RESEARCH COMMUNICATION

Suppressive Effects of Fruit-juice Concentrate of Prunus Mume Sieb. et Zucc. (Japanese apricot, Ume) on Helicobacter Pylori-induced Glandular Stomach Lesions in Mongolian Gerbils

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Abstract

Helicobacter pylori **(*Hp*) infection is an important factor in human gastric disorders, including chronic active gastritis, peptic ulcers, intestinal metaplasia and cancer. Since epidemiologic studies overwhelmingly agree on a protective influence of fruits and vegetables in reducing the risk of gastric neoplasia and processed foods made from Prunus mume Sieb. et Zucc. (Japanese apricot or "Ume" in Japanese) are traditionally known for their miscellaneous medical effects, in the present study we investigated the efficacy of a fruit-juice concentrate of Japanese apricot (CJA) in the glandular stomach of *Hp*-infected Mongolian gerbils. *Hp*-inoculated gerbils were given CJA in their drinking water at concentrations of 1 and 3% for 10 weeks. The microscopic scores for gastritis and mucosal hyperplasia in the CJA groups were significantly lower than in the *Hp*-inoculated control group, with dose-dependence. Real-time PCR was performed to quantitate *Hp* by demonstrating urease A gene amount using gerbils' glyceraldehyde-3-phophate dehydrogenase (GAPDH) gene as an internal control. Average relative urease A gene dosage in the glandular stomach in the 1 and 3% CJA and *Hp*-inoculated control groups was 26.6 ± 11.6% (average ± SE), 30.3 ± 10.5%, 100 ± 40.9%, respectively, the fruit-juice concentrate causing significant lowering (P<0.01 and P<0.05, respectively, with 1 and 3%). These findings suggest that suppressive effects on gastric cancer development might also be expected as a result of decreased numbers of *Hp* and improvement of *Hp*-induced chronic active gastritis on administration of CJA.**

Key Words: Helicobacter pylori - Mongolian gerbils - Prunus mume Sieb. et Zucc. (Japanese apricot, Ume) - glandular stomach - inflammation

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Introduction

Helicobacter pylori (*Hp*) is a major causative factor for gastric disorders and epidemiological evidence has accumulated indicating a significant relationship with chronic active gastritis, peptic ulcer, atrophic gastritis, intestinal metaplasia, and lymphoma or cancer development (Marshall and Warren, 1984; Nomura et al., 1991; Uemura et al., 2001). In 1994, the World Health Organization/ International Agency for Research on Cancer concluded that *Hp* is a 'definite carcinogen' based on the epidemiological findings (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). However the pathogenic roles of *Hp* are still not fully understood. Eradication of *Hp* reduces the relapse rate of peptic ulcers and also results in histological resolution of chronic active gastritis (Hunt, 1996). The standard regimen for this purpose is adoption of triple therapy with a proton pump inhibitor in combination with two antibiotics, clarithromycin and amoxicillin (Misiewicz et al., 1997). Although the currently most effective treatment regimens cure about 90% of infections, 10% of patients remain *Hp* positive. Several factors contribute to treatment failure. These include patient compliance, bacterial resistance to antibiotics, and treatment related issues (Graham, 1998; Huang and Hunt, 1999). Therefore, it is important to find alternative approaches to

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control which are both effective and safe in terms of gastrointestinal protection from *Hp* associated diseases.

Epidemiologic studies overwhelmingly agree on the protective effect of fruits and vegetables in reducing the risk of gastric cancer (Serafini et al., 2002). In Japan, processed foods made from fruits of Prunus mume Sieb. et Zucc. (Japanese apricot or "Ume" in Japanese) are popular and traditionally considered to have miscellaneous medical benefit, such as antibacterial and fungicidal properties (Fujita et al., 2002; Maitani et al., 1985). Nomura et al. (Nomura et al., 1982) previously reported a significant negative association of ume (pickled plum) intake with intestinal metaplasia of the human stomach.

