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Effect of the flavonoid quercetin on inflammation and lipid peroxidation induced by *Helicobacter pylori* in gastric mucosa of guinea pig

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Background. *Helicobacter pylori* infection induces an inflammatory response in the gastric mucosa. Activation of polymorphonuclear leukocytes can produce oxidative damage to gastric tissue through intermediary radicals of oxygen and nitrogen. Vegetable extracts containing polyphenols of the flavonoid family have antibacterial activity, and the flavonoid quercetin possesses anti-*H. pylori* activity in vitro. The aim of this study was to analyze the effect of oral administration of pure quercetin on inflammation and lipid peroxidation induced by *H. pylori* in the gastric mucosa of the guinea pig. ***Methods.*** Sixty days after oral infection with *H. pylori* guinea pigs received 200 mg/kg of quercetin daily by mouth for 15 days. The infiltration index of inflammatory cells and bacterial density in both the pyloric antrum and corpus were histologically determined by myeloperoxidase histochemistry, hematoxylin-eosin, and modified Giemsa stains. The lipid hydroperoxide content was assessed by the orange xylenol spectrophotometric method. ***Results.*** Quercetin significantly reduced the infiltration index of mononuclear cell and bacterial colonization in the pyloric antrum and corpus. In the antrum of infected quercetin-treated animals, a significant diminution of neutrophil leukocyte infiltration was observed compared with the infected non-quercetin-treated animals. In the antrum, the lipid hydroperoxide concentration was significantly decreased in infected animals treated with quercetin, whereas in the corpus no significant differences were observed. ***Conclusions.*** Our results indicate that in vivo oral quercetin administration decreases *H. pylori* infection in the gastric mucosa and reduces both the inflammatory response and lipid peroxidation.

Key words: neutrophil leukocytes, myeloperoxidase, oxidative stress, gastritis

Introduction

Colonization of the gastric mucosa by *Helicobacter pylori* induces, through its bacterial derivatives such as CagA, cytotoxin VacA, and heat shock protein 60, the production of proinflammatory factors such as interleukin (IL)-8, IL-1, and tumor necrosis factor (TNF)-α.[1–3] The bacterial derivatives cause inflammatory infiltration in the stomach tissue consisting of neutrophil and eosinophil leukocytes, lymphocytes, plasma cells, and macrophages.[4–6] Several studies have indicated that *H. pylori* infection is accompanied by an inflammatory response associated with gastric mucosal damage through the activation of polymorphonuclear neutrophil leukocytes.[7] In these cells the activation of enzymatic systems such as NADPH oxidase, inducible nitric oxide synthase, and myeloperoxidase form reactive species of oxygen and nitrogen, precursors of the production of hypochlorite, monochloramine, and peroxinitrite, with oxidative and cytolytic effects on the gastric mucosa.[8,9]

There is evidence that vegetable extracts possess antibacterial activity against *H. pylori*. Vegetal polyphenols containing flavonoids have antibacterial activity, as has been demonstrated in vitro by using crude plant preparations from *Origanum vulgare* and *Cistus laurifolius*.[10–12] However, few in vivo studies using pure flavonoids have been published.

Quercetin is a natural flavonoid present in fruits and vegetables.[13] Several authors have reported that this compound has antiulcer action in the stomach and decreases lipid peroxidation in rats.[14,15] To our knowledge there are no in vivo studies of the antibacterial effect of quercetin on *H. pylori* infection. The aim of

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the present study was to analyze the effect of oral administration of the flavonoid pure quercetin on inflammation, lipid peroxidation, and *H. pylori* eradication in the gastric mucosa of the infected guinea pigs.

