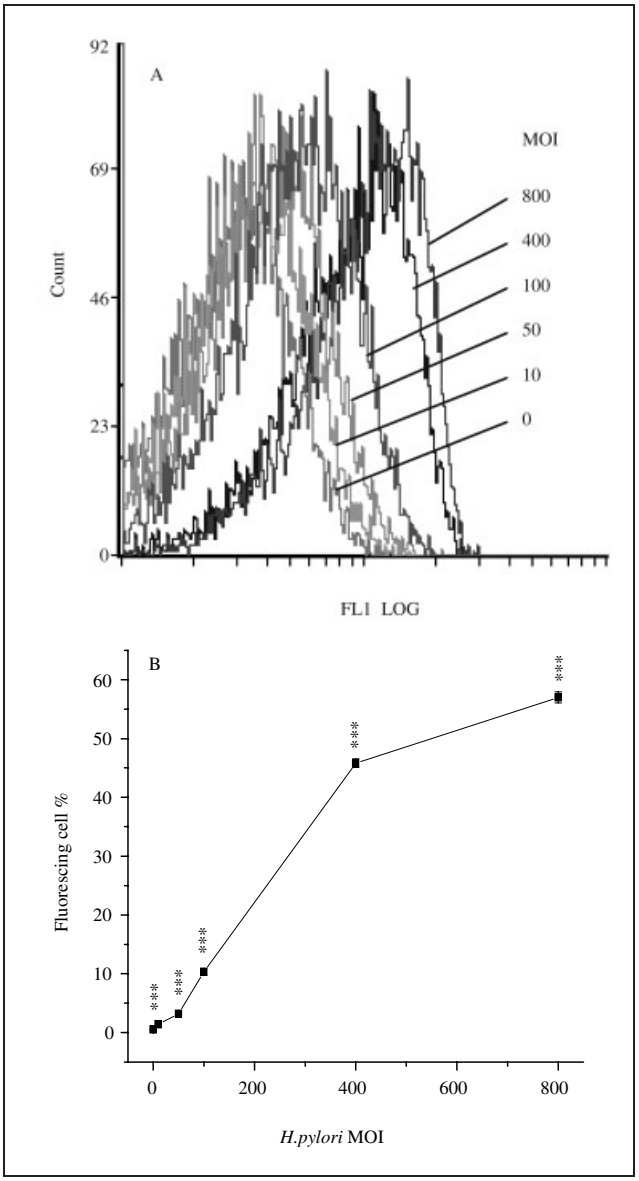



Fig. 3: Increase of ROS formation in SGC-7901 cells by *H. pylori*. Cells were incubated for 1 h in RPMI 1640 medium alone or with *H. pylori* at indicated multiple of infection (MOI). ROS formation was determined by DCF fluorescence (A) and the percent of fluorescing cell (B). Data represent means ± SD of triplicate experiments. *** $p < 0.001$ vs control.

