Effective Prevention and Treatment of *Helicobacter pylori* Infection Using a Combination of Catechins and Sialic Acid in AGS Cells and BALB/c Mice\(^{1,2}\)

Jyh-Chin Yang,\(^{3,6}\) Chia-Tung Shun,\(^4\) Chiang-Ting Chien,\(^{5,6,*}\) and Teh-Hong Wang\(^3\)

Departments of \(^{2}\)Internal Medicine, \(^{4}\)Forensic Medicine and Pathology, and \(^{3}\)Medical Research and \(^{6}\)Graduate Institute of Clinical Medicine, Hospital and College of Medicine, National Taiwan University, 10043 Taipei, Taiwan

### Abstract

The increasing emergence of *Helicobacter pylori* strains resistant to antibiotics may cause unsuccessful treatment. An alternative agent or mixture with anti-*H. pylori* effect is urgently required to reduce *H. pylori* infection. We explored the preventive and therapeutic potential of a combination of catechins and sialic acid on *H. pylori*-infected human gastric cells in vitro and in mice in vivo. We evaluated the anti-*H. pylori* activity of catechins and/or sialic acid using the agar dilution and checkerboard methods. The effect of catechins and/or sialic acid on *H. pylori* infection-induced oxidative stress and apoptosis/autophagy in cell culture was explored using an ultrasensitive chemiluminescence analyzer, immunocytochemistry, and Western blotting. Specific pathogen-free BALB/c mice were divided into uninfected control, infected control, pretreated, and post-treated groups. The effects of catechins/sialic acid were determined by histology and immunocytochemistry. The combination of catechins and sialic acid showed synergistic or additive anti-*H. pylori* activity and significantly reduced inducible nitric oxide synthase expression and Bax/Bcl-2-mediated apoptosis but enhanced Beclin-1-mediated autophagy. All mice infected with *H. pylori* displayed gastritis and accumulation of 3-nitrotyrosine and 4-hydroxynonenal. Pretreatment with catechins/sialic acid completely prevented *H. pylori* infection and resulted in normal histology. Post-treatment with catechins/sialic acid decreased the bacterial load and gastritis score and eradicated up to 60% of *H. pylori* infections in a dose-dependent manner. This is the first demonstration to our knowledge of a nonprobiotic, nonantibiotic treatment that is 100% effective in preventing and has promising possibilities for treating *H. pylori* infection. Further studies are needed to confirm this result in humans. J. Nutr. 138: 2084–2090, 2008.

### Introduction

*Helicobacter pylori* is strongly associated with chronic active type B gastritis, peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma (1). Currently, a 1-wk combination therapy of a proton pump inhibitor and antibiotics is used as the treatment of choice for *H. pylori* infection (2). However, poor compliance and the increasing emergence of *H. pylori* strains resistant to some of these agents lead to eradication failure in some patients (3). Following failure of the initial treatment, 2nd-line therapies, including alternative triple and quadruple regimens, have been recommended. However, these drugs still cannot solve the problem of the rising trend in antibiotic resistance. An alternative agent or mixture with preventive and therapeutic effects is urgently required to reduce *H. pylori* infection.

The initial step in *H. pylori* infection is the penetration and adherence of the bacterium to mucin and gastric epithelial cells through several different adhesion molecules (4). Antiadhesive therapy using 3′-sialyllactose has been shown to prevent the binding of *H. pylori* to various human gastrointestinal epithelial cells in vitro (5) and to decrease *H. pylori* colonization in rhesus monkeys without side effects (6). After adhering to the gastric mucosa, *H. pylori* causes gastric epithelial cell damage and atrophy via oxidative stress and the type I apoptotic or type II autophagic programmed cell death-related pathway (7,8). Catechins belong to well-characterized flavanol group of polyphenols (9). Catechins and their major active component, epigallocatechin-3-gallate (EGCG),\(^7\) have antioxidative, antin...

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\(^{*}\) To whom correspondence should be addressed. E-mail: ctchien@ntuh.gov.tw.