Mongolian gerbils can be easily infected with *Hp*, providing a good experimental animal to clarify the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric cancer (Hirayama et al., 1996). We have established a gastric carcinogenesis model using these animals, and demonstrated that gastric cancer development is enhanced by *Hp* infection when they are treated with chemical carcinogens, like *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N'*-nitrosoguanidine (MNNG) (Shimizu et al., 1999; Tatematsu et al., 1998). *Hp* eradication reduces the enhancing effect of *Hp* on gastric carcinogenesis (Cao et al., 2002; Nozaki et al., 2003).

In the present study, we therefore, investigated the efficacy of fruit-juice concentrate of Japanese apricot (CJA) in the glandular stomach of *Hp*-infected Mongolian gerbils.

Materials and Methods

Animals and Samples

A total of 60 specific pathogen-free male, four-week-old Mongolian gerbils (Meriones unguiculatus; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka, Japan) were housed in steel cages on hardwood-chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. They were given food (Oriental CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) irradiated with 30 Gy γ-rays and autoclaved distilled water. The experimental design was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute, and the animals were cared for in accordance with institutional guidelines. CJA was obtained from Minabegawa Village Office (Wakayama, Japan). CJA dissolved in distilled water at concentrations of 1 and 3% was freshly prepared three times per week for administration as drinking water.

Bacteria

Hp strain ATCC 43504 (American Type Culture Collection, Rockville, MD) was inoculated on Brucella agar plates (Becton Dickinson Co., Cockeysville, MD) containing 7% v/v heat-inactivated fetal bovine serum and incubated at 37˚C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo) at high humidity. Two days later, the bacteria grown on the plates were introduced into Brucella broth (Becton Dickinson Co.) supplemented with 7% v/v heat-inactivated fetal bovine serum and incubated under the same conditions for 24 h. The broth cultures of *Hp* were checked under a phase contrast microscope for bacterial shape and mobility. Samples containing about 1.0×10^8 colony-forming units per milliliter were used as the inoculum and delivered intra-gastrically (i.g.) using an oral catheter to gerbils fasted for 24 h. Uninfected gerbils underwent sham inoculation using the same sterile Brucella broth.

Experimental Protocol

The experimental design is illustrated in Fig. 1. Sixty gerbils were divided into 5 groups. *Hp* was inoculated into three of these groups at 1 experimental week. The other 2 groups received Brucella broth. CJA was administrated to *Hp*-inoculated and *Hp*-free animals in drinking water at the concentrations of 0, 1 or 3%, in all cases until the end of experiment at week 10. The gerbils were killed humanely at the end of the study period. All animals were subjected to deep ether anesthesia after 24 h fasting, laparotomized, and exsanguinated from the inferior vena cava, followed by excision of their stomachs. One half of each glandular stomach was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and routinely processed for histopathological examination, and the other half was quick frozen at -70^{o}C for genomic DNA analysis.

Histopathological Analyses

Tissue sections were stained with hemotoxylin and eosin (H&E), Giemsa, and by immunohistochemistry for examination of *Hp* (anti-*Hp* serum, Dako Cytomation, Copenhagen, Denmark). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System (Dixon et al., 1996) by scoring the following parameters: mononuclear cell infiltration (0-3; 0, normal; 1, mild infiltration into lamina propria; 2, moderate infiltration into lamina propria; 3, marked infiltration into lamina propria and multiple lymphoid follicle formation); neutrophil infiltration (0-3; 0, none; 1, number of neutrophils in the pyloric mucosa in a line from the forestomach to the

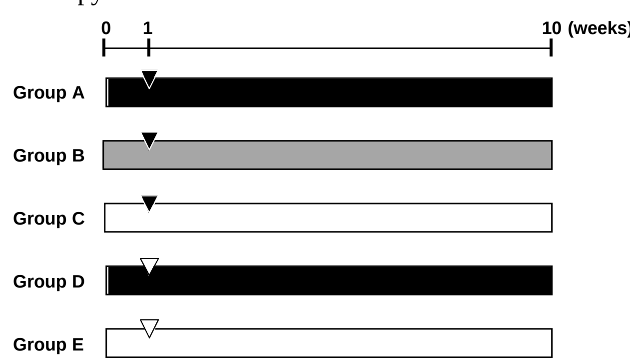

Figure 1. Experimental Design. Four week-old male Mongolian gerbils were used. Intragastric inoculation of Hp (closed triangles) or Brucella broth (open triangle). 3% (closed bar) or 1% (hatched bar) fruit-juice concentrate of Japanese apricot (CJA) was given in the drinking water. Control groups received unsupplemented water (open bar).