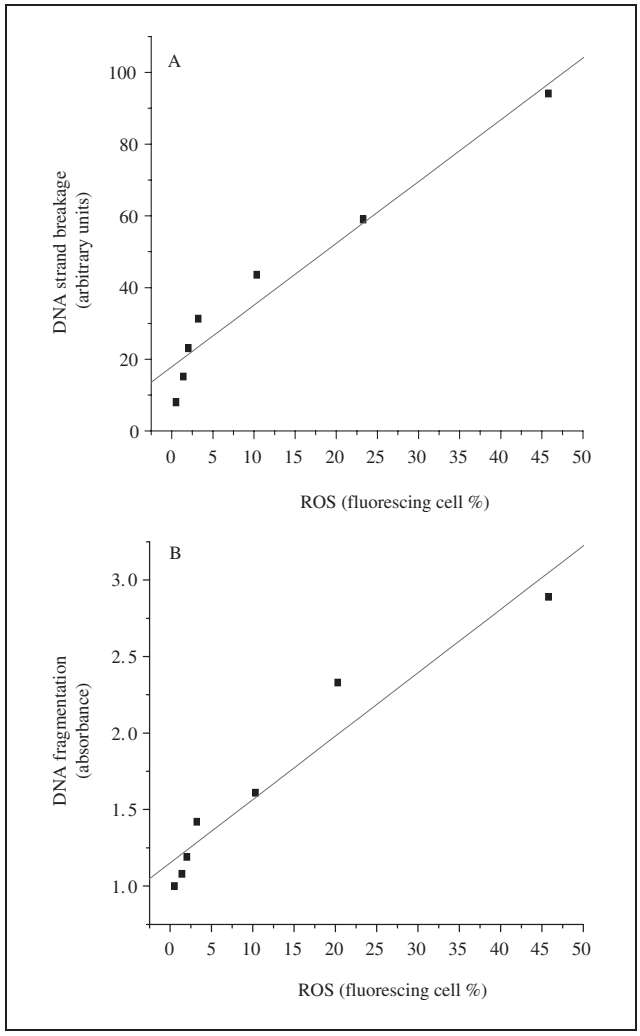



Fig. 4: Correlations between ROS formation and DNA strand breakage (A) and fragmentation (B) in *H. pylori*-infected gastric epithelial cells SGC-7901.

ROS can cause formation of oxidized bases, strand break and crosslinking of DNA (Halliwell and Aruoma 1991). *H. pylori* infection stimulated ROS formation in gastric mucosa due to neutrophil infiltration (Davies et al. 1994; Zhang et al. 1997; Drake et al. 1998). *H. pylori* also stimulated lymphocytes and gastric cells to produce ROS *in vitro* (Shimoyama et al. 2003; Bagchi et al. 1996 and 2002). Our results showed that *H. pylori* stimulated gastric cells to produce ROS. Furthermore, ROS formation was positively correlated with both DNA strand breakage ($r = 0.97$) (Fig. 4A) and fragmentation ($r = 0.96$) (Fig. 4B), indicating that DNA damage may be caused by *H. pylori*-induced oxidative stress in gastric epithelial cells.

*H. pylori* also decreased GSH content in a MOI dependent manner. GSH content declined to about half that of the control at a MOI of 32, and was almost completely depleted when the MOI was higher than 400 (Fig. 5).

Reduced glutathione (GSH) is the most important intracellular antioxidant and plays an essential role in protecting cells from oxidative damage. The GSH level in *H. pylori*-positive mucosa has been observed lower than that in *H. pylori*-negative mucosa (Shirin et al. 2001; Verhulst et al. 2000; Jung et al. 2001), but a higher GSH level was found in Mongolian gerbils with *H. pylori* infection (Suzuki et al. 1999). *In vitro* studies implied that *H. pylori* reduced intracellular GSH in cultured cells (Beil et al.

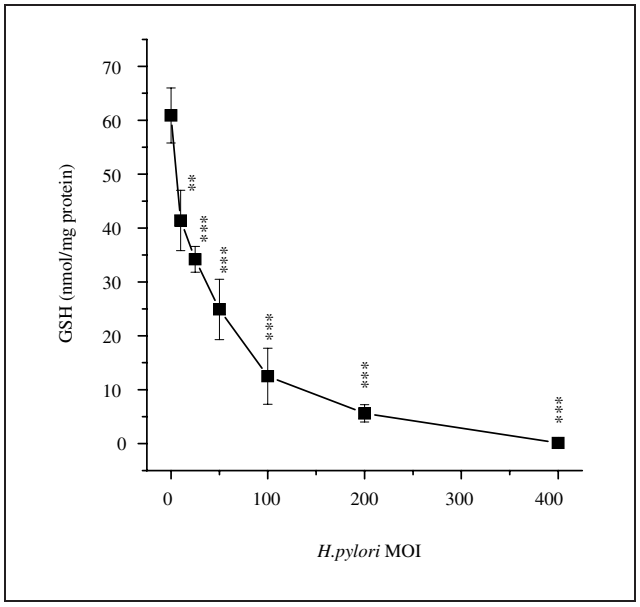



Fig. 5: Decrease of GSH content in SGC-7901 cells by *H. pylori*. Cells were incubated for 24 h in RPMI 1640 medium alone or with *H. pylori* at indicated multiplicity of infection (MOI). GSH was determined with o-phthaladehyde by measuring fluorescence and was expressed as nmol/mg protein. Data represent means ± SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs control.

