When the COP of ≥80 cm was applied, the differences decreased to 12.8 cm and 4.1 kg/m^2, respectively.

Table 4 shows the odds ratios and 95% confidence interval (CI) for fatty liver according to the number of MetS components other than central obesity by WC status. Regardless of sex and the WC COP selected, a strong linear trend was observed for the association (trend $P < 0.0001$) with the number of components. The odds ratio for subjects without central obesity and with all three components of MetS was 9.69 (95% CI 3.1130.2) in men and 55.3 (6.34–483) in women. Using the ≥90 and ≥80 cm COP criterion for central obesity in men and women, respectively, the odds ratio was 55.3 (6.34–483) and 62.4 (6.23–626). These point estimates of odds ratios were higher than those of MetS subjects with two risk factors other than obesity among women, and even among men, they were higher than those of the risk group for MetS who satisfied the central obesity criterion.

Figure 1 shows the ROC curves for the diagnosis of fatty liver according to MetS status by the JMetS criteria and by our criteria. The AUC for the JMetS criteria and our criteria 1 and 2 in men was 0.638, 0.681, and 0.655, respectively. In women, the AUC for our criteria using ≥90 and ≥80 cm COPs for central obesity were 0.625 and 0.681, respectively, whereas that for the JMetS criteria was only 0.570. Based on the findings of our study, the largest AUC was recorded using our criterion 1 (≥85 cm) in men and our criteria 2 in women (≥80 cm). The shapes of the ROC curves of our criterion 2 for men and our criterion 1 for women were very similar, with the coordinates (false positive rate, true positive rate) for MetS and the risk group for MetS being (0.030, 0.188) and (0.204, 0.543), respectively, for men and (0.023, 0.181) and (0.205, 0.537), respectively for women. In addition, when WC was considered as a component, the COP for the largest AUC among men and women was ≥82 cm (0.701) and ≥77 cm (0.699), respectively. We therefore conclude that it would be both practical and appropriate to take WC into consideration, with WC COPs of ≥85 cm for men and ≥80 cm for women. In our study population, 26.7% of the men and 36.6% the women satisfied the criteria.

Table 4 Odds ratio and 95% confidence interval for fatty liver according to the number of the components of MetS other than obesity by waist circumstance status

Number of the components[a]	Odds ratio	95% confidence interval	Odds ratio	95% confidence interval
Men				
	Waist circumference <85 cm		*Waist circumference ≥85 cm*	
0	1.00	Reference	5.49	3.25–9.27
1	1.99	1.32–3.01	7.09	4.51–11.1
2	5.34	3.26–8.74	18.4	9.78–34.4
3	9.69	3.11–30.2	99.7	12.6–786
P for trend	<0.0001		<0.0001	
	Waist circumference <90 cm		*Waist circumference ≥90 cm*	
0	1.00	Reference	7.66	3.59–16.32
1	1.88	1.33–2.66	11.91	6.34–22.39
2	5.17	3.40–7.85	19.96	7.67–51.93
3	14.71	5.45–39.72	31.53	3.62–274.34
P for trend	<0.0001		<0.0001	
Women				
	Waist circumference <90 cm		*Waist circumference ≥90 cm*	
0	1.00	Reference	9.59	4.32–21.3
1	2.32	1.56–3.46	7.37	3.80–14.3
2	5.42	3.10–9.48	17.4	6.45–46.8
3	55.3	6.34–483	44.2	4.85–403
P for trend	<0.0001		<0.0001	
	Waist circumference <80 cm		*Waist circumference ≥80 cm*	
0	1.00	Reference	6.67	3.82–11.7
1	2.67	1.45–4.92	8.63	5.04–14.8
2	6.02	2.70–13.4	26.0	12.5–54.1
3	62.4	6.23–626	125	14.4–1084
P for trend	<0.0001		<0.0001	

[a] Number of the components of metabolic syndrome other than abdominal obesity

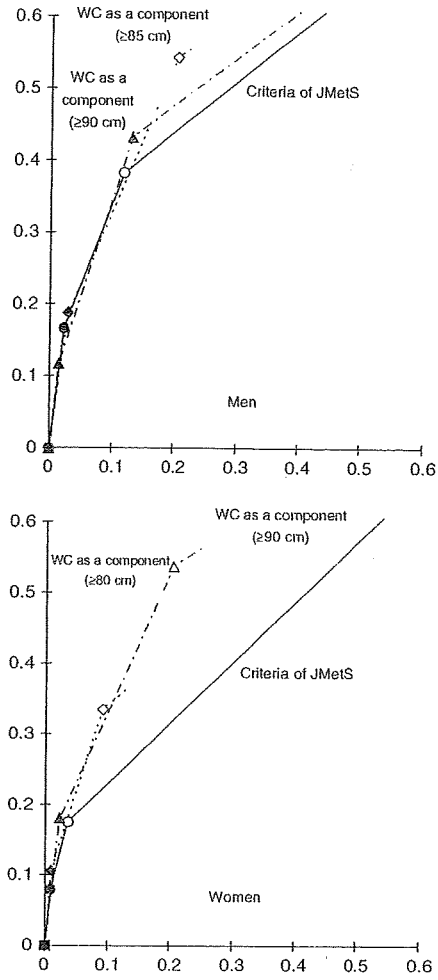

Fig. 1 Receiver operating characteristic curves for fatty liver diagnosis by metabolic syndrome status of several criteria. *JMetS* Japanese metabolic syndrom, *WC* waist circumference

Discussion

In the present study, we considered concurrent fatty liver to be a specific example of a disease in the metabolic domino of MetS and observed that the accumulation of MetS components was associated with higher odds ratios, even without the central obesity component. Taking these results as a whole, we observed stronger associations between MetS and fatty liver in men and women when we considered central obesity as a component rather than an essential requirement for the diagnosis of MetS. We therefore suggest that individuals with an accumulation of components should be regarded as having MetS even in the absence of central obesity, since fatty liver is a component of the metabolic domino. In addition, these individuals may belong to a risk group for other metabolic diseases, including cardiac arrest and cerebrovascular diseases. We also suggest that the optimal COP for WC should be ≥85 cm for men and ≥80 cm for women.

Although the main concepts of MetS are consistent, the COPs for defining central obesity for MetS are controversial, especially in Japan [21]. Several studies have been performed to elucidate the optimal COPs in which ROC analyses with obesity and two or more MetS components other than obesity [22–25] were used. The results suggested that the optimal cut-offs for men and women are 84–90 and 78–82 cm, respectively. Our results are consist with these reported values. However, these earlier studies were based on the internal consistency of obesity and MetS components other than obesity. Further ROC analyses need to be performed to establish the optimal COP for WC, and these should include certain diseases not currently included in MetS. This study is one such analysis.

An important question is whether central obesity should be considered as a requirement for the diagnosis of MetS or as a component of MetS. To answer this question, we need to examine the association between the number of MetS components and particular diseases stratified by central obesity. To date, there have been only two prospective cohort studies [26, 27] from Japan on cardiovascular diseases. Results from NIPPON DATA [26] show the existence of risk accumulation among non-obese subjects, whereas those from Hisayama-cho [27] indicate there is no risk accumulation in such subjects. Data from many studies, including those from our study, are required to facilitate further discussion on this question. However, before the absence of risk accumulation can be established among non-obese individuals, it is possible to treat central obesity as a component of MetS as a precautionary measure.

In general, if a factor is considered to be an essential requirement for the diagnosis of a certain disease, then that factor should not only be etiologically essential but also amenable to accurate measurement in practice; at the very least, the COP should be a sensitive measure. Otherwise, a considerable number of cases would not be detected by the criterion. In fact, the COPs based on the IDF criteria (≥94 cm for men and ≥80 cm for women), with central obesity as a requirement, are more sensitive than those of the NCEP-ATP III criteria (≥102 cm for men and ≥88 cm for women), wherein central obesity is considered a component. Although the JMetS definition is similar to the IDF definition, the JMetS COP for WC in women (≥90 cm) is much less sensitive than the COP of the IDF (≥80 cm). The COP for central obesity for the diagnosis of JMetS is based on the association between visceral fat area and WC [16]. The committee reported that simple correlation analysis of the regression line in women indicated that a WC corresponding to 100 cm^2 of visceral fat was 92.5 cm. However, the correlation coefficient was only 0.65, and more than half of the women with a visceral fat area

≥ 100 cm^2 would not be found using the WC COP of ≥ 90 cm (meaning that sensitivity is <0.5). The poor sensitivity of the WC in detecting abdominal adiposity is directly linked to the poor sensitivity of the JMetS criteria, in which WC is an essential requirement.