Table 1. PCR Primer Sequnces used in the Light Cycler Analysis

Description	Gene	Sequences	Product length (bp)	Accession No.
ua1	Urease A	5'-TGTTGGCGACAGACCGGTTCAAATC-3' (sense)	120	M60398
ua2		5'-GCTGTCCCGCTCGCAATGTCTAAGC-3' (antisense)		
ga1	GAPDH[a]	5'-AACGGCACAGTCAAGGCTGAGAACG-3' (sense)	118	AB040445
ga2		5'-CAACATACTCGGCACCGGCATCG-3' (antisense)		

[a] glyceraldehyde-3-phophate dehydrogenase

duodenum<50/mm; 2, 50-100/mm; 3, >100/mm); *Hp* density (0-3; 0, none; 1, mild *Hp* density; 2, moderate; 3, marked). The thickness of the pyloric mucosa was also measured at five randomly selected points in the foveolar epithelium.

Serology

Serum samples were used to measure the titer of anti-*Hp* IgG antibodies (GAP-IgG; Biomerica, Newport Beach, CA) by enzyme-linked immunosorbent assay (ELISA) using anti-gerbil IgG antibodies. The antibody titer was expressed by means of an arbitrary index (AI). A value greater than 1.37 AI was considered to be positive for *Hp* infection in both the infection and the control groups, as described earlier (Kumagai et al., 2001). Serum gastrin levels were measured using a gastrin radioimmunoassay kit (Gastrin-RIAkit II; Dainabot Co., Ltd., Tokyo).

Real-time Polymerase Chain Reaction and Relative Quantitative Analysis

Genomic DNA was extracted from glandular stomach tisue of gerbils using a DNeasy tissue kit (QIAGEN, Hilden, Germany). For *Hp* quantification, *Hp* specific urease A gene dosage within glandular stomachs of *Hp*-inoculated gerbils, relative quantitative real-time polymerase chain reaction (PCR) of Urease A was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using gerbil specific glyceraldehyde-3-phophate dehydrogenase (GAPDH) gene as an internal control. This was performed basically as described (Tsukamoto et al., 2001; Tsukamoto et al., 2004) using QuantiTect SYBR Green PCR (QIAGEN) with the optimal Mg^{2+} concentration at 2.5mM. The 5'-and 3'-primer sequences are listed in Table 1. Specificity of the PCR reaction was confirmed using the melting program provided with the LightCycler software. To further confirm that there was no obvious primer dimer formation or amplification of any extra bands, the samples were electrophoresed in 3% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Relative quantitative analysis of *Hp* urease A gene expression was performed as earlier established using an internal control without the necessity of external standards (Tsukamoto et al., 2001; Tsukamoto et al., 2004), with values expressed as the percentage urease A gene expression, relative to the 100% in the *Hp*-inoculated control group (group C).

Statistics Analysis

The Mann-Whitney *U* test was applied to establish the significance of differences in urease A gene expression for corrected crossing points, microscopic score for gastritis, mucosal hyperplasia, titers of anti-*Hp* IgG antibodies, serum gastrin levels. *P* values <0.05 were considered to be statistically significant.

Results

Intake of CJA

Data for total intake of CJA per animal are shown in Table 2. CJA administration did not affect food intake or body weights.