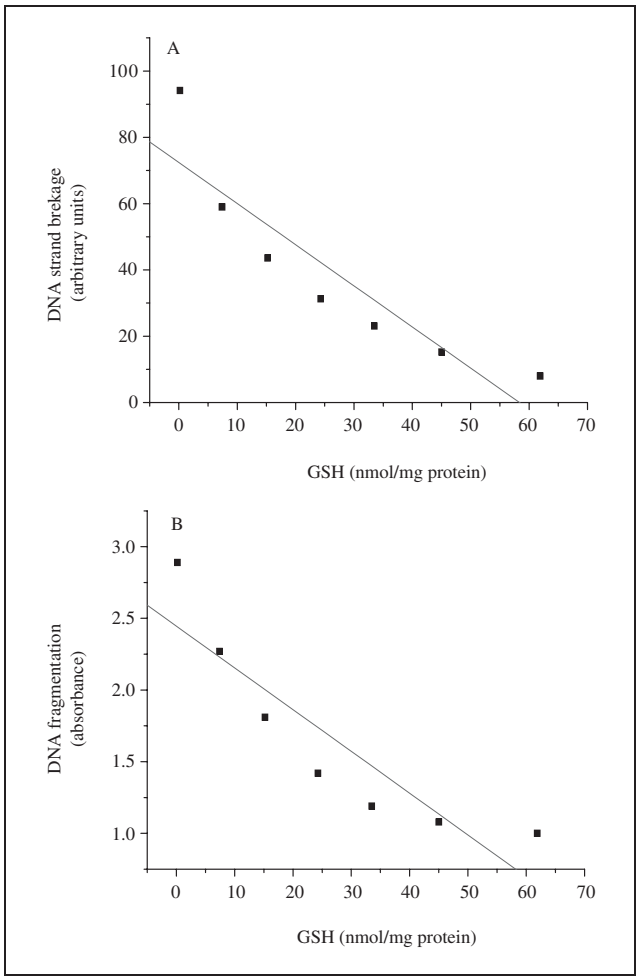



Fig. 6: Correlations between GSH content and DNA strand breakage (A) and fragmentation (B) in *H. pylori*-infected gastric epithelial cells SGC-7901.

2000; Obst et al. 2000). In our experiment, the decrease of GSH content was negatively and closely correlated with both DNA strand breakage ($r = -0.91$) (Fig. 6A) and DNA fragmentation ($r = -0.90$) (Fig. 6B), indicating that reduced antioxidant level may be one of the important pathogenetic factors, and may also contribute to DNA damage in *H. pylori*-infected gastric cells.

### *2.3. Effect of vitamin C and sodium selenite on GSH content and ROS formation in H. pylori-infected gastric epithelial cells*

Vitamin C and sodium selenite are important antioxidants. Either vitamin C (within the range $50 \sim 200\ \mu M$) or sodium selenite (within the range $0.01 \sim 2\ \mu M$) increased GSH content which decreased by *H. pylori* (Fig. 7). The increase of GSH content displayed significance compared with *H. pylori*-infected control when the vitamin C con-