Methods

Bacterial and culture conditions

Helicobacter pylori were obtained from biopsies of human patients with a diagnosis of gastroduodenal disease associated with *H. pylori* infection. The bacteria were cultured under microaerophilic conditions (10% CO_2 and 5% O_2) in a plate of blood agar (Becton Dickinson, Mexico D.F., Mexico) supplemented with defibrinated lamb's blood (5% v/v) and antibiotics: vancomycin (2.5 mg/l), trimethoprim (1.25 mg/l), and amphotericin B (1.25 mg/l) (Oxoid, Hampshire, UK) at 37°C. The bacterium was characterized by morphology, urease assay, Gram stain, catalase assay, and *ureC* polymerase chain reaction (PCR) amplification.[16] Positivity to CagA and VacA (s1/m1) virulence factors were determinate for PCR amplification.[17,18] The *H. pylori* inoculums were obtained from three patients. Cultured bacteria were grown under the same incubation conditions, pooled, and suspended in phosphate-buffered saline containing 1×10^8 bacteria/ml, employing the McFarland standard for bacterial density.

Animals and experimental protocol

Female short-hair guinea pigs weighing 250–350 g were kept on a 12-h light/dark schedule and fed water and complete guinea pig food (Purina; Nestle Purina, St. Louis, MO, USA) ad libitum. Experiments were performed according to the experimental guidelines of the local ethics committee. Twenty animals were divided into four groups: (1) *H. pylori*-infected animals (HP-S group); (2) *H. pylori*-infected animals treated with quercetin (Sigma-Aldrich, Carlsbad CA, USA) (HP-T group); (3) control animals treated with quercetin but not *H. pylori*-infected (C-T group); and (4) untreated control animals without *H. pylori* infection (C-S group). Animals were orally inoculated with 1 ml of *H. pylori* suspension once per day for 3 days. The experiment was terminated at 75 days. On day 60 after inoculation animals were orally treated with quercetin (100 mg/kg every 12 h for 15 days). Quercetin was dissolved in 1% gum arabic. The C-T group was treated with quercetin in the same way as the HP-T group, whereas the C-S group received 1% arabic gum (1 ml every 12 h for 15 days) like the HP-S group.

Gastric tissue collection and processing

Animals were killed by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Stomachs were removed and washed with saline solution. Samples from the pyloric antrum and corpus were fixed in 10% buffered formalin or frozen to –70°C for lipid peroxidation analysis.

Measurement of lipid hydroperoxide

Total lipid hydroperoxide (LOOH) concentrations in gastric tissue were measured by a FOX2 assay with minor modification.[19] The FOX2 reagent was prepared with xylenol orange (Sigma-Aldrich). The gastric tissue (0.2 g) was homogenized in 20 mM of cold Tris HCl with a manual homogenizer. The test reaction (quantification of total hydroperoxide) was performed in triplicate for each sample. The spectrophotometric readings were obtained at 560 nm. The concentration of LOOH was calibrated in a base of 1 mg of protein from the homogenized sample. The concentration of protein was obtained with the Bradford assay.[20]

Histological processing

The pyloric antrum and corpus were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin, and 5-μm-thick sections were cut. To examine inflammation, the sections were stained with myeloperoxidase as described by Williams et al.[21] with minor modifications. Briefly, sections were incubated in a water bath at 37°C with peroxidase indicator reagent solution. Subsequently, they were rinsed with distilled water, air-dried, and counterstained with hematoxylin-eosin (H-E). Neutrophil leukocytes were identified by their myeloperoxidase positivity (color, blackish brown). Mononuclear cells and eosinophil leukocytes were identified on the basis of their morphology. *Helicobacter pylori* bacteria were recognized by using the modified Giemsa stain[22] and identified by their characteristic spiral or curved morphology. The presence of *H. pylori* was confirmed by immunohistochemical staining, using the streptavidin–biotin peroxidase complex method (Dako Cytomation, LSAB + System-HRP, Carpinteria, CA, USA). Sections were incubated for 24 h at 4°C with primary polyclonal antibody specific to rabbit anti-*H. pylori* IgG (Cell Marque, Rocklin, CA, USA). Diaminobenzidine hydrochloride was used as the chromogen. For the negative control, the primary antibody was omitted. Sections were counterstained with Harris hematoxylin.

Assessment of mucosal inflammatory infiltration and density of H. pylori

The acute inflammatory response was evaluated by manually counting the number of neutrophil or eosinophil leukocytes. Each tissue sample was divided into three sections; in each section 15 measurements were made and the means of the three sections reported. High magnification (1000×) was used, and the number of cells/field was assessed.