\(^{7}\) Abbreviations used: Bax/Bcl-2, proapoptotic Bcl-2 family proteins; CFU, colony-forming unit; CS, water containing glucose and catechins/sialic acid; EGCG, epigallocatechin gallate; FIC, fractional inhibitory concentration; 4-HNE, 4-hydroxynonenal; H2O2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; MIC, minimal inhibitory concentration; NO, nitric oxide; 3-NT, 3-nitrotyrosine; O2\(^{−}\), superoxide; ROS, reactive oxygen species.
flammatory, antiapoptotic, and cancer prevention activities (10–12). Moreover, catechins and EGCG have antibacterial activity against various food-borne pathogenic bacteria and against *H. pylori* by inhibiting *H. pylori* urease and vacuolating cytotoxin A activity (13–16). These data indicate that catechins and 3’-sialyllactose have an inhibitory effect on *H. pylori* infection in vitro. However, they fail to effectively control infection in animal models in vivo when each is used alone (7,15,16).

As far as we know, the effect of combined catechins/sialic acid treatment on *H. pylori* infection has not yet been determined. To search for a treatment with preventive and therapeutic potential against *H. pylori*, we studied the combined effect of catechins and sialic acid in the control of *H. pylori* infection in gastric epithelial cell in vitro and in mice in vivo.

**Materials and Methods**

**Bacterial strains and drugs.** The standard strain ATCC 43504 and 20 clinical isolates (TA1–TA20) of *H. pylori* were used. The clinical isolates were obtained from gastric biopsy specimens from patients with gastritis and peptic ulcer after getting the informed consents. Decaffeinated green tea extract was purchased from Vinho Biochemistry; this consisted of 328 mg/g of epigallocatechin gallate, 152 mg/g of epicatechin gallate, 148 mg/g of gallolecatechin gallate, 132 mg/g of epicatechin, 108 mg/g of epigallocatechin, 104 mg/g of gallolecatechin, and 44 mg/g of catechin. Sialic acid was obtained from Sigma.

**In vitro antibacterial activity.** The test strains were grown as described previously (17) and stored at −80°C until required. They were recovered at 37°C for 3 d under microaerophilic conditions (5% O2, 10% CO2, 85% N2), then suspended in 10 mL of Brucella broth for 24 h until they reached an optical density at 450 nm of 0.5 units, corresponding to a concentration of ~10⁸ colony-forming units (CFU)/L. The minimal inhibitory concentrations (MIC) of catechins and sialic acid were determined by the agar dilution method as described previously (18). The effect of a combination of catechins and sialic acid was determined by the checkerboard method and evaluated using the fractional inhibitory concentration (FIC) index as described previously (19).

**Cell culture system.** A cytotoxin-associated gene A-lesaculating cytotoxin A-positive strain of *H. pylori* (TA1) was recovered from frozen stock by seeding on Columbia agar plate containing 5% sheep blood at 37°C for 3 d under microaerophilic conditions. The human gastric cancer cell line ATCC CRL 1739 (AGS cells) was cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% IsoVitaleX, and antibiotics and maintained for 48 h, then the concentration was adjusted to ~10⁸ CFU/mL. After the bacteria were washed off the plates and resuspended in PBS to an OD at 430 nm of 1.0 units, corresponding to a bacterial concentration of 2 × 10¹¹ CFU/mL, and added to wells containing 2 × 10⁷ gastric epithelial cells at an *H. pylori*:AGS cell ratio of 100:1 and were then coccultured for 4 h in the absence or presence of 128 mg/L of catechins and/or 32 mg/L of sialic acid in a cell culture incubator.

**Oxidative stress measurement.** The nitric oxide (NO) concentration was measured using an NO chemiluminesence probe and a Chemiluminesence Analyzing System (CLD-110, Tohoku Electronic) (21). For coculture of *H. pylori* and AGS cells, the bacteria were washed off the plates and resuspended in PBS to an OD at 430 nm of 1.0 units, corresponding to a bacterial concentration of 2 × 10¹¹ CFU/mL, and added to wells containing 2 × 10⁷ gastric epithelial cells at an *H. pylori*:AGS cell ratio of 100:1 and were then coccultured for 4 h in the absence or presence of 128 mg/L of catechins and/or 32 mg/L of sialic acid in a cell culture incubator.