Conclusion

Based on the findings of our study, we suggest that a WC of ≥ 85 cm for men and ≥ 80 cm for women would be optimal COPs for central obesity for the diagnosis of MetS in the Japanese population. We also suggest that central obesity should be used as a component of MetS rather than an essential requirement for the diagnosis of MetS. No definite conclusion has yet been reached regarding the most appropriate diagnostic criteria for MetS. However, within the framework of our study in which fatty liver was considered to be an independent variable, we found that defining abdominal circumference as a component of MetS was less likely to cause errors of oversight and was thus more appropriate than considering abdominal circumference to be a required criterion. The challenge for the future is to identify pathologic conditions that are responsible for MetS and to find better diagnostic criteria through further similar studies that consider factors, other than fatty liver, involved in the metabolic domino effect [11, 12] as independent variables.

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Inhibitory effect of nordihydroguaiaretic acid, a plant lignan, on *Helicobacter pylori*-associated gastric carcinogenesis in Mongolian gerbils

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Recent epidemiological studies have demonstrated that consumption of certain natural products can lower cancer risk in humans. For example, plant-derived lignans have been shown to exert chemopreventive effects against cancer *in vitro* and *in vivo*. In the present study, the effects of three such lignans, termed arctiin, arctigenin, and nordihydroguaiaretic acid (NDGA), on the proliferation of *Helicobacter pylori* and the prevention of *H. pylori*-associated gastric cancer were investigated in Mongolian gerbils. To examine the effects of arctigenin and NDGA on stomach carcinogenesis, specific pathogen-free male, 5-week-old gerbils were infected with *H. pylori*, administered 10 p.p.m. *N*-methyl-*N*-nitrosourea in their drinking water and fed diets containing various concentrations of lignans until they were killed after 52 weeks. At a dietary level of 0.25%, NDGA significantly decreased the incidence of gastric adenocarcinomas. Arctigenin, in contrast, failed to attenuate neoplasia at a level of 0.1%. Both NDGA and arctigenin significantly reduced serum 8-hydroxy-2′-deoxyguanosine levels at doses of 0.25 and 0.05% (NDGA), and 0.1% (arctigenin). Administration of 0.25% NDGA significantly suppressed the formation of intestinal metaplasia both in the antrum and the corpus. Although all three lignans dose-dependently inhibited the *in vitro* proliferation of *H. pylori*, there were no differences in the titers of anti-*H. pylori* antibodies or the amount of the *H. pylori*-specific *urease A* gene among all *H. pylori*-infected groups. These results suggest that NDGA might be effective for prevention of gastric carcinogenesis. The possible mechanisms appear to be related to inhibitory effects on progression of gastritis and antioxidative activity rather than direct antimicrobial influence. (*Cancer Sci* 2007; 98: 1689–1695)

Lignans, one of the main groups of plant compounds classified as phytoestrogens, are characterized by possession of a diphenolic structure and have attracted interest as possible chemopreventive materials for cancer in recent years.[1] A number of epidemiological, *in vitro* and animal model studies have provided evidence that naturally occurring plant products, including lignans, are effective for cancer prevention in parts of the body such as the breast, colon and prostate gland.[1–3] The mechanisms of the anti-carcinogenic effects of plant lignans may involve the hormonal influence on the estrogen-mediated carcinogenic pathway, antioxidative activity to scavenge free radicals and block generation of carcinogenic precursors, and/or anti-proliferative/pro-apoptotic effects.[1]

Nordihydroguaiaretic acid (NDGA) is a plant lignan derived from the creosote bush (*Larrea tridentata* DC Coville, Zygophyllaceae), a common shrub of North America and traditionally used in folk medicine.[4] NDGA is a potent antioxidant and has been used as an additive to preserve foods and oils. Several studies have demonstrated that NDGA can prevent tumor cell growth *in vitro* and inhibit *in vivo* carcinogenesis in the skin, bladder and colon.[5–7] In addition to its antioxidative effects, NDGA has been shown to inhibit the activity of lipoxygenase, which is an important enzyme in the arachidonic acid cascade along with cyclooxygenase.[8] While cyclooxygenase-2 inhibitors have been reported to exert suppressive effects on gastric carcinogenesis in rodents,[9,10] the influence of lipoxygenase inhibitors in animal models remains unclear. Arctiin and arctigenin are generally derived from *Arctium* and *Artemisia* species (Compositae) and possess similar structures. Several studies have indicated that they may exhibit inhibitory effects *in vitro* or *in vivo* on skin, pancreas and lung carcinogenesis.[11–13]

Helicobacter pylori is a major causative factor for gastric disorders and epidemiological evidence has accumulated that indicates a significant relationship with chronic active gastritis, peptic ulcers, atrophic gastritis, intestinal metaplasia, and lymphoma or cancer development.[14,15] In 1994, the World Health Organization/International Agency for Research on Cancer concluded that *H. pylori* is a 'definite carcinogen' based on the epidemiological findings.[16] Triple therapy with a proton pump inhibitor and two antimicrobials, amoxicillin and clarithromycin, is usually recommended as the general therapy for *H. pylori* eradication.[17] However, considering the occurrence of strains resistant to these antimicrobial drugs and the persistence of gastric inflammation even after eradication of *H. pylori*, the search for new agents for alternative therapies continues to be very important.[18] The major determining factor of stomach carcinogenesis is the severity of *H. pylori*-induced gastritis. It has been suggested that oxidative stress associated with inflammation plays an important role in gastric carcinogenesis as a mediator of DNA damage and carcinogenic compound formation.[19] Therefore, prevention of *H. pylori*-induced gastritis and oxidative stress is a possible approach by which to inhibit gastric carcinogenesis.

Mongolian gerbils can readily be infected with *H. pylori*, and the resultant chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric cancer resemble the lesions that are apparent in humans.[20,21] The authors have previously demonstrated that a fruit-juice concentrate of Japanese apricot has suppressive effects on *H. pylori*-induced gastritis in the gerbil model.[22] Several natural products, such as turmeric, garlic and green tea extract, have been also found to inhibit *H. pylori*-associated gastric disorders.[23–25] Therefore, in the present study, the effects of three

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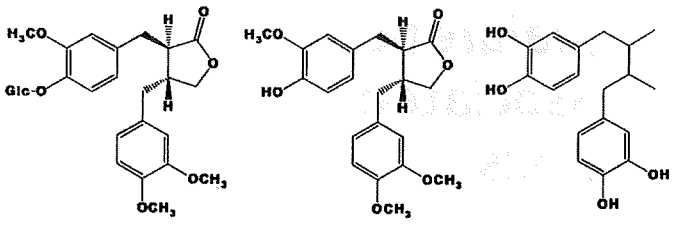

Fig. 1. Chemical structures of the plant lignans used in the present study. Arctiin is a glycosidic form of arctigenin, a dibenzylbutyrolactone lignan. Nordihydroguaiaretic acid (NDGA) is a member of the dibenzylbutane lignans. Glc, glucose.

plant lignans, arctiin, arctigenin, and NDGA, on *H. pylori* proliferation *in vitro* and *H. pylori*-associated gastric carcinogenesis were investigated in Mongolian gerbils.