Inflammation Score

Table 2 summarizes data for the efficacy of CJA in the glandular stomach of *Hp*-infected Mongolian gerbils. All animals of the *Hp*-inoculated control group (group C)

Table 2 Effects of Fruit-juice Concentrated of Japanese apricot (CJA) on Gastric Lesion of Mongolian Gerbils

Group	Administration	No. of gerbils	Microscopic score [SD]	Mucosal hyperplasia (mm) [SD]	Anti-*Hp* titer titer (AI) [SD]	Serum gastrin (pg/ml) [SD]	Total CJA intake (g/gerbil) [SD]
A	3 % CJA + Hp	20	3.00[a,b] [1.95]	0.34[c] [0.11]	4.01 [2.86]	101.13[d,e] [22.90]	10.54 [0.67]
B	1% CJA + Hp	21	4.38[a] [1.91]	0.42 [0.23]	5.89 [3.36]	133.19 [29.46]	4.76 [0.60]
C	Hp	10	8.00 [1.25]	0.50 [0.23]	6.47 [4.14]	150.31 [40.00]	0
D	3 % CJA	4	0	0.21 [0.02]	0.48 [0.17]	117.88 [18.54]	10.68 0
E	Control	5	0	0.23 [0.03]	0.18 [0.08]	140.88 [26.28]	0

[a] P<0.0001 vs. group C [b] P<0.05 vs. group B [c] P<0.05 vs. group C [d] P<0.005 vs. group C [e] P<0.001 vs. group B

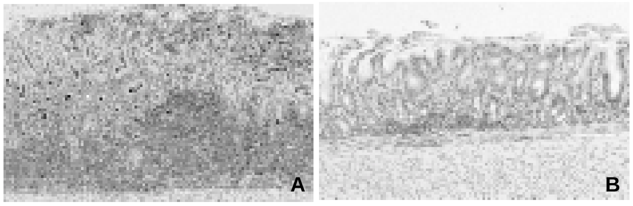

Figure 2. Histopathological Findings in the Pyloric Mucosa of Mongolian Gerbils Inoculated with *Hp*. (A) *Hp*-inoculated control group (group C). The glandular stomach shows hyperplastic change and severe infiltration of inflammatory cells (H&E, Original magnification, x50). (B) *Hp*-inoculated 3% CJA group (group A). The glandular stomach shows mild infiltration of inflammatory cells and mucosal hyperplasia (H&E, Original magnification, x50).

microscopically demonstrated severe gastritis with moderate to marked infiltration of inflammatory cells, mucosal hyperplasia with hemorrhagic erosion and moderate to marked *Hp* density mainly in the pyloric mucosa of glandular stomachs (Fig. 2A). The microscopic scores for the 1 and 3% CJA administrated group (groups A and B) were significantly lower than for the *Hp*-inoculated control group, with dose-dependence (Table 2). The thickness of the pyloric mucosa was also reduced dose-dependently in CJA administrated group, reaching significance in the 3% CJA group (Fig. 2B). No evidence of gastritis and mucosal hyperplasia was found in any *Hp*-free animals.

Antibody Titer and Serum Gastrin Level

Titer of anti-*Hp* antibodies in all *Hp*-inoculated groups were greater than the cut off values expect in one animal in group A, which was excluded from the analysis. There were no significant differences in antibody titers among groups A-C (Table 2). The values for serum gastrin were reduced dose-dependently in the CJA groups, and significantly with the 3% dose (group A) (Table 2).

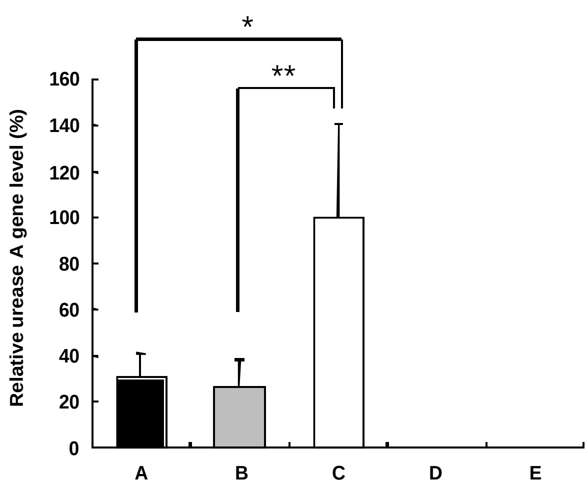

Figure 3. Relative Expression Levels of the Urease A Gene in Glandular Stomachs of Mongolian Gerbils. Values were set at 100% in group C and expressed as mean ± SE. Note decrease in relative urease A gene levels in groups A and B as compared to group C. *P<0.05 and **P<0.01, by the Mann-Whitney *U* test.