**Apoptosis and autophagy assay.** *H. pylori*-induced AGS cell apoptosis was assayed in triplicate using the terminal deoxynucleotidyl transferase-mediated nick-end labeling method (23). Autophagic vacuoles were labeled in triplicate with 0.05 mmol/L monodansylcadaverine (24). After labeling, the cells were washed 4 times with PBS and immediately fixed with 4% paraformaldehyde and observed under a fluorescence microscope (Leica model DMRED).

**Table 1** In vitro antimicrobial activities of catechins or sialic acid against 20 *H. pylori* strains

<table>
<thead>
<tr>
<th>Drug</th>
<th>Range</th>
<th>MIC mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechins</td>
<td>32–1024</td>
<td>128</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>&gt;4000</td>
<td>256</td>
</tr>
</tbody>
</table>

1 These clinical isolates consisted of 10 strains double resistant to metronidazole (MIC ≥ 24 mg/L) and clarithromycin (MIC ≥ 8 mg/L) and 10 strains sensitive to both antibiotics.

**Western blots.** After 4-h treatment of cells with *H. pylori* or catechins and/or sialic acid, proteins were extracted from the cells and electrophoresed on 10% SDS-PAGE, then transferred to polyvinylidene difluoride membranes using a semidry transfer system (Hoeffer Pharmacia Biotech) (23). The membranes were blocked for 2 h at room temperature in PBS containing 5% skim milk (blocking buffer), then incubated for 1 h at room temperature in triplicate with blocking buffer containing antibodies against inducible NO synthase (iNOS) (Chemicon), Bax, Bcl-2, caspase 3, poly-(ADP-ribose)-polymerase (all from Cell Signaling Technology), or Belcin-1 (BD Biosciences). The membranes were then washed 3 times and incubated for 1 h at room temperature with blocking buffer containing horseradish peroxidase-conjugated rabbit anti-IgG antibody (Pierce). The signals were detected by enhanced chemical luminescence (Amersham Biosciences) and exposure to X-ray film.

**Animal model.** Five-wk-old male specific-pathogen-free BALB/c mice were obtained from the National Laboratory Animal Center, Taiwan, and housed at the Experimental Animal Center, National Taiwan University, at a constant temperature. Mice consumed food [picolab mouse diet 20, PMIEnter National Nutrition (20.5% of protein, 18.5% of fat, 53% of carbohydrate, 2.7% of fiber, 4.8% of mineral)] and water ad libitum. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine and were in accordance with the guidelines of the National Science Council of Taiwan.

**Table 2** Combined effects of catechins and sialic acid against *H. pylori* strains

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Strains, n</th>
<th>Synergistic</th>
<th>Additive</th>
<th>Indifferent</th>
<th>Antagonistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 S, Antibiotic-sensitive isolates; R, isolates resistant to both metronidazole and clarithromycin.

2 Significant difference between the groups with FIC ≤ 1 and the group with FIC > 1, *P* < 0.01.
inoculation, then had free access to drinking CS solution for 5 d. The infected controls received 1% glucose water orally for 3 d before to 5 d after infection. All procedures other than those described above were the same in all 4 study groups. The daily water/solution intake was ~25 mL per mouse.

Four wk after H. pylori inoculation, the mice were killed by anesthesia with 0.2–0.5 mL of 50% urethane and their stomachs removed and longitudinally divided into 2 equal parts for histological and microbiological examination. H. pylori was positively identified after 3–5 d culture and the CFU of H. pylori counted after culturing. Gastritis was graded by the pathologist without knowledge of the treatment protocol according to the updated Sydney system (26). Confirmation of H. pylori status in gastric tissue was adapted by PCR (17,27).

Using the same procedure as in the post-treated and infected control groups of the first experiment, the effects of different concentrations of catechins/sialic acid on the eradication of H. pylori infection were further investigated. Sixty mice divided into 3 groups were post-treated, respectively, with the same dose or with 2× and 5× doses. Another 10 mice served as infected control. The eradication rate of H. pylori in each group was evaluated 4 wk after post-treatment of catechins/sialic acid.