Materials and Methods

Lignans. NDGA was purchased from Cayman Chemicals (Ann Arbor, MI, USA; Fig. 1). Arctiin was kindly donated from Alps Pharmaceutical Ind. Co. Ltd. (Gifu, Japan), and arctigenin was obtained by acid hydrolysis of arctiin at Yomeishu Seizo, Co. Ltd. (Nagano, Japan; Fig. 1). Identification of isolated arctigenin was performed using high-performance liquid chromatography (HPLC), infrared spectrometry, $^{1}H/^{13}C$ nuclear magnetic resonance analysis and thin-layer chromatography and the purity was determined to be more than 98% using HPLC. The lignans were all prepared as 100 mM solutions in dimethyl sulfoxide (DMSO) immediately before use for *in vitro* experimentation. NDGA and arctigenin were pelleted into AIN93G diet (CLEA Japan, Tokyo, Japan) for the *in vivo* carcinogenesis experiment at the following concentrations: NDGA, 0.25%, 0.05%, and 0.01%; arctigenin, 0.1%.

Bacterial culture. *H. pylori* were prepared using the same method as described previously.[26] Briefly, *H. pylori* strain ATCC43504 (American Type Culture Collection, Rockville, MD, USA) was inoculated on Brucella agar (Merck, Darmstadt, Germany) plates containing 7% v/v heat-inactivated fetal calf serum (FCS) and incubated at 37°C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co. Inc. Tokyo, Japan) at high humidity. Two days later, the bacteria grown on the plates were introduced into Brucella broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with 7% FCS and incubated under the same conditions for 24 h. The broth cultures of *H. pylori* were checked under a phase contrast microscope for bacterial shape and mobility.

Colony forming units (c.f.u.) of *H. pylori*. To assess the influence of lignans on *H. pylori* proliferation, the c.f.u. were determined for the various concentrations of lignans. *H. pylori* grown on Brucella agar plates for 2 days were introduced into Brucella broth with 7% FCS containing arctiin, arctigenin, or NDGA (1, 10 and 100 μM) or 0.1% DMSO as the vehicle control and incubated as mentioned above. After 24 h, serial diluted broth cultures were seeded on segregating agar plates for *H. pylori* (Nissui Pharmaceutical, Tokyo, Japan) and incubated as described above for 5 days. Then, the c.f.u. was determined for each group by counting numbers of colonies.

***In vivo* carcinogenesis.** The experimental design is illustrated in Fig. 2. A total of 178 specific pathogen-free male, 5-week-old Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Kyudo, Fukuoka, Japan) were used in the present study. They were housed in plastic cages on hardwood-chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle,

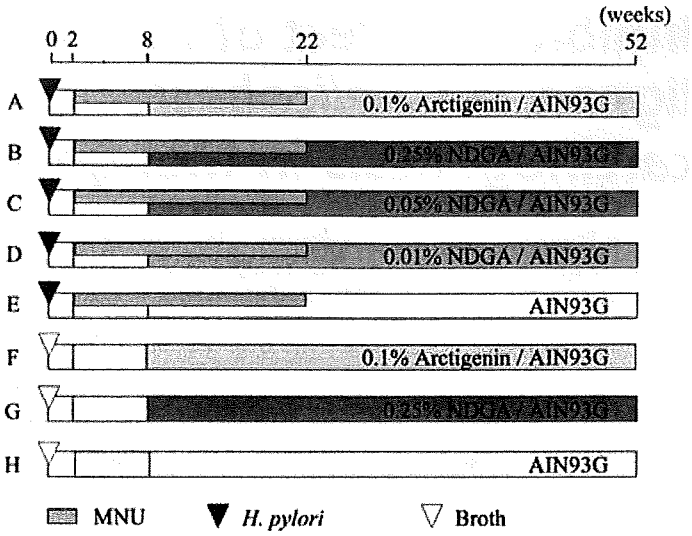

Fig. 2. Experimental design for *in vivo* carcinogenesis. Five-week-old male Mongolian gerbils were inoculated with *Helicobacter pylori* (ATCC43504; groups A–E) or broth (groups F–H). After 2 weeks, animals of groups A–E were administered 10 p.p.m. *N*-methyl-*N*-nitrosourea (MNU) in their drinking water for 20 weeks. The animals were given AIN93G diets containing 0.1% arctigenin (groups A and F), 0.25% nordihydroguaiaretic acid (NDGA; groups B and G), 0.05% NDGA (group C) and 0.01% NDGA (group D) from weeks 8–52.

and were allowed free access to food and water. The gerbils were divided into eight groups (groups A–H). Animals of groups A–E were inoculated with 1.0 mL of broth culture containing *H. pylori* (1×10^8 c.f.u./mL) intragastrically and given a chemical carcinogen, *N*-methyl-*N*-nitrosourea (MNU; Sigma Chemical, St Louis, MO, USA) in their drinking water at the concentration of 10 p.p.m. for 20 weeks, while gerbils of groups F–H were inoculated with Brucella broth. From weeks 8–52, the animals in groups A and F, B and G, C, and D received AIN93G diet containing 0.1% arctigenin, 0.25% NDGA, 0.05% NDGA, and 0.01% NDGA, respectively. Groups E and H were maintained on normal AIN93G diet. At week 52, all animals were killed under deep anesthesia and had their stomachs resected and blood samples collected from the inferior vena cava. Internal organs, including the liver, spleen, kidney, heart, lung, pancreas and testis of groups F–H were also excised for morphological observation. 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.

Histological and serological examination. The excised stomachs were fixed in 10% neutral-buffered formalin and sliced along the longitudinal axis into 4–8 strips of equal width, and embedded in paraffin. Tissue sections were stained with HE. The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System,[27] by scoring the infiltration of neutrophils and mononuclear cells, intestinal metaplasia, and heterotopic proliferative glands, on a four-point scale (0–3: 0, normal; 1, mild; 2, moderate; 3, marked). Blood samples were centrifuged and separated sera were stored at –80°C until use. The titers of anti-*H. pylori* antibodies were measured as described earlier.[28] The sera were also centrifuged (10 000*g* for 50 min at room temperature) through centrifugal filter devices (Microcon YM-10; Millipore, Bedford, MA, USA) and used for the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) using enzyme-linked immunosorbent assay (high-sensitive 8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan).[9]

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Table 1. **Primer sequences for relative quantitative real-time polymerase chain reaction using the LightCycler**

Gene	Sequences	Product length (bp)	EMBL/GenBank/DDBJ Accession no.
GAPDH	5′-AACGGCACAGTCAAGGCTGAGAACG-3′ 5′-CAACATACTCGGCACCGGCATCG-3′	118	AB040445
Urease A	5′-TGTTGGCGACAGACCGGTTCAAATC-3′ 5′-GCTGTCCCGCTCGCAATGTCTAAGC-3′	120	M60398

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Real-time polymerase chain reaction and relative quantitative analysis. Genomic DNA was extracted from glandular stomach mucosa at the border between the antrum and corpus using a DNeasy tissue kit (Qiagen, Hilden, Germany). For *H. pylori* quantification, relative quantitative real-time polymerase chain reaction (PCR) of *urease A* was performed using a LightCycler system (Roche Diagnostics, Mannheim, Germany) with the gerbil-specific *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as an internal control. This was performed basically as described earlier, using QuantiTect SYBR Green PCR (Qiagen) with the optimal Mg^{2+} concentration at 2.5 mM.[22,29] The primer sequences of each marker are listed in Table 1. Specificity of the PCR reaction was confirmed using a 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 quantification of the *H. pylori urease A* gene was performed as previously established using the internal control without the necessity for external standards.[29] The amounts of the *H. pylori urease A* gene were expressed relative to 100% in the *H. pylori*-infected control group (group E).

Statistical analysis. Fisher's exact test was used to assess the incidence of gastric adenocarcinomas. The Mann–Whitney *U*-test was applied to determine the significance of differences in the c.f.u., microscopic scores for gastritis, body weights, serological results, and the amount of *H. pylori* genomic DNA using *urease A* locus. *P*-values <0.05 were considered to be statistically significant.