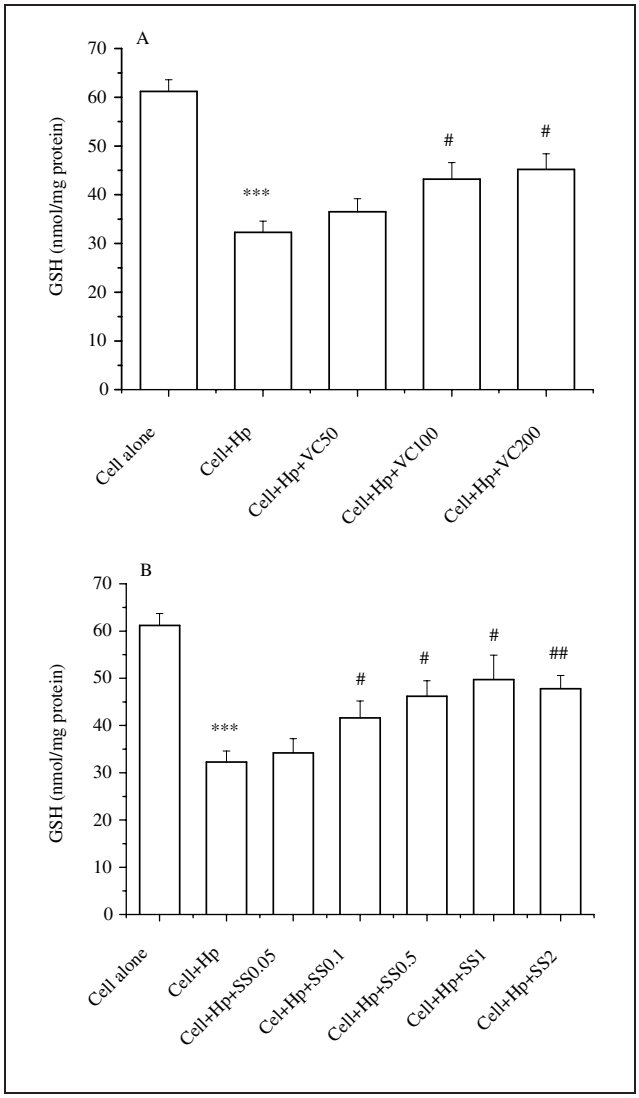
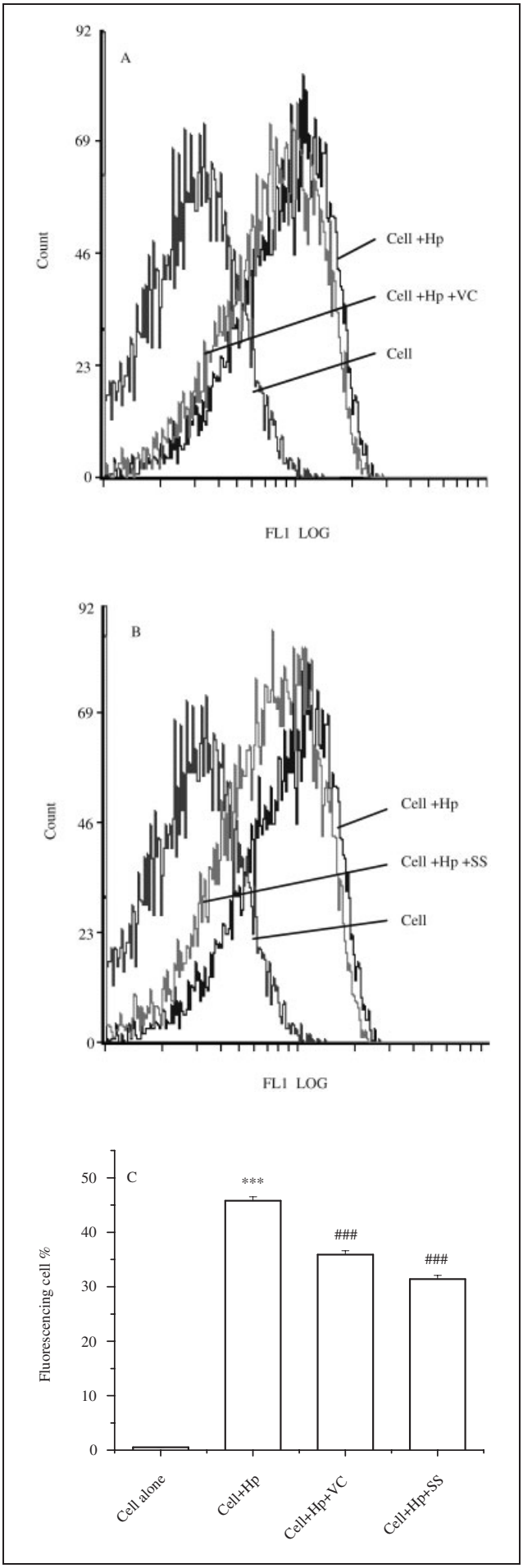