**In situ demonstration of 3-nitrotyrosine and 4-hydroxynonenal in H. pylori-infected gastric tissue.** We immunostained the oxidative markers 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) in paraffin-embedded sections (28). They were incubated overnight at 4°C with rabbit anti-nitrotyrosine IgG antibodies (NITT12-A) or rabbit anti-HNE antibodies HNE11-S (both from Alpha Diagnostic, both diluted 1:50 in PBS). The sections were stained by an avidin-biotinylated horseradish peroxidase procedure using a commercially available kit (ABC Elite, Vector Laboratories). The signal was visualized by incubating the sections with liquid diaminobenzidine tetrahydrochloride. Hematoxylin was used to counter-stain the sections.

**Statistical analysis.** The Score test (29) and binomial test (30) were used to test the equality of 4 proportions and reveal the significant differences between the groups in the combined effects of anti-H. pylori. One-way ANOVA and Duncan’s multiple-range test were used to examine differences among groups in the cell culture system. One-way ANOVA or the Kruskal-Wallis test and Dunnett’s multiple comparison test were used to test the equality of 4 proportions and reveal the significant differences between the groups in the combined effects of anti-H. pylori.

**Results**

**In vitro antibacterial activity of catechins and sialic acid.** The catechins possessed antibacterial activity against all clinical isolates of H. pylori in vitro regardless of the sensitivity of these isolates to antibiotics. The MIC of the catechins for 90% of isolates (MIC90) was 256 mg/L (Table 1). Sialic acid alone did not show any anti-H. pylori effect. All clinical isolates were susceptible to the combination of catechins and sialic acid, which had either an additive or a synergistic effect (Table 2). These data show that sialic acid enhanced the antibacterial activity of catechins against most clinical isolates tested. Our checkerboard study (data not shown) demonstrated that the combination of 128 mg/L catechins and 32 mg/L sialic acid completely inhibited the growth of all isolates tested in vitro. Antibiotic-sensitive and -resistant isolates did not differ in susceptibility to the combination of catechins and sialic acid.

**Antioxidant activity in cell culture.** H. pylori infection increased O$_2^-$, H$_2$O$_2$, NO production, and iNOS expression in AGS cell cultures (Fig. 1). This effect was noted after 1 h of H. pylori infection and persisted until 4 h after infection. These effects were significantly suppressed by the presence of catechins and/or sialic acid in the 4-h H. pylori-AGS cell cocultures. H. pylori infection increased Bax expression and decreased Bcl-2 expression, suggesting that the increased Bax:Bcl-2 ratio enhanced apoptosis in H. pylori-infected AGS cells (Fig. 2). Catechins and/or sialic acid suppressed Bax expression and increased Bcl-2 expression, suggesting that catechins and/or sialic acid could reduce the apoptotic effect of H. pylori infection. Both the expression of caspase 3 and the expression of poly-(ADP-ribose)-polymerase were also significantly in-
H. pylori infection decreased the expression of the Beclin-1 (Fig. 3), but catechins and/or sialic acid stimulated the production of Beclin-1 and enhanced Beclin-1-dependent autophagy. H. pylori infection of AGS cells for 4 h induced morphologic changes, apoptosis formation, and inhibition of autophagy (Fig. 4B,E,I) compared with control cells (Fig. 4A,E,I). Combined catechins/sialic acid treatment resulted in preservation of cell morphology, inhibition of apoptosis, and maintenance of autophagy (Fig. 4C,G,K). Combined catechins/sialic acid treatment of uninfected AGS cells had no harmful effects (Fig. 4D,H,L).