Results

Inhibitory effects of lignans on *H. pylori* proliferation. All three lignans decreased the numbers of *H. pylori* colonies in a dose-dependent manner, and the suppressive effects of each lignan were significant at the dose of 100 μM (Fig. 3). Arctigenin showed the highest inhibitory effect of all three lignans, and colony formation was also significantly inhibited at the dose of 10 μM. Inhibition by NDGA was slightly stronger than that by arctiin.

Average body weights, total lignan intake, titers of anti-*H. pylori* antibodies and serum 8-OHdG levels in each experimental group. Data for average body weights at week 52, total lignan intake, titers of anti-*H. pylori* antibodies and serum 8-OHdG levels are summarized in Table 2. The average body weights for 0.25% NDGA-treated groups (groups B and G) were significantly lower than those of the corresponding control groups (groups E and H, respectively). Total lignan intake by each group essentially corresponded to the proportion of lignan in their food. All *H. pylori*-infected groups (groups A–E) demonstrated significantly higher values for anti-*H. pylori* antibody titers than the non-infected groups (groups F–H). There were no significant differences between the *H. pylori*-infected groups. Serum 8-OHdG levels in the 0.1% arctigenin-treated and *H. pylori*-infected group (group A), 0.25% NDGA-treated and *H. pylori*-infected group (group B) and 0.05% NDGA-treated and *H. pylori*-infected group (group C) were significantly lower than that in the *H. pylori*-infected control group (group E; $P < 0.01$).

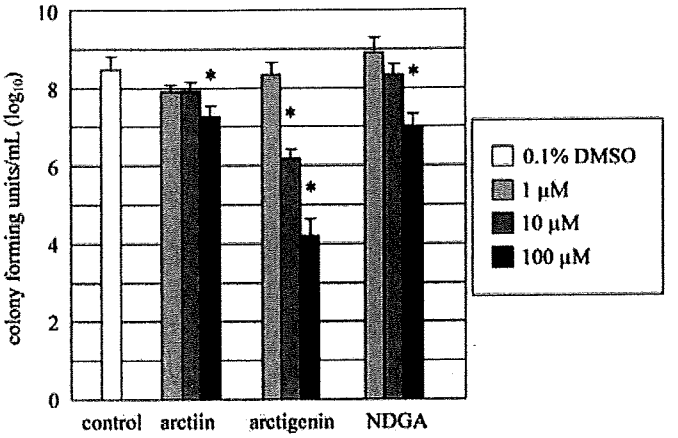

Fig. 3. Inhibitory effects of arctiin, arctigenin and nordihydroguaiaretic acid (NDGA) on *Helicobacter pylori* proliferation, as assessed by counting the numbers of c.f.u. Each value represents the mean ± SD of three independent experiments. *$P < 0.05$ compared with vehicle control (0.1% dimethyl sulfoxide [DMSO]).

Incidences of glandular stomach adenocarcinomas. The incidences of gastric adenocarcinomas are summarized in Table 2. The value for group B (39.4%) was significantly lower than that in group E (65.5%, $P < 0.05$). In contrast, there was no significant difference in the incidence between groups A and E. In groups F–H, no gastric tumors were observed. Both differentiated and undifferentiated adenocarcinomas were found in groups A–E (Fig. 4). All of the glandular stomach adenocarcinomas generated in the present study developed in the pyloric gland area. No macroscopic lesions were observed in liver, spleen, kidney, heart, lung, pancreas and testis of all groups. In the histological examination for groups F–H, no pathological findings were recognized in the internal organs except for a renal hemangioma in 0.25% NDGA-treated group (group G).

Status of gastritis. Data for the status of gastritis in each group are summarized in Table 3. The gastric mucosa of groups A–E was generally thickened and edematous, occasionally with erosions, ulcers, and polypoid lesions. Such macroscopic findings were not recognized in the stomachs of groups F–H. Groups A–E showed significantly higher scores for infiltration of neutrophils and mononuclear cells, intestinal metaplasia, and heterotopic proliferative glands than those of groups F–H. Scores for intestinal metaplasia both of antrum and corpus in group B and that for heterotopic proliferative glands of antrum in group A were significantly lower than those of group E ($P < 0.05$). There were no significant differences in scores for infiltration of neutrophils and mononuclear cells between lignan-treated groups (groups A–D) and group E.

Quantification of *H. pylori*. Average relative *urease A* gene levels in the glandular stomachs in each group are shown in Fig. 5. There were no significant differences in the amount of *H. pylori* genomic DNA levels at the *urease A* gene locus among groups A–E. No amplification of the *urease A* gene was detected in groups F–H.

Table 2. Summary of general data and incidences of gastric carcinomas in Mongolian gerbils

Group	Treatment	Effective number	Body weight (g)	Total lignan intake (g)	Anti-*Hp* IgG titer (AI)	Serum 8-OHdG level (ng/mL)	Carcinoma†		
							Differentiated	Undifferentiated	Incidence (%)
A	*Hp* + MNU + 0.1% Arctigenin	30	91.8 ± 14.0	0.942 ± 0.036	370.8 ± 35.6	0.448 ± 0.086**	13	4	17/30 (56.7)
B	*Hp* + MNU + 0.25% NDGA	33	79.0 ± 12.7*	2.078 ± 0.054	306.0 ± 20.0	0.467 ± 0.123**	11	2	13/33 (39.4)*
C	*Hp* + MNU + 0.05% NDGA	29	90.5 ± 15.2	0.513 ± 0.056	280.9 ± 18.9	0.449 ± 0.064**	15	1	16/29 (55.2)
D	*Hp* + MNU + 0.01% NDGA	28	95.2 ± 14.2	0.108 ± 0.012	258.9 ± 38.9	0.583 ± 0.233	16	1	17/28 (60.7)
E	*Hp* + MNU	29	92.0 ± 14.8	0	278.4 ± 25.5	0.590 ± 0.132***	17	2	19/29 (65.5)
F	Broth + 0.1% Arctigenin	7	108.5 ± 10.6	1.094 ± 0.201	2.51 ± 0.81	n.d.	0	0	0/7 (0)
G	Broth + 0.25% NDGA	8	94.8 ± 6.70***	2.573 ± 0.105	1.85 ± 0.19	n.d.	0	0	0/8 (0)
H	Broth	14	106.6 ± 7.46	0	1.54 ± 0.29	0.485 ± 0.107	0	0	0/14 (0)

†Classification based on the histopathology. 'Differentiated' includes tubular types, whereas 'undifferentiated' consists of signet-ring cell and poorly differentiated types. *$P < 0.05$ versus group E, **$P < 0.01$ versus group E, ***$P < 0.05$ versus group H. Values for results are expressed as averages ± SD. 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AI, arbitrary index; *Hp*, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; n.d., not determined; NDGA, nordihydroguaiaretic acid.

Fig. 4. Histology of gastric adenocarcinomas. (a) Well differentiated adenocarcinoma and (b) poorly differentiated adenocarcinoma from group E.

Fig. 5. Quantitation of the *Helicobacter pylori* using DNA specific for *urease A* in glandular stomachs of Mongolian gerbils. The value was set at 100% in group E and data are means ± SE.