Fig. 7: Increase of intracellular GSH content by vitamin C (A) and sodium selenite (B) in *H. pylori*-infected SGC-7901 cells. Cells were incubated for 24 h in RPMI 1640 medium alone, or with *H. pylori* (MOI 25) in combination with or without vitamin C or sodium selenite. GSH content was determined with o-phthaladehyde by measuring fluorescence and was expressed as nmol/mg protein. Data represent means ± SD of triplicate experiments. *** $p < 0.001$ vs cell alone control. # $p < 0.05$, ## $p < 0.01$ vs *H. pylori*-infected control. Hp: *H. pylori*; VC: vitamin C; SS: sodium selenite. The number presented concentration (μM) of vitamin C and sodium selenite.



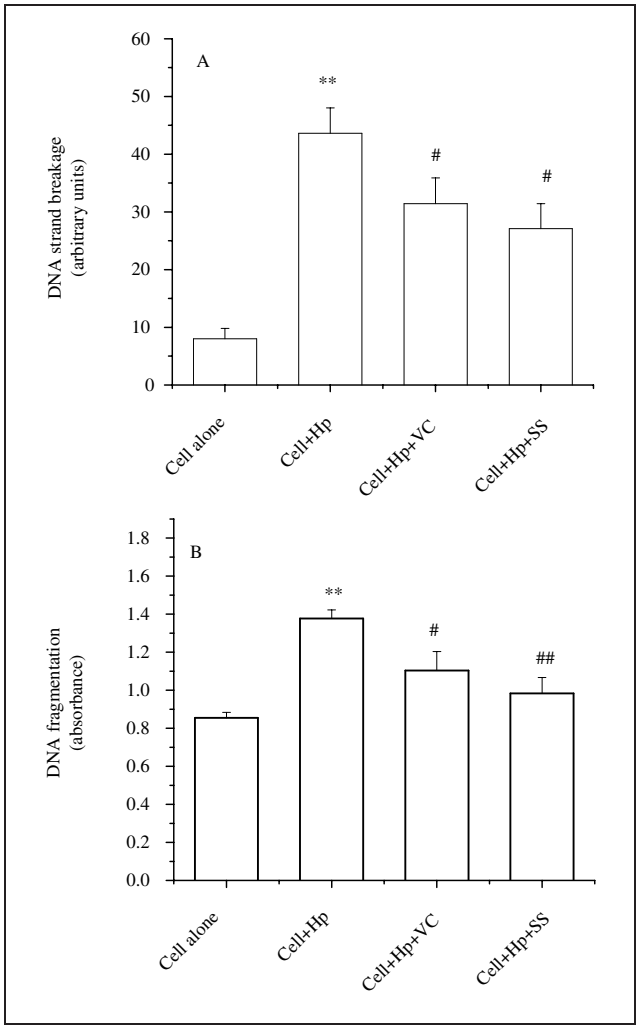




Fig. 9: Decrease of DNA strand breakage (A) and fragmentation (B) by vitamin C and sodium selenite in *H. pylori*-infected SGC-7901 cells. Cells were incubated for 24 h in RPMI 1640 medium alone, with *H. pylori* in combination with or without vitamin C or sodium selenite. DNA strand breakage were determined by comet assay and expressed as arbitrary units. DNA fragmentation was determined by quantifying the amount of oligonucleosome-bound DNA in the supernatant of cell lysate and expressed as absorbance. Hp: *H. pylori* (MOI 100); VC: vitamin C (200 μM); SS: sodium selenite (1 μM). Data represent means ± SD of triplicate experiments. ** $p < 0.01$ vs cell alone control, # $p < 0.05$, ## $p < 0.01$ vs *H. pylori*-infected control.