The chronic inflammatory cell (CIC) response and density of *H. pylori* were scored using the scale variation of the Sydney system proposed by Aydin et al.[23] Chronic inflammation (mononuclear cells) was scored as follows: 0, no CICs; 1, <10 CICs; 2, >10 CICs; 3, several areas with dense CICs; 4, diffuse infiltration with dense CICs; 5, nearly the entire mucosa contains dense CICs; 6, entire mucosa contains dense CIC infiltrate. The index of bacterial colonization of *H. pylori* was scored as follows: 0, no *H. pylori*; 1, *H. pylori* found only in one place; 2, only a few *H. pylori* found; 3, scattered *H. pylori* found in separate areas; 4, numerous *H. pylori* found in separate areas; 5, nearly the entire gastric surface covered by a layer of *H. pylori;* and 6, continuous gastric surface coverage by a thick layer of *H. pylori*.

Statistical analysis

The data are presented as means ± SEM. Statistical significance of the differences was determined by two-way analysis of variance, followed by the Tukey-Kramer multiple comparisons test. Values of $P < 0.05$ were considered statistically significant.

Results

Index of bacterial colonization

Gastric tissue slides stained by the Giemsa modified method were used to determine the effect of quercetin administration on bacterial colonization in the gastric mucosa of the infected animals. The identity of the bacteria on the gastric mucosa was corroborated by immunohistochemical staining (Fig. 1A).

The index of bacterial colonization in the pyloric antrum area of the HP-S group was 1.91 ± 0.9, whereas in the corpus the index was significantly less (0.63 ± 0.2, $P < 0.001$). In the quercetin-treated group (HP-T), the pyloric antrum bacterial colonization index was significantly lower (0.21 ± 0.1) than in the HP-S group ($P < 0.001$) (Fig. 1B). Comparison between the index of bacterial colonization of the corpus in the HP-S and HP-T groups showed that the bacterial density was significantly decreased in the quercetin-treated group (0.052 ± 0.09, $P < 0.001$) (Fig. 1B).

Gastric mucosal inflammation

H-E-stained gastric tissue slides showed signs of chronic inflammation with prominent lymphoid follicular structures on the antral submucosa (Fig. 2C). Typical inflammatory infiltration from an animal with *H. pylori* infection is shown in Fig. 2B. For comparison, Fig. 2A shows a normal gastric mucosa.

In the pyloric antrum of the HP-S group, the mononuclear cell score was 1.04 ± 0.25, whereas the number of neutrophil leukocytes was 1.9 ± 0.6; both variables were significantly higher than in the C-S control group (mononuclear cells, 0.18 ± 0.07 and neutrophil leucocytes, 0.38 ± 0.1, respectively, $P < 0.05$ for both variables) (Fig. 3A and B). No differences were found between HP-S and C-S in eosinophil leukocyte numbers (1.36 ± 0.52 vs. 1.74 ± 0.63, respectively, not significant) (Fig. 3A).

In the antral mucosa of the HP-T group, quercetin administration decreased neutrophil leukocyte infiltration compared with in the HP-S group (0.3 ± 0.06 vs. 1.9 ± 0.6 respectively, $P < 0.05$) (Fig. 3A). The mononuclear cell score was decreased in the HP-T group compared with in the HP-S group (0.42 ± 0.07 vs. 1.04 ± 0.25, respectively, $P < 0.05$) (Fig. 3B). It is noteworthy that in the quercetin-treated group (HP-T), although the inflammatory reaction was significantly decreased, it was still higher than in the C-S group (0.42 ± 0.07 vs. 0.18 ± 0.07, $P < 0.05$).