Discussion

In the present study, it was demonstrated that arctigenin and NDGA exert inhibitory effects on *H. pylori* proliferation *in vitro*, and NDGA was found to decrease the incidence of *H. pylori*-associated gastric adenocarcinomas in Mongolian gerbils. In

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Table 3. Histopathological evaluation of gastritis

Group	Effective number	Infiltration of neutrophils		Infiltration of mononuclear cells		Intestinal metaplasia		Heterotopic proliferative glands	
		Antrum	Corpus	Antrum	Corpus	Antrum	Corpus	Antrum	Corpus
A	30	2.37 ± 0.11	2.40 ± 0.11	2.97 ± 0.03	3.00 ± 0.00	1.90 ± 0.15	1.87 ± 0.13	2.57 ± 0.10*	2.87 ± 0.06
B	33	2.61 ± 0.12	2.45 ± 0.14	2.91 ± 0.05	2.91 ± 0.05	1.40 ± 0.14*	1.12 ± 0.09*	2.73 ± 0.08	2.70 ± 0.08
C	29	2.66 ± 0.10	2.79 ± 0.09	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.16	1.62 ± 0.13	2.90 ± 0.06	2.86 ± 0.07
D	28	2.43 ± 0.11	2.71 ± 0.10	2.93 ± 0.07	2.93 ± 0.05	1.54 ± 0.14	1.61 ± 0.15	2.79 ± 0.09	2.79 ± 0.08
E	29	2.38 ± 0.15	2.48 ± 0.15	3.00 ± 0.00	3.00 ± 0.00	1.97 ± 0.14	1.79 ± 0.13	2.90 ± 0.06	2.83 ± 0.07
F	7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
G	8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
H	14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

*$P < 0.05$ versus group E. Values for results are expressed as averages ± SD.

in vitro culture, all three lignans reduced the c.f.u. of *H. pylori* in a dose-dependent manner, with arctigenin being the most effective. Arctiin is a glycosidic form of arctigenin and its relatively low effect might be explained by low permeability due to the conjugated glucose. The present result is consistent with previous reports that arctigenin has stronger suppressive effects than arctiin on heat shock responses in mammalian cells and 4-nitroquinoline-*N*-oxide/glycerol-induced mouse pulmonary tumors.[11,30] The weak suppressive effect of arctiin at the highest concentration might reflect spontaneous hydrolysis and conversion to arctigenin rather than actual influence of the glycosidic form.

Based on the results of inhibitory effects *in vitro*, the chemopreventive effects of NDGA and arctigenin on *H. pylori*-associated gastric carcinogenesis in Mongolian gerbils were investigated, and it was found that administration of a 0.25% NDGA diet significantly decreased the incidence of gastric adenocarcinomas at week 52. NDGA concentrations in the non-toxic range of ≤0.25% were chosen because a previous study in rats demonstrated no significant toxicity at 0.5% and 1.0% NDGA given for 2 years.[31] We also set a dose of 0.1% for arctigenin, based on the amount in the dried seed of *Arctium lappa* (0.05–0.6%), which is traditionally used as a folk medicine.[13,30] To the authors' knowledge, this is the first demonstration that NDGA can prevent stomach carcinogenesis. Although the basic mechanisms for the inhibitory effects of NDGA remain unclear, the present results are in line with previous epidemiological studies suggesting that antioxidants reduce the risk of gastric cancer.[32,33]

NDGA has a long history as an antioxidant to preserve foods and oils and is known to be a potent scavenger of peroxynitrite, singlet oxygen, hydroxyl radicals, and hypochlorous acid.[34] *H. pylori* infection has been demonstrated to cause production of reactive oxygen species in human gastric epithelial cells, and antioxidative supplements such as vitamin C or E lead to protective effects on *H. pylori*-induced gastric lesions.[35,36] Several studies have pointed to the suppressive effects of NDGA on *in vivo* carcinogenesis in the skin, urinary bladder, kidney, and colon in rodent models.[4–6,37] In these studies, the antioxidative ability of NDGA was thought to be responsible for the cancer preventive effect. In the present case, although both NDGA and arctigenin reduced the serum 8-OHdG levels, a marker of oxidative stress, only 0.25% NDGA-treatment exhibited a preventive effect on gastric cancer development in Mongolian gerbils. In contrast, 0.25% NDGA significantly inhibited development of intestinal metaplasia both in the antrum and corpus, which has been extensively studied as a pre-neoplastic lesion in the human stomach, and is strongly associated with gastric cancer development.[38] The multistep morphogenesis from *H. pylori* infection to gastric cancer development includes sequential stages of chronic atrophic gastritis, intestinal metaplasia and focal dysplasia.[39,40] Therefore, the present results indicate that the inhibitory mechanism of NDGA against gastric carcinogenesis in gerbils might be associated not only with antioxidative activity, but also with inhibitory effects on the progression of gastritis.

NDGA is a well-known inhibitor of lipoxygenase that converts arachidonic acid and other polyunsaturated fatty acids into biologically active molecules, including leukotriene and hydroxylated arachidonic acid derivatives, associated with inflammatory responses and carcinogenesis. These arachidonic acid metabolites have been identified as mediators of tumor development and progression in various organs.[8] Therefore, lipoxygenase has been proposed as a putative target for cancer chemoprevention.[41] A previous study has suggested that *H. pylori*-induced gastritis is associated with an increased capacity to generate leukotriene.[42] In addition, Park *et al.* recently reported that *H. pylori* increased the biosynthesis of leukotriene by the 5-lipoxygenase pathway in a gastric epithelial cell line, and that NDGA suppressed this *H. pylori*-mediated 5-lipoxygenase signaling.[43] Thus, one of the underlying mechanisms of NDGA against gastric carcinogenesis might be considered to be an inhibitory effect on progression of *H. pylori*-induced gastritis through the lipoxygenase signaling pathway. Further studies are required to clarify the association between progression of *H. pylori*-induced gastritis and lipoxygenase-mediated leukotriene synthesis.

The incidence of gastric cancer in women is approximately half that recorded in men. Recent epidemiological studies have showed that postmenopausal women are at increased risk of gastric cancer, suggesting an inverse association between estrogenic activity and stomach carcinogenesis,[44,45] although this hypothesis is still controversial.[46] Furukawa *et al.* reported that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastric cancer in rats was also predominant in males, and that estrogens reduced the incidence.[47] Plant lignans, generally not estrogenic themselves, are converted to mammalian lignans (enterodiol and enterolactone) that have weak estrogenic activities, through a series of metabolic reactions by the intestinal microflora.[1,48] Furthermore, previous studies have demonstrated that NDGA itself can bind to the sex steroid binding protein, as well as estrogen receptors α and β.[49,50] These observations indicate a possible hormonal effect of NDGA and/or its derivatives on gastric carcinogenesis, although this remains to be confirmed.

Arctigenin failed to reduce the incidence of gastric adenocarcinomas at the dose used in this study, despite the strong inhibitory effect of *H. pylori* proliferation *in vitro*. Serological examination showed that there were no significant differences in the titers of anti-*H. pylori* antibodies among all *H. pylori*-infected groups. Moreover, relative quantitative analysis for *H. pylori* using DNA specific for *urease A* in the gastric mucosa, known to be a highly sensitive and specific marker for the detection and quantification of *H. pylori*,[51,52] also supported this observation. More continuous

and/or highly concentrated exposure to NDGA and arctigenin might be necessary to inhibit *H. pylori* proliferation directly *in vivo*. It is well established that arctiin is rapidly transformed to arctigenin by intestinal microflora of rat and human, and the arctigenin is then also converted to enterolactone through a stepwise reaction.[53,54] Thus, drug-specific pharmacokinetic differences might account for the lack of effects and it is possible that a higher dietary dose of arctigenin might exhibit anti-carcinogenic activity against *H. pylori*-associated gastric cancer development.

Although NDGA has been used as a food and pharmaceutical preservative for its antioxidative effect, it has been banned in some countries because of reports of toxicity in the liver and kidney with high-dose use.[55,56] The reported LD_{50} (oral) of NDGA is 0.8–5.5 g/kg body weight in rodents.[4] In the present study, the average body weights of 0.25% NDGA-treated groups (groups B and G) were significantly lower than those of the control groups (groups E and H, respectively). However, the total food intake of group B (831.4 ± 21.8 g; means ± SD) was also significantly reduced compared with that of group E (1073.7 ± 35.2 g; $P < 0.01$). Similarly, the total food intake of group G (1029.2 ± 42.1 g) showed a decreasing tendency compared with that of group H (1133.5 ± 39.9 g; $P = 0.064$). In addition, histological examination for groups F–H revealed no pathological findings in the liver, spleen, kidney, heart, lung, pancreas, and testis, except for a microscopic renal hemangioma in group G, which has been reported as a spontaneous neoplasm in aging Mongolian gerbils.[57] No macroscopic lesions in the internal organs, including kidneys, were observed in any other groups. Therefore, it was considered that NDGA toxicity was relatively low at the dose used in the present study. The body-weight loss of group B was unlikely to influence the incidence of gastric tumors because previous epidemiological studies have demonstrated that body weight is not associated with risk of non-cardiac gastric adenocarcinoma.[58,59]

In conclusion, the present study showed a chemopreventive effect of NDGA on MNU-initiated and *H. pylori*-promoted gastric carcinogenesis in Mongolian gerbils. While NDGA failed to reduce *H. pylori* proliferation *in vivo*, *H. pylori*-associated intestinal metaplasia was suppressed by NDGA treatment. The results indicate that the anti-carcinogenic effects of NDGA might be due to inhibition of the progression of gastritis and to antioxidative properties, rather than to direct antimicrobial activity.