centration was 100 and 200 μM, and the sodium selenite concentration was more than 0.1 μM. At the same time, vitamin C (200 μM) and sodium selenite (1 μM) respectively and significantly diminished *H. pylori*-enhanced ROS formation in gastric epithelial cells (Fig. 8). The results suggest that both antioxidants decrease *H. pylori*-induced oxidative stress in SGC-7901 cells.

Fig. 8: Decrease of ROS formation by vitamin C (A) and sodium selenite (B) in *H. pylori*-infected SGC-7901 cells. Cells were incubated for 1 h in RPMI 1640 medium alone, with *H. pylori* in combination with or without vitamin C or sodium selenite. ROS formation was determined by DCF fluorescence (A and B) and the percent of fluorescing cell (C). Hp: *H. pylori* (MOI 400); VC: vitamin C (200 μM); SS: sodium selenite (1 μM). Data represent means ± SD of triplicate experiments. *** $p < 0.001$ vs cell alone control. ### $p < 0.001$ vs *H. pylori*-infected control.

### *2.4. Vitamin C and sodium selenite protected gastric epithelial cells against H. pylori-induced DNA damage*

Vitamin C 200 μM and sodium selenite 1 μM significantly decreased DNA strand breakage to 72.0% and 62.2% respectively (Fig. 9A) as well as DNA fragmentation to 80.1% and 71.4% respectively (Fig. 9B) compared with a *H. pylori*-infected control. The results suggest that both antioxidants protect DNA against *H. pylori*-induced damage, further demonstrating that DNA damage may be derived from oxidative stress in *H. pylori*-infected gastric cells.

In our experiment, vitamin C and sodium selenite, at the concentrations tested, did not affect *H. pylori* growth (data not shown). The protection by vitamin C and sodium selenite against *H. pylori*-related gastric cell damage indicated that both antioxidants at appropriate concentrations may have a preventive or therapeutic role against diseases associated with *H. pylori*. These data suggest that appropriate dietary supplementation with vitamin C and sodium selenite may prevent *H. pylori*-induced gastric cell injuries, which could have important implications for human nutrition.

In conclusion, *H. pylori* was found to induce DNA damage and oxidative stress in gastric epithelial cells SGC-7901. The antioxidants, vitamin C and sodium selenite, prevented *H. pylori*-associated cell injuries. The results suggest that DNA damage caused by *H. pylori*-induced oxidative stress may be a factor in the pathogenesis of *H. pylori*, and that vitamin C and sodium selenite may have a preventive or therapeutic role against *H. pylori*-associated gastric diseases.

## 3. Experimental

### *3.1. Reagents*

RPMI1640 and Mueller Hinton agar were purchased from Gibco Laboratories, Detroit, USA. Newborn bovine serum (NBS) was a product of Zhengzhou Bai'an Bioengineering Corporation Ltd., Zhengzhou, China. 2′,7′-dichlorofluorescin diacetate (DCFH-DA), *o*-phthalaldehyde, low and normal melting point agarose, ethidium bromide and sodium lauroyl sarcosine were purchased from Sigma Chemical Co., St. Louis, USA. Vitamin C and sodium selenite were purchased from Sino-American Biotechnology Company, Beijing, China. All other reagents used in the study were of high analytical purity.

