Effective Prevention and Treatment of Helicobacter pylori Infection Using a Combination of Catechins and Sialic Acid in AGS Cells and BALB/c Mice

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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.

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, antiin-
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 or α-D-mannose and α-D-galactose 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 Vignour Biochemistry; this consisted of 328 mg/g of epigallocatechin gallate, 152 mg/g of epicatechin gallate, 148 mg/g of galloallocatechin gallate, 132 mg/g of epicatechin, 108 mg/g of epigallocatechin, 104 mg/g of galloallocatechin, 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)/mL. 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 (cagA)-vacuolating 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 ~1011 CFU/mL, and added to wells containing 2 × 105 gastric epithelial cells at an H. pylori/AGS cell ratio of 100:1 and were then cocultured 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 chemiluminescence probe and a Chemiluminescence Analyzer System (CLD-110, Tohoku Electronic) (21). For measurement of O2·− and hydrogen peroxide (H2O2), a 0.2-mL culture sample and 0.5 mL of 0.1 mmol/L lucigenin or 0.2 mmol/L luminol in PBS, pH 7.4, was injected into the chamber for chemiluminescence assay. 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.

The H. pylori infection mouse model was modified from the previous study (25). Forty mice were divided into 4 groups of 10 mice each. The H. pylori strain TA1 was used to i.g. infect mice. The recovered bacterial colonies were transferred to Brucella broth supplemented with 5% fetal bovine serum, 1% IsoVitaleX, and antibiotics and maintained for 48 h, then the concentration was adjusted to ~1011 bacteria/mL. Three groups of mice were inoculated i.g. 2 times on successive days with 0.5 mL of bacterial suspension. Uninfected control mice received distilled water only. The mice in the pretreatment group were pretreated with 0.5 mL of distilled water containing 128 mg/L of catechins and 32 mg/L of sialic acid 72 h before H. pylori inoculation, then had free access to drinking water containing 1% glucose and a mixture of 128 mg/L of catechins and 32 mg/L of sialic acid (CS solution) for 3 d. Mice in the post-treatment group were post-treated with 0.5 mL of distilled water containing 128 mg/L of catechins and 32 mg/L of sialic acid at 2 wk after H. pylori infection.

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Range</th>
<th>For 50% of strains</th>
<th>For 90% of strains</th>
</tr>
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<tbody>
<tr>
<td>Catechins</td>
<td>32–1024</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>&gt;4000</td>
<td>&gt;4000</td>
<td>&gt;4000</td>
</tr>
</tbody>
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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 Beclin-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.

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Strains, n</th>
<th>FIC ≤ 0.5</th>
<th>0.5 &lt; FIC ≤ 1</th>
<th>1 &lt; FIC ≤ 2</th>
<th>FIC &gt; 2</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 groups with FIC > 1, P < 0.01.

Catechins/sialic acid prevent and treat Helicobacter pylori 2085
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 infection. Both the expression of caspase 3 and the expression of poly-(ADP-ribose)-polymerase were also significantly increased in the animal model. We applied simple logistic regression to reveal the dosage effect on eradication rate. Differences with a P-value < 0.05 were considered significant.

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 25.6 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 O₂⁻, H₂O₂, 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-

FIGURE 1 Effect of catechins (C) and/or sialic acid (S) on O₂⁻ (A), H₂O₂ (B), and NO (C) production and iNOS expression (D) in AGS cells at 4 h after infection with H. pylori (HP). In D, a western blot is shown and the untreated, noninfected control was set at 1.0. Values are means ± SEM, n = 3. *Different from the untreated, noninfected control, P < 0.05.
Inhibitory effect of catechins and sialic acid on microscopic gastritis was lower (had gross mucosal injury, the average score of 0.8 for *H. pylori* infection. Although most uneradicated mice in this group observed in all mice in this group (with infiltration of many mononuclear cells and neutrophils was edema and hemorrhage. Microscopically, prominent gastritis infected (all mice in the inoculated control group were successfully 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* eradicated by the post-treatment of catechins/sialic acid in a dose-dependent manner. The eradication rates were 0% in the noninfected 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.

**FIGURE 2** Effect of catechins (C) and/or sialic acid (S) on apoptosis in AGS cells at 4 h after infection with *H. pylori* (HP). (A) Western blot for protein levels. (B) The Bax:Bcl-2 ratio. The untreated, noninfected control was set at 1.0. Each column with a vertical line represents the mean ± SEM, *n* = 3. *Different from the untreated noninfected control, *P* < 0.05; #Different from the *H. pylori* infection alone, *P* < 0.05.

**FIGURE 3** Effect of catechins (C) and/or sialic acid (S) on autophagy in AGS cells at 4 h after infection with *H. pylori* (HP). A Western blot is shown in the upper panel and the lower panel shows the folds of Beclin-1 levels expressed relative to the noninfected control. The untreated noninfected control was set at 1.0. Each column with a vertical line represents the mean ± SEM, *n* = 3. *Different from *H. pylori* infection alone, *P* < 0.05.

**FIGURE 4** Effects (A,B,C), autophagy (D,E,F), and (G,H) upregulation of Beclin-1 in AGS cells at 4 h after infection with *H. pylori*. (A) Western blot for protein levels. (B) The untreated, noninfected control was set at 1.0. Each column with a vertical line represents the mean ± SEM, *n* = 3. *Different from the untreated noninfected control, *P* < 0.05; #Different from the *H. pylori* infection alone, *P* < 0.05.
Apoptosis 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 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 H. pylori infection.

Previous study has shown that 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 H. pylori and that sialic acid reinforced this effect. Although this additive/synergistic effect was independent of the antibiotic susceptibility of H. pylori, the mechanism of this effect is still unclear. The catechins/sialic acid combination significantly decreased the epithelial cell damage induced by 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 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, 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 H. pylori and its host.

The attenuation of chronic longstanding 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 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 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, ~25 mL of CS solution was taken for each mouse per day. With the assumption that the response of human to this treatment is especially in glycoproteins and gangliosides (5,6,39). In this study, ~25 mL of CS solution was taken for each mouse per day. With the assumption that the response of human to this treatment is...

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Literature Cited