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4-Vinyl-2,6-dimethoxyphenol (canolol) suppresses oxidative stress and gastric carcinogenesis in *Helicobacter pylori*-infected carcinogen-treated Mongolian gerbils

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Oxidative stress is linked to gastric carcinogenesis because of its ability to damage DNA. Here we examined antioxidative and anti-inflammatory effects of 4-vinyl-2,6-dimethoxyphenol (canolol), a recently identified potent antioxidative compound obtained from crude canola oil, on *Helicobacter (H.) pylori*-induced gastritis and gastric carcinogenesis using a Mongolian gerbil model. The animals were allocated to *H. pylori*-infection alone (12 weeks) or *H. pylori* + *N*-methyl-*N*-nitrosourea (MNU) administration (52 weeks). After oral inoculation of *H. pylori*, they were fed for 10 and 44 weeks with or without 0.1% canolol. *H. pylori*-induced gastritis, 5′-bromo-2′-deoxyuridine (BrdU) labeling and scores for cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) immunohistochemistry were attenuated in the canolol-treated groups. Expression of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), COX-2 and iNOS mRNA in the gastric mucosa, and serum 8-hydroxy-2′-deoxyguanosine (8-OHdG), anti-*H. pylori* IgG and gastrin levels were also significantly lower in canolol-treated groups. Furthermore, the incidence of gastric adenocarcinomas was markedly reduced in the *H. pylori* + MNU + canolol-treated group [15.0% (6/40)] compared to the control group [39.4% (13/33)] ($p < 0.05$). These data indicate canolol to be effective for suppressing inflammation, gastric epithelial cell proliferation and gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils. Interestingly, the viable *H. pylori* count was not changed by the canolol containing diet. Thus, the data point to the level of inflammation because of *H. pylori* rather than the existence of the bacteria as the determining factor. Importantly, canolol appears to suppress induction of mRNAs for inflammatory cytokines.

Key words: canolol; antioxidant; canola oil; *Helicobacter pylori*; Mongolian gerbils

Helicobacter pylori (*H. pylori*) is now considered as the most important etiological agent for chronic gastritis and peptic ulcer disease, as well as a cause of gastric carcinoma.[1,2] There is accumulating evidence that eradication of *H. pylori* in the stomach by administration of oral antimicrobial agents results in the resolution of *H. pylori*-infected chronic active gastritis and peptic ulceration and significantly lowers the risk of stomach tumor development in patients without precancerous lesions.[3,4] However, such bacterial eradication treatment has not been always successful. The occurrence of antibiotic-resistant *H. pylori* has been reported, and it is occasionally associated with adverse effects.[5] Therefore, it is still desirable to develop alternative approaches for cancer prevention and a number of studies have demonstrated protective effects of plant extracts against *H. pylori* infection, such as green tea catechins[6] and a garlic extract.[7] In a previous study, we also found fruit-juice concentrate of Japanese apricot (ume) (CJA) to decrease the number of *H. pylori* and suppress chronic active gastritis in gerbils,[8] although the mechanism was unclear. *H. pylori* infection has been reported to cause production of H_2O_2 in AGS human gastric epithelial cells, which might contribute to carcinogenesis.[9] Correa *et al.* reported antioxidants like vitamin C or vitamin E have protective effects against *H. pylori*-induced lesions due to their free radical scavenging activity.[10,11]

4-Vinyl-2,6-dimethoxyphenol (canolol), was recently identified as a potent antioxidative compound in crude canola oil, exhibiting more potent antialkylperoxyl [ROO•] radical activity than well-known antioxidants, like α-tocopherol, vitamin C, β-carotene, rutin and quercetin.[12] We previously reported strong scavenging capacity against the endogenous mutagen, peroxynitrite ($ONOO^-$), and suppression of bacterial mutation, consistent with the earlier observed protection from DNA damage, and prevention of oxidation of lipids and proteins.[13]

The Mongolian gerbil (*Meriones unguiculatus*) provides a useful animal model of *H. pylori*-induced chronic active gastritis that allows investigation of morbidity-related pathological epithelial alterations in gastric mucosa, and their development into intestinal metaplasia and gastric neoplasia.[14] The purpose of our study was to evaluate the effectiveness of canolol for inhibition of *H. pylori*-infected chronic gastritis and gastric carcinogenesis. An investigation of effects on induction of cytokines in mouse peritoneal macrophages *in vitro* was included.

Material and methods

Chemicals

Canolol, 4-vinyl-2,6-dimethoxyphenol (molecular weight, 180) (Fig. 1), is a novel and potent antioxidant, contained in crude canola oil. In our study, it was synthesized to at least 95% purity (confirmed by nuclear magnetic resonance) and mixed with 2,6 di-tert-butyl-4-methylphenol (butylhydroxytoluene, BHT, Sigma Chemical, St Louis, MO) at final concentration of 300 ppm at Junsei Chemical, Tokyo, Japan. The preparation was sealed under helium or nitrogen, and also kept as an ethanol stock solution in nitrogen at −80°C.

Diets

AIN93G was purchased from Clea Japan, (Tokyo, Japan). Tert-butylhydroquinone (tBHQ), contained in the standard recipe of AIN93G, was excluded to facilitate analysis of subtle changes because of canolol. Soybean oil was replaced with canola oil containing 443.7 ppm tocopherol (158.7 ppm α-tocopherol, 279.6 ppm γ-tocopherol, 5.4 ppm δ-tocopherol and no β-tocopherol) (Showa Sangyo, Funabashi, Japan). Canolol powder containing 300 ppm BHT was dissolved in ethanol and mixed with the modi-

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fied AIN93G described earlier to give a final concentration of 0.1% canolol and 0.3 ppm BHT. Since no information was available regarding *in vivo* administration of canolol, previous literature for other antioxidants was referenced for a suitable dose; 0.1–0.5% tocopherol successfully suppressed formation of colon aberrant crypt foci[15] and mammary adenocarcinomas.[16] Since canolol was revealed to be a more potent oxidative radical scavenger than tocopherol,[13] the lowest concentration (0.1%) was applied here. For controls, 0.3 ppm BHT alone or no antioxidant supplement in the basal diet were used. The diets sealed under vacuum were stored in a freezer at −30°C and thawed for use everyday. Leftovers were measured on the next day and the new diet supplied given again to prevent excessive oxidation.

Carcinogen

N-methyl-*N*-nitrosourea (MNU) (Sigma Chemical, St Louis, MO) was dissolved in distilled water at the concentration of 10 ppm and administered *via* light-shielded bottles in drinking water *ad libitum*. MNU solutions were freshly prepared 3 times per week.

Inoculation of H. pylori

ATCC43504 (American Type Culture Collection, Manassas, VA) was grown in Brucella broth (Becton Dickinson, Cockeysville, MD), containing 7% (v/v) heat-inactivated fetal bovine serum, at 37°C under microaerophilic conditions, at high humidity for 24 hr. After 24 hr fasting, gerbils were inoculated *via* an oral catheter with 1.0 ml aliquots of *H. pylori* culture containing 1.0×10^8 colony-forming units/ml of the organisms. Four hours later, the animals were again allowed free access to food.

Animals and experimental protocol

One hundred seventy-eight specific-pathogen-free male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan), 6 weeks old, were used in this study. They were housed in an air-conditioned biohazard room designed for experimentally infected animals, with a 12-hr light/12-hr dark cycle and were allowed free access to food. The experimental design is illustrated in Figure 2. The animals were allocated to Experiments I or II.