### *3.2. H. pylori culture*

*H. pylori* type strain NCTC11637, a CagA-positive and cytotoxin-producing strain, was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, P.R. of China. *H. pylori* was grown on Mueller Hinton agar plates supplemented with 10% defibrinated horse blood and 0.2% glucose at 37 °C in a microaerophilic atmosphere (5% $O_2$, 10% $CO_2$ and 85% $N_2$) for 68 ~ 72 h, for a minimum of two and a maximum of six passages from frozen stocks. Bacteria were harvested from the plates by using sterile cotton swabs and suspended in RPMI 1640, washed twice, pelleted by centrifugation and resuspended in RPMI 1640 containing 10% NBS. To estimate bacteria titers, optical density measurements were performed at 600 nm and were correlated to viable colony counts (colony forming unit, cfu), 1 $OD_{600}$ corresponding to $2.5 \times 10^8$ cfu/ml.

### *3.3. Culture of gastric epithelial cell SGC-7901 and H. pylori infection*

The human gastric epithelial cell line SGC-7901 (Lin et al. 1981), purchased from the Cell Bank of the Chinese Academy of Science, Beijing, was used in the study. SGC-7901 cells were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere in RPMI 1640 containing 10% NBS in 25 $cm^2$ flasks (Corning Coster Corp., Cambridge, MA, USA) at a seeding density of $5 \times 10^4$ cells/ml, for a minimum of two and a maximum of eight passages from frozen stocks.

After SGC-7901 cells were cultured in RPMI 1640 containing 10% NBS for adhering 24 h at 37 °C, the medium was discarded and the cells were washed three times with RPMI 1640. Then *H. pylori* suspension was added to the cells according to the multiplicity of infection (MOI, i.e. the ratio of the number of bacteria to the number of host cells) and the cells continued to incubate for 24 h at 37 °C in a humidified 5% $CO_2$ atmosphere. Under this coculture condition, *H. pylori* does not grow (data not shown). For investigating the effects of antioxidants on *H. pylori*-related gastric cell injury, vitamin C and sodium selenite (dissolved in 10 mM PBS pH7.2, respectively) were added to the SGC-7901 cells before adding *H. pylori*. The cells were incubated in RPMI 1640 medium alone, or with *H. pylori* (at the MOI indicated), or with *H. pylori* in combination with antioxidant, vitamin C or sodium selenite, for 24 h at 37 °C in a humidified 5% $CO_2$ atmosphere.

### *3.4. Assessment of DNA strand breakage (Comet assay)*

DNA single strand breakage was assessed by the comet assay (Singh et al. 1988) with some modifications.

After incubation of SGC-7901 cells with *H. pylori* combined with or without antioxidants for 24 h, the cells were washed, harvested and centrifuged. The cell pellet was suspended in PBS at a concentration about $2 \times 10^5$ cells/ml and the cells were analyzed by comet assay as quickly as possible, after no more than 4 h stored at 4 °C. 25 μl cell suspension were mixed with 50 μl 1% w/v low melting point agarose dissolved in PBS at 37 °C, and then placed on a microscope slide which was precoated with 0.5% w/v normal melting point agarose dissolved in distilled water and covered with a coverglass. The slides were kept at 4 °C for 5 min for solidification of the agarose. After removing the coverglass, the slides were immersed in lysing solution (1% sodium lauroyl sarcosine, 2.5 M NaCl, 100 mM $Na_2$-EDTA, 10 mM Tris, pH 10, and 1% Triton X-100, 10% dimethylsulfoxide added freshly) for 70 min to lyse the cells and to permit DNA unfolding. They were then placed in an electrophoretic buffer (1 mM $Na_2$-EDTA and 300 mM NaOH, pH 13) for 20 min for DNA unwinding, followed by 20 min electrophoresis at a constant voltage of 25 V, 300 mA. Afterwards, the slides were carefully removed from the tank and washed gently with ice cold neutralizing buffer (0.4 M Tris, pH 7.5) twice for 5 min, dried and stained with 25 μl of 20 μg/ml ethidium bromide each, and then each covered with a coverglass. All of the steps described above were conducted in subdued light to prevent DNA damage from UV light.