In the corpus mucosa signs of chronic inflammation were noted in the HP-S but not in the C-S group (0.7 ± 0.1 vs. 0.12 ± 0.1, $P < 0.001$) (Fig. 3B). In several groups the histological analysis of the corpus showed no neutrophilic leukocytes (Fig. 3A). Comparison of the HP-S and C-S groups showed that eosinophil leukocyte infiltration was significantly higher in the HP-S group (0.56 ± 0.1 vs. 0.07 ± 0.07, $P < 0.05$) (Fig. 3A). This is different from the pyloric antrum, where no correlation was noted between eosinophil infiltration and the presence of *H. pylori*.

In the corpus of the HP-T group, quercetin administration decreased the level of mononuclear cell infiltration compared with in the HP-S group (0.24 ± 0.1 vs. 0.7 ± 0.1, $P < 0.05$) (Fig. 3B), whereas no differences were found in eosinophil leukocyte infiltration between the HP-T and HP-S groups (0.5 ± 0.2 vs. 0.56 ± 0.1, not significant) (Fig. 3B).

Lipid peroxidation in the gastric mucosa

Compared with in the C-S group, in the pyloric antrum of the HP-S group an increase in lipid hydroperoxides was observed (26.2 ± 2.5 vs. 38.1 ± 3.7 nmol, $P < 0.05$) (Fig. 4A). In infected animals, quercetin reduced lipid oxidation (22.8 ± 2.2 nmol) to a level comparable to that

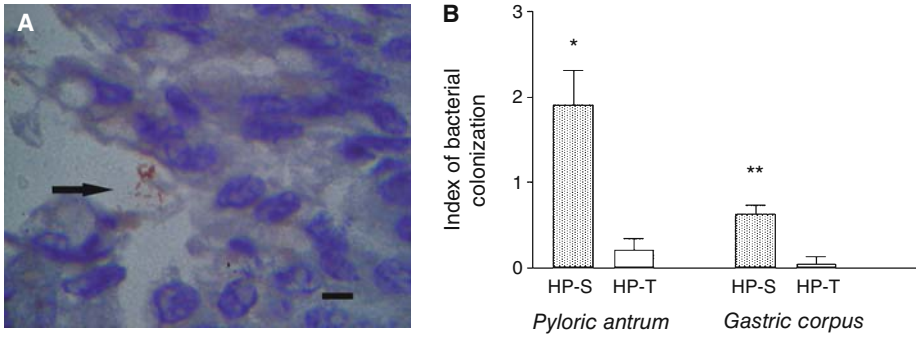

Fig. 1. A Immunohistochemical analysis of *Helicobacter pylori* (*arrow*) on the gastric mucosa of infected guinea pig (bar = 10 μm). **B** Colonization of *H. pylori* on a histological section of antral mucosa and corpus of an infected animal with and without quercetin administration (*HP-T* and *HP-S*, respectively). Values are expressed as means ± SEM (n = 5); *P = 0.0006; **P = 0.0001

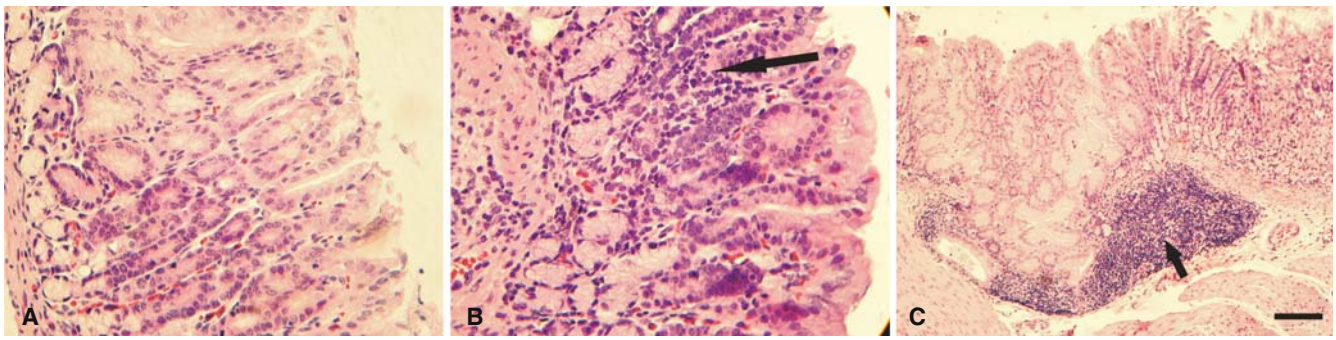

Fig. 2A–C. Experimental *H. pylori* infection causes chronic inflammation in the gastric mucosa. **A** Control gastric mucosa (antrum) without infection; **B** gastric mucosa (antrum) infected with *H. pylori* shows leukocyte infiltration (*arrow*); **C** Lymphofollicular organization (*arrow*) on antral submucosa of infected animal (bar = 140 μm)