In Experiment I, 58 gerbils were divided into 6 groups (A–F): [Group A, $n = 10$]: *H. pylori*-infected, canolol + BHT-treated animals; [Group B, $n = 10$]: *H. pylori*-infected, BHT-treated animals, no canolol; [Group C, $n = 10$]: *H. pylori*-infected, untreated animals; [Group D, $n = 10$]: Broth-inoculated (no *H. pylori*), canolol + BHT-treated animals; [Group E, $n = 10$]: Broth-inoculated, only BHT-treated animals; and [Group F, $n = 8$]: Broth-inoculated, untreated animals. Animals were killed at 18 weeks of age for midterm examination.

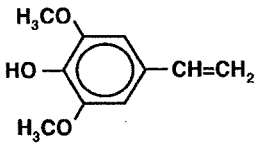

FIGURE 1 – Structure of 4-vinyl-2,6-dimethoxyphenol (canolol). Molecular weight, 180.

In Experiment II, 120 gerbils were divided into 4 groups (G to J). Two weeks after inoculation of *H. pylori*, Groups G, H and I were administrated with MNU for 20 weeks. Group J was given broth and autoclaved distilled water as control. Groups G, H, I and J were given, (*i*) canolol + BHT, (*ii*) BHT, (*iii*) control diet and (*iv*) canolol + BHT diet, respectively, from the 8th experimental week to the end of the experimental period. BrdU at a dose of 100 mg/kg was injected intraperitoneally 60 min before the sacrifice at experimental week 52. The study was approved by the Animal Care Committee of Aichi Cancer Center Research Institute.

Bacterial cultures

To assess bacterial colonization in the gastric mucosa, half of each glandular stomach from gerbils in Experiment I was homogenized with 1.0 ml of phosphate buffered saline (PBS) for culture of *H. pylori*. One hundred-microliter aliquots were inoculated onto *H. pylori* agar plates (Nissui Pharmaceutical, Tokyo, Japan), which were then incubated at 37°C under microaerophilic conditions. The numbers of *H. pylori* colonies were counted after 5–7 days.

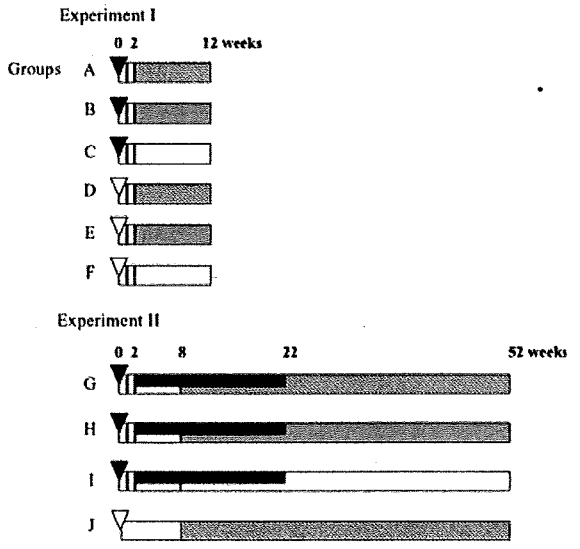

FIGURE 2 – Experimental design. Animals, 6-week-old male Mongolian gerbils; ▼, *H. pylori* inoculation (i.g.); ▽, Broth only, control. ■, MNU in drinking water at the concentration of 10 ppm. ▨, 0.1% canolol/0.5 ppm 2,6 Di-tert-butyl-4-methylphenol (BHT)/AIN93G diet; ▩, 0.5 ppm BHT/ AIN93G diet, control; □, AIN93G diet, control.

TABLE I – PCR PRIMERS USED FOR LIGHTCYCLER ANALYSIS

Gene	Sequence	Product size (bp)
GAPDH	F: 5′-AACGGCACAGTCAAGGCTGAGAACG-3′ R: 5′-CAACATACTCGGCACCGGCATCG-3′	118
IL-1β	F: 5′-TGACTTCACCTTGGAATCCGTCTCT-3′ R: 5′-GGCAACAAGGGAGCTCCATCAC-3′	91
TNF-α	F: 5′-GCTGCCCCCACCTCGTGCTC-3′ R: 5′-CTTGATGGCAGACAGGAGGCTGACC-3′	89
COX-2	F: 5′-GCCGTCGAGTTGAAAGCCCTCTACA-3′ R: 5′-CCCCGAAGATGGCGTCTGGAC-3′	97
iNOS	F: 5′-GCATGACCTTGGTGTTTGGGTGCC-3′ R: 5′-GCAGCCTGTGTGAACCTGGTGAAGC-3′	110

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

TABLE II – HISTOPATHOLOGICAL RESPONSES IN GERBILS

Experiments	Groups	No.	Treatments	Antrum						
				Infiltration of neutrophils	Infiltration of mononuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)	Score of COX-2 immunohistochemistry	Score of iNOS immunohistochemistry
Experiment I	A	10	*Hp* -> Canolol + BHT	1.2 ± 0.4[1]	2.2 ± 0.4[1]	1.2 ± 0.6[1]	0.0 ± 0.0	12.5 ± 2.5[1]	0.9 ± 0.2[1]	0.9 ± 0.1[1]
	B	10	*Hp* -> BHT	2.9 ± 0.3	3.0 ± 0.1	2.2 ± 0.3	0.0 ± 0.0	20.3 ± 3.7	2.2 ± 0.2	1.7 ± 0.2
	C	10	*Hp*	3.0 ± 0.1	3.0 ± 0.1	2.3 ± 0.4	0.0 ± 0.0	20.9 ± 3.3	2.3 ± 0.3	1.5 ± 0.3
	D	10	Broth -> Canolol + BHT	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	5.2 ± 0.8	0.2 ± 0.1	0.2 ± 0.2
	E	10	Broth -> BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.8	0.3 ± 0.1	0.3 ± 0.1
	F	8	Broth	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 1.1	0.3 ± 0.1	0.2 ± 0.1
Experiment II	G	40	*Hp* + MNU -> Canolol + BHT	1.9 ± 0.4[3]	2.3 ± 0.4[3]	1.9 ± 0.4[3]	0.9 ± 0.5[3]	10.4 ± 2.3[3]	1.0 ± 0.4[3]	1.2 ± 0.5[3]
	H	33	*Hp* + MNU -> BHT	2.6 ± 0.3	3.0 ± 0.0	2.5 ± 0.3	1.7 ± 0.4	18.5 ± 5.4	1.7 ± 0.5	2.1 ± 0.3
	I	36	*Hp* + MNU	2.7 ± 0.2	3.0 ± 0.0	2.6 ± 0.4	1.8 ± 0.4	20.6 ± 4.6	1.8 ± 0.5	1.7 ± 0.5
	J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 0.6	0.0 ± 0.0	0.2 ± 0.1

Experiments	Groups	No.	Treatments	Corpus						
				Infiltration of neutrophils	Infiltration of mononuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)	Score of COX-2 immunohistochemistry	Score of iNOS immunohistochemistry
Experiment I	A	10	*Hp* -> Canolol + BHT	0.9 ± 0.2[1]	0.9 ± 0.2[1]	0.7 ± 0.2[1]	0.0 ± 0.0	6.8 ± 1.7[2]	0.6 ± 0.2[2]	0.5 ± 0.1[2]
	B	10	*Hp* -> BHT	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.0 ± 0.0	9.6 ± 2.6	1.0 ± 0.2	0.8 ± 0.2
	C	10	*Hp*	1.5 ± 0.2	1.5 ± 0.2	1.2 ± 0.1	0.0 ± 0.0	10.4 ± 2.7	1.1 ± 0.1	0.8 ± 0.2
	D	10	Broth -> Canolol + BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 0.6	0.2 ± 0.1	0.2 ± 0.1
	E	10	Broth -> BHT	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.4	0.2 ± 0.1	0.2 ± 0.0
	F	8	Broth	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.5	0.3 ± 0.1	0.3 ± 0.1
Experiment II	G	40	*Hp* + MNU -> Canolol + BHT	2.2 ± 0.5[3]	2.5 ± 0.3[4]	2.1 ± 0.5[4]	0.8 ± 0.3[3]	7.9 ± 2.4[3]	0.6 ± 0.3[3]	1.0 ± 0.4[3]
	H	33	*Hp* + MNU -> BHT	2.8 ± 0.3	3.0 ± 0.1	2.6 ± 0.3	1.6 ± 0.5	12.3 ± 2.9	1.2 ± 0.4	1.6 ± 0.5
	I	36	*Hp* + MNU	2.7 ± 0.3	3.0 ± 0.2	2.8 ± 0.3	1.7 ± 0.3	13.8 ± 1.9	1.3 ± 0.4	1.4 ± 0.3
	J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.7	0.0 ± 0.0	0.3 ± 0.2

Values for results are expressed as means ± SD.
[1] $p < 0.01$ *vs.* Groups B and C.–[2] $p < 0.05$ *vs.* Groups B and C.–[3] $p < 0.01$ *vs.* Groups H and I.–[4] $p < 0.05$ *vs.* Groups H and I.