Cells were viewed at magnification × 200 with a fluorescent microscope (BH-2 Olympus, excited by green light and a barrier filter of 590 nm) within 24 h. Photomicrographs were taken using Lucky ASA 400 black and white film. For each sample, three slides were scored visually as described by Collins (2004). 50 images per slide were classified into 5 categories according to the intensity of the fluorescence in the comet tail and given a value of either 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4). In this way, the total score per slide could range from 0 to 200, and DNA damage (strand breakage) was expressed in arbitrary units. The results from this method correlate very well with % tail DNA from computer image analysis (Collins 2004).

### *3.5. Assessment of DNA fragmentation*

DNA fragmentation was measured quantitatively by an ELISA assay that is used to detect histone-associated DNA fragmentation enriched in the cytoplasm (Wanger et al. 1997). The test was performed with the Cell Death Detection ELISA$^{plus}$ kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The absorbance value of the ELISA reaction reflects the amount of DNA fragmentation. Each test kit contained a DNA-histone-complex positive control. After incubation of SGC-7901 cells alone, or with *H. pylori* combined with or without antioxidant for 24 h, the cells were lysed and centrifuged, and the supernatants were used to detect DNA fragmentation by quantifying the amount of oligonucleosome-bound DNA. Absorbance was measured at a wavelength of 405 nm and a reference wavelength of 690 nm, and DNA fragmentation was expressed as absorbance value.

### *3.6. Measurement of reactive oxygen species formation in SGC-7901*

After SGC-7901 cells were cultured in RPMI 1640 containing 10% NBS for adhering 24 h at 37 °C in 5% $CO_2$, the cells were washed three times with RPMI 1640, then DCFH-DA was added at a final concentration of 20 μM and they were incubated for 15 min at 37 °C. *H. pylori* suspension was added to the cells having been loaded with DCFH-DA and the cells were incubated for another 1 h in 5% $CO_2$ at 37 °C. Cells were harvested by 0.02% EDTA dissociation, washed twice with PBS, and resuspended in PBS. The fluorescence intensities of intracellular DCF formed by the reaction of DCFH-DA with ROS were analyzed using a Coulter Epics® XL Flow Cytometer and System II version 3.0 software (Excitation wavelength 488 nm, emission wavelength 525 nm). In each sample, 10000 cells were examined. The results were expressed as percentage of cells containing DCF fluorescence to total cells (Bass et al. 1983; Wolber et al. 1987; LeBel et al. 1992; Obst et al. 2000).

### 3.7. Measurement of intracellular GSH content

GSH was determined using a fluorometric assay described by Hissin and Hilf (1976) with some modifications.

After incubation of SGC-7901 cells alone, or with *H. pylori* combined with or without antioxidant for 24 h, the cells were harvested, centrifuged, lysed with 400 μL of 0.5% Triton X-100 in PBS (pH 7.2) and divided into two equal parts: one part was used for determining protein content by Lowry's method (Lowry et al. 1951); another part was used for determining GSH content. After the protein in the latter part was precipitated with 20 μL of 50% trichloroacetic acid and centrifuged for 20 min at $4{,}000 \times g$, the supernatant was used for determining GSH content with *o*-phthalaldehyde by a fluorometric method (Hissin and Hilf, 1976) with some modification. Briefly, 100 μl acid GSH standard solution or acid supernatant of cell lysate was mixed with 200 μl methanol solution, then 2.5 ml 100 mM $Na_2HPO_4$ − 5 mM EDTA buffer and 200 μl of 0.1% (wt/vol) *o*-phthalaldehyde in methanol were added, mixed and kept for 45 min at room temperature in subdued light conditions. The fluorescence was measured on a Shimadzu RF5000 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) at an excitation wavelength of 350 nm and an emission wavelength of 425 nm. The GSH level was expressed as nmol/mg protein.

### 3.8. Statistical analysis

Results were expressed as means ± SEM of at least triplicate experiments. Student's t-test was used for statistical analysis as appropriate. $p < 0.05$ were taken to imply statistical significance, $p < 0.01$ were considered to be extremely significant.

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