Quantification of Hp

Real-time PCR was performed to demonstrate expression of the urease A gene of *Hp*-inoculated groups using GAPDH as an internal control. Average relative urease A gene levels of glandular stomach in 1 and 3% CJA and *Hp*-inoculated control groups were 26.6±11.6% (average ±SE), 30.3±10.5% and 100±40.9%, respectively. The lowering by CJA was significant (P<0.01 and P<0.05, respectively, of 1 and 3%) (Fig. 3). Furthermore, no amplification of the urease A gene was detected in 4 of 20 animals (20%) in group A and 1 of 21 animals (4.8%) in group B, in addition to all the *Hp*-free animals.

Discussion

Our present data provide clear evidence that a fruit-juice concentrate of Japanese plums administered in the drinking water can suppress chronic active gastritis in the glandular stomachs of *Hp*-infected Mongolian gerbils in a dose-dependent manner, reducing urease A gene amount in the *Hp*-inoculated glandular stomach. In the 20% of 3% CJA and 4.8% of 1% CJA administered gerbils without detectable urease A gene, histological examination for *Hp* also proved negative, indicating the possibility that *Hp* had been eradicated in these animals. Rokbi et al. have previously demonstrated that real-time PCR is a powerful tool for the detection and quantification of *Hp* gene expression in the gastric mucosa (Rokbi et al., 2001) and PCR amplification of the *Hp* urease A gene is a highly sensitive and specific method for the diagnosis of *Hp* infection (Clayton et al., 1992).

The Japanese plum (ume), Prunus mume Sieb. et Zucc. (Rosaceae), has been traditionally used as a medical food in Japan and in Chinese traditional medicine, various parts of the plant are used. Although a number of reports have been published with concrete evidence that Japanese apricots are effective against diseases (Maitani et al., 1985), information on the mechanisms, for example of its antibacterial and fungicidal properties, is limited. It has been postulated that antioxidants may reduced cancer risk by modulating red-ox status, by preventing biologic oxidant, and by inhibiting the formation of carcinogen (Serafini et al., 2002). Utsunomiya et al. previously reported that fruit-juice concentrate of Japanese plum possesses a potent antioxidant activity (Utsunomiya et al., 2002). Iimuro et al. have shown that antioxidative effects of garlic may have suppressive effects on *Hp*-induced gastritis in Mongolian gerbils (Iimuro et al., 2002). We therefore hypothesize that antioxidative effects of CJA may have contributed to the suppression of chronic active gastritis in glandular stomach of *Hp*-infected Mongolian gerbils.

In addition, CJA harbors strong acids, including citric and malic acid (Chuda et al., 1999; Fujita et al., 2002), which may exert antibacterial action and cause environmental change in the stomach. Suppressive effects on gastric cancer development would be expected as a result of the decrease of quantity of *Hp* and improvement of *Hp*-induced chronic active gastritis by administration of CJA. Actual ingredients which might be effective for *Hp*-induced chronic active gastritis have not been clarified but warrant further examination. Studies are now in progress to clarify the suppressive effect of gastric cancer development in gastric carcinogenesis model using Mongolian gerbils.

In conclusion, in this present study, we found CJA to suppress chronic active gastritis in the glandular stomachs of *Hp*-infected Mongolian gerbils. Therefore, CJA may have potential as a safe and inexpensive agent to control *Hp*-associated gastric disorders in Japan, including gastric neoplasia.

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