**Inhibitory effect of catechins and sialic acid on H. pylori colonization and H. pylori-related gastric injury in mice.** All mice in the inoculated control group were successfully infected (Table 3). Most showed gross mucosal injury with edema and hemorrhage. Microscopically, prominent gastritis with infiltration of many mononuclear cells and neutrophils was observed in all mice in this group (Fig. 5F–H). The mean gastritis score was 2.0. In the pretreatment group, none of the mice were infected with *H. pylori* and had no gross mucosal injury. There was only minimal histological change microscopically (Fig. 5C,D). The mean gastritis score was 0.3, the same as in noninfected mice treated with distilled water only (Fig. 5A,B). In the post-treated group, some mice (20%) were cleared of *H. pylori* infection. Although most uneradicated mice in this group had gross mucosal injury, the average score of 0.8 for microscopic gastritis was lower (*P* < 0.01) than that in the infected control group (Fig. 5E). Uninfected, pretreated, and post-treated groups differed from the infected control group (all *P* < 0.01). Gastric accumulation of 3-NT and 4-HNE adducts was pronounced in the proximal part of the stomach of *H. pylori*-infected mice and was much lower in those of mice pretreated with the catechins/sialic acid (Fig. 5I–N). *H. pylori* eradication was the post-treatment of catechins/sialic acid in a dose-dependent manner. The eradication rates were 0% in the nontreated group, 20% in the 1× group, 30% in the 2× group, and 60% in the 5× group. The dosage effect on eradication rate was significant (*P* < 0.01), with odds ratio = 1.695 for every fold of standard dose added.

**Discussion**

The current antibiotic-based therapies are generally effective but may fail due to antibiotic resistance or low compliance. Efforts to find an effective method for the nonantibiotic control of *H. pylori* infection are therefore urgently required. Some strains of *Lactobacillus* and *Bifidobacterium* can inhibit *H. pylori* growth. However, a systematic review of clinical trials has suggested that probiotics do not eradicate *H. pylori* but maintain a lower level of this pathogen in the stomach (31). A vaccine can be used either prophylactically or therapeutically for *H. pylori* infection (32). In the mice, vaccination can result in significantly reduced *H. pylori* colonization but cannot achieve satisfactory eradication or prevention of this infectious disease (32). Several nonantibiotic compounds can inhibit the growth of *H. pylori* (33). Among these, adhesion receptor antagonists, such as 3'-sialyllactose, and antioxidants, such as tea catechins, have shown promising results (6,8,13). Although catechins, 3'-sialyllactose, or sialic acid inhibit *H. pylori* infection in vitro, infection could not be completely controlled in mice models in vivo when either was used alone in our pilot studies (data not shown) and other reports (6,13,14). In the present study, we found that this combination was very efficient in prevention of *H. pylori* infection in vitro and in vivo when given as a pretreatment and also had a dose-dependent effect on *H. pylori* eradication in infected mice when given after infection.
Apoposis and autophagy are 2 tightly regulated biological processes that play a central role in tissue homeostasis and disease development. Recently, we reported that increased production of reactive oxygen species (ROS) results in severe type I programmed cell death, including increased DNA fragmentation and apoptotic cell number in damaged tissue (10,23,34). Autophagy is type II programmed cell death and is a major lysosomal catabolic pathway for cytoplasmic macromolecules and organelles. Beclin-1, a novel Bcl-2-interacting protein, promotes autophagocytosis (35). Autophagy seems to play a role in promoting a cell survival response (36). In this study, we found that \textit{H. pylori}-induced AGS cell damage was caused by increased Bax/Bcl-2-related proapoptotic cell death and decreased autophagy survival and/or repair and that application of catechins and sialic acid ameliorated these responses. We suggest that the catechins/sialic acid combination causes downregulation of apoptosis and upregulation of autophagy to protect AGS cells against \textit{H. pylori} infection.