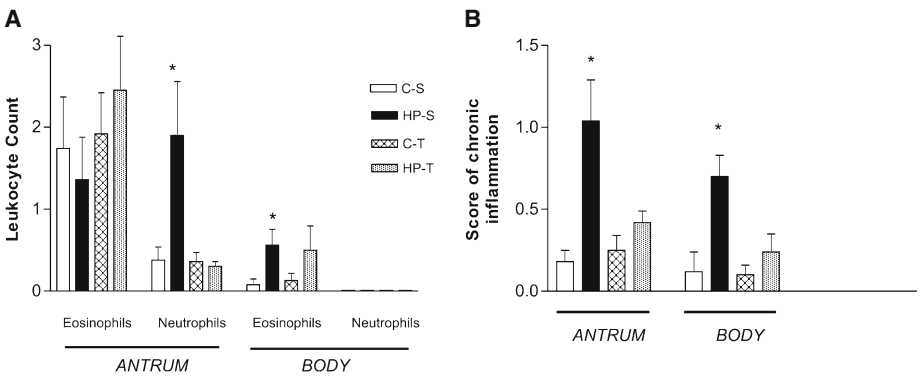

Fig. 3A, B. Histological assessment of the grade of inflammation in the pyloric antrum (*ANTRUM*) and corpus (*BODY*) in guinea pig: **A** Acute inflammation; polymorphonuclear leukocytes. **B** Chronic inflammation. *HP-S*, *H. pylori* infection without quercetin treatment; *HP-T*, *H. pylori* infection with quercetin treatment; *C-S*, control without quercetin treatment; *C-T*, control with quercetin treatment. Values are expressed as means ± SEM (n = 5); *P < 0.05 (versus *HP-S* grup)

in the C-S group (not significant) (Fig. 4A). The level of lipid peroxidation in the corpus showed no significant differences among the groups (Fig. 4B).

Discussion

In our experimental model of *H. pylori* infection, more bacteria were located in the pyloric antrum than in the corpus. This finding has been reported in other animal species such as Mongolian gerbils and rats.[24,25] Our study also demonstrated for the first time that in the mucosa of the pyloric antrum and corpus quercetin induced a significant decrease in bacterial density, indicating that besides an in vitro antibacterial effect,[26] quercetin also possesses a significant antibacterial effect in vivo. Comparative in vitro antibacterial assays of several polyphenols, including quercetin, shows that quercetin has low to moderate antibacterial effects on *H. pylori*.[27] In our animals treated with quercetin, a significant decrease in the number of *H. pylori* was observed in the antrum and corpus mucosa (89% and 92%, respectively). It has been reported that triple antibacterial therapy (omeprazole, metronidazole, and clarithromycin) results in

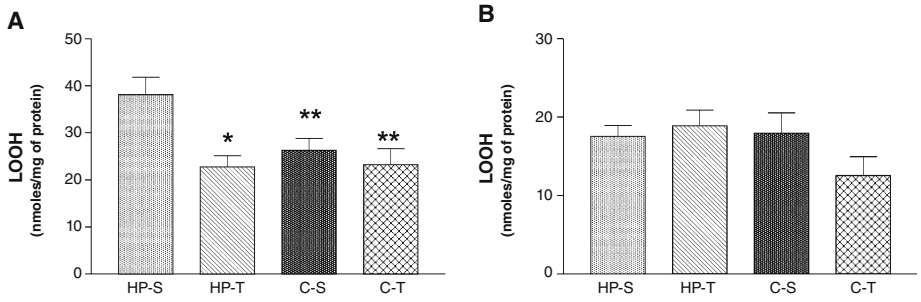

Fig. 4A, B. Comparison of lipid hydroperoxide content of the gastric mucosa from the pyloric antrum (**A**) and corpus (**B**) of guinea pigs. Values are expressed as means ± SEM (n = 5); *$P < 0.01$, **$P < 0.05$ (versus *HP-S* grup)