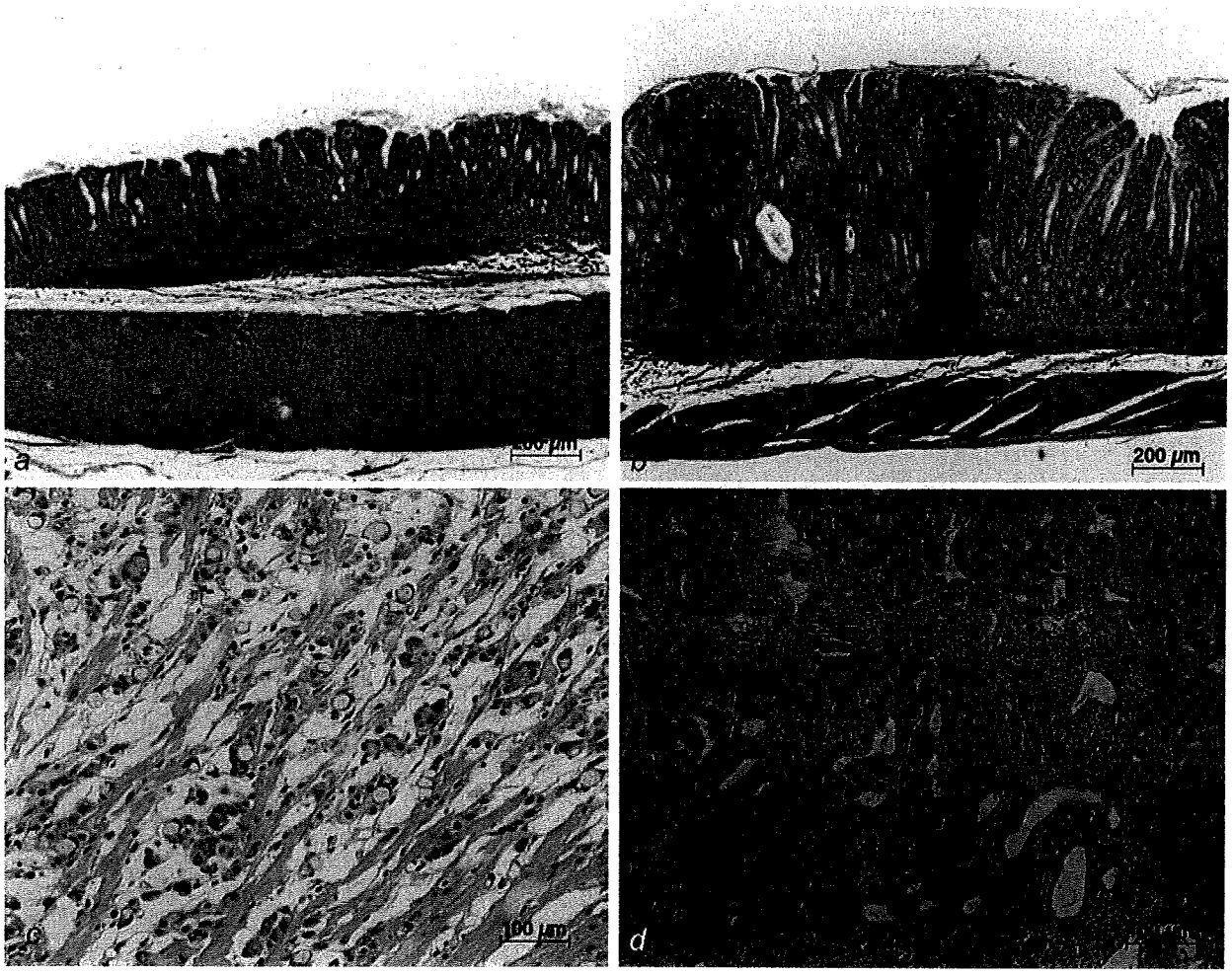

FIGURE 3 – Histopathological findings and histology of adenocarcinomas in the gastric mucosa. (*a*) Mild gastritis in a Group A gerbil at 12 weeks postinfection with canolol treatment. (H&E; ×25); (*b*) Marked infiltration of inflammatory cells and hyperplasia is seen in a Group B gerbil with control diet (H&E; ×25); (*c*) Signet-ring cell carcinoma at 52 weeks in a Group H gerbil (H&E; ×200). (*d*) Well differentiated adenocarcinoma in a glandular stomach at 52 weeks in a Group I gerbil (H&E; ×80).

Histopathology and immunohistochemistry

For histological and immunohistochemical examination, the stomachs were fixed in 10% neutral buffered formalin for 24 hr and embedded in paraffin. Serial paraffin sections (4-μm thick) were prepared and stained with hematoxylin and eosin (H&E) for morphological observation, and immunohistochemistry for iNOS and COX-2. Mucosal inflammation in the gastric mucosa was analyzed on H&E-stained sections. The inflammatory responses of glandular mucosa were graded according to the following morphological criteria: Grade 0 (normal), Grade 1 (mild), Grade 2 (moderate) and Grade 3 (marked) (Supplementary Table I). Tissue sections were immunostained for BrdU labeling with a mouse monoclonal anti-BrdU antibody (1:50, DAKO) as described previously.[17] Labeling indices (LI) were calculated as the percentages of BrdU-positive epithelial cells within glands. For this purpose 10 different arbitrarily selected points in the antrum and corpus mucosa were selected for quantitation. Immunohistochemical analyses of COX-2 and iNOS were carried out as previously described,[18] using an anti-COX-2 mouse monoclonal antibody (diluted 1:200; BD Biosciences, San Jose, CA) and an anti-iNOS mouse polyclonal antibody (diluted 1:500, EMD Biosciences, San Diego, CA). To quantitate the degree of staining, a grading system was employed with the following criteria: Grade 0, no immunoreactivity; Grades 1–3, increasing degrees of intermediate immunoreactivity and Grade 4, extensive immunoreactivity.[19]

Effects of canolol on production of nitric oxide (NO), interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) in peritoneal macrophages from mice

Mouse peritoneal macrophages were obtained after intraperitoneal injection of 3 ml of 10% proteosepeptone and Griess assays for nitrate/nitrite measurement were conducted to assess the production of NO. Amounts of IL-12 and TNF-α were measured with enzyme-linked immunosorbent assay (ELISA). Macrophages were incubated with 0–300 μM canolol and 10% FBS at 37°C, then 1.0 μg/ml lipopolysaccharide (LPS) or 1.0 μg/ml LPS plus 0.2 μg/ml interferon-γ (IFN-γ) were added. After incubation for 20 hr, NO, IL-12 and TNF-α production from macrophages was measured.

Analysis of mRNA expression of cytokines by real time quantitative PCR

Total RNA was extracted from the antrum and corpus in the glandular stomach (Experiment I) or in the border region between the 2 regions (Experiment II), using a RNA extraction kit (Isogen,