Previous study has shown that \textit{H. pylori} induces DNA damage and apoptosis with considerable production of ROS and iNOS in several experimental backgrounds (37). EGCG has a direct scavenging activity and can therefore prevent DNA damage by various noxious stimulants (10,38). In the present study, we demonstrated that catechins had a bactericidal effect against \textit{H. pylori} and that sialic acid reinforced this effect. Although this additive/synergistic effect was independent of the antibiotic susceptibility of \textit{H. pylori}, the mechanism of this effect is still unclear. The catechins/sialic acid combination significantly decreased the epithelial cell damage induced by \textit{H. pylori}-related ROS in a cell culture system. Furthermore, in a mouse animal model, the catechins/sialic acid combination also decreased the severity of gastritis and gastric mucosal damage and the accumulation of gastric-oxidized protein and lipid products, such as 3-NT and 4-HNE. Given that a heavy bacterial load and damaged epithelia are the crucial variables for \textit{H. pylori} infection, our results indicate that the combination of catechins and sialic acid can enhance the ability of the gastric epithelium to fight against adhesion of, and colonization and persistent infection by, \textit{H. pylori}. Although the treatment using catechins or using sialic acid alone was as effective as the treatment using the combination of these 2 compounds in in vitro study (Fig. 2 and 3), the effectiveness of treatment using each alone was much less effective in in vivo study. This discrepancy between the in vitro and in vivo results may be related to the influences of the bioavailability of these compounds and the interactions between \textit{H. pylori} and its host.

The attenuation of chronic longstanding \textit{H. pylori} infection might be associated with the prevention of chronic atrophic gastritis or gastric carcinogenesis (1). Based on the results of our in vitro study of antibacterial activity, we choose the mixture of 128 mg/L of catechins and 32 mg/L of sialic acid as the standard doses to be used in all subsequent studies in the cell culture system and mice. We found that these doses completely prevented \textit{H. pylori} infection in mice and that the combination was much more effective than either alone. Because the infection is generally acquired during childhood (1), regular intake of these 2 compounds in children might constitute a low-cost, large-scale solution for reducing \textit{H. pylori} infection worldwide. The main sources of catechins include tea, red wine, fruit, and some plants, whereas sialic acid is found widely distributed in animal tissues such as gastrointestinal mucins and milk, especially in glycoproteins and gangliosides (5,6,39). In this study, \(\approx\)25 mL of CS solution was taken for each mouse per day. With the assumption that the response of human to this treatment is

\begin{table}
\centering
\caption{Effects of the catechins/sialic acid combination on gastric lesions in \textit{H. pylori}-inoculated BALB/c mice\(^1\)}
\begin{tabular}{cccc}
\hline
Groups & \multicolumn{2}{c}{\textit{H. pylori} infection} & \multicolumn{2}{c}{Gastritis score} \\
& Macroscopic & Bacterial & score \\
& damage & count & \\
\hline
Uninfected & 0 (0) & 0 (0) & 0.3 ± 0.5 \(^*\) \\
Post-treated & 0 (0) & 0 (0) & 0.3 ± 0.5 \(^*\) \\
Infected & 10 (100) & 6 (60) & 1.0 ± 0.8 \(^*\) \\
\hline
\end{tabular}
\end{table}

\(^1\) Values are means ± SD, \(n = 10\) or \(n (%)\). *Different from the uninfected control in that column; \# different from the infected control in that column, \(P < 0.01\).
similar to the response of mice, then a dose of 5.6 g of catechins and 1.4 g of sialic acid would be needed for a 70-kg human. The flavonoids are the most common and the largest plant polyphenolics obtained from the typical plant-source diet and sialic acid is an important component of gastrointestinal mucus and milk. Therefore, these 2 compounds are widely accepted to be very safe to humans (5,6,39). By showing a promising eradication rate (up to 60%) in a dose-dependent manner, this combination may have potential as an alternative or adjuvant regimen for the treatment of \textit{H. pylori} infection, particularly in cases colonized by multiple antibiotic-resistant strains. The optimal dosing method for these purposes deserves further investigation.

In summary, \textit{H. pylori} infection causes detrimental injury, including oxidative stress, inflammation, and apoptosis formation but inhibits the autophagy survival pathway in AGS cell cultures in vitro and in mice in vivo. A nontoxic combination of catechins and sialic acid can reverse these damaging processes, enhance the repair system, and efficiently prevent and treat \textit{H. pylori} infection in vivo. This is the first demonstration of a nonprobiotic, non-antibiotic treatment that is 100% effective in preventing and has promising results in treating \textit{H. pylori} infection in mice. Further studies are needed to confirm this result in humans.

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Literature Cited