100% eradication of *H. pylori* from the gastric mucosa of rodents.[28] The comparison of triple antibacterial therapy with quercetin treatment of *H. pylori*-infected animals in our experiment suggests that quercetin has similar anti-*H. pylori* activity. However, further experiments are required to test the effectiveness of alternative therapy in gastroduodenal diseases associated with *H. pylori*.

The mechanism of action of quercetin on *H. pylori* is not clear. It is possible that quercetin, like other flavonoids, interferes with the vacuolization of VacA toxin, as been suggested by Tombola et al.[29] This toxin induces the formation of specific anion channels in the plasma membrane associated with the release of bicarbonate and organic anions from the cellular cytosol of the gastric epithelium, thus permitting adequate bacterial growth.[30] Alternatively, it is possible that quercetin reduces the action of urease on *H. pylori*, since it has been shown that several polyphenolic compounds can modify the activity of this enzyme, which is essential for bacterial survival under gastric acid stress.[31] In addition, the protective action of quercetin may be related to its being a strong chelator of iron.[32] Iron is an important enzymatic cofactor required for bacterial growth, including that of *H. pylori*,[33] and a lack of this metal in gastric juice caused by quercetin may alter the growth of the bacteria.

Quercetin administration to *H. pylori*-infected animals reduced both the bacterial number on the gastric mucosa and the inflammatory process; thus, loss of bacteria can eliminate the stimuli associated with inflammatory response. The decreased inflammatory process has been observed in previous studies employing standard antibacterial therapies.[34,35] Consistent with these results, our experiment showed that decreased bacterial number was associated with a diminution of neutrophil leukocyte infiltration. Alternatively, it is possible that quercetin has a direct anti-inflammatory property through degradation of the Iκβα factor, preventing NF-κβ nuclear translocation, as a result of the decreased genetic expression and production of relevant inflammatory mediators such as TNF-α, IL-1β, IL-6, and IL-8, as suggested by Min et al.[36] and Ruiz et al.[37]

Because neutrophil leukocytes play an important role in elimination of pathogens we investigated the number of these cells in the gastric mucosa of infected animals using myeloperoxidase histochemistry. It is noteworthy that in all *H. pylori*-infected animals treated or untreated with quercetin, neutrophil leukocytes were absent in the gastric mucosa of the corpus, whereas eosinophil leukocytes were increased in number in tight association with bacterial colonization. It is possible that this change in leukocyte type could compensate for the absence of neutrophil leukocytes in the control of corpus infection. Previous work has demonstrated a correlation between a high level of eosinophil leukocyte infiltration and bacterial density in the gastric mucosa of infected humans and guinea pigs.[38–40] In our experiment, eosinophil leukocyte infiltration was independent of bacterial colonization in the pyloric antrum. Although we cannot explain the cause, it is possible that factors not related to the gastric colonization of *H. pylori* are responsible.

Quercetin administration induced a decrease in lipid peroxidation in the pyloric antrum of infected animals. This finding could be a result of decreased neutrophil leukocyte infiltration and free radicals of neutrophil leukocytes in this gastric region. It is also conceivable that the antioxidant property of quercetin is involved in the decrease of lipid peroxidation, as suggested by Martín et al.[15] who described a decreased index of lipid peroxidation in gastric injury caused by ethanol. In these studies no changes were noted in neutrophil leukocyte infiltration.[15]

Our results provided novel information regarding reduction in *H. pylori* infection by quercetin on gastric mucosa in vivo. We propose that the flavonoid quercetin may be considered a candidate treatment agent for gastroduodenal disease associated with *H. pylori* in humans, but further in vitro and in vivo studies is necessary.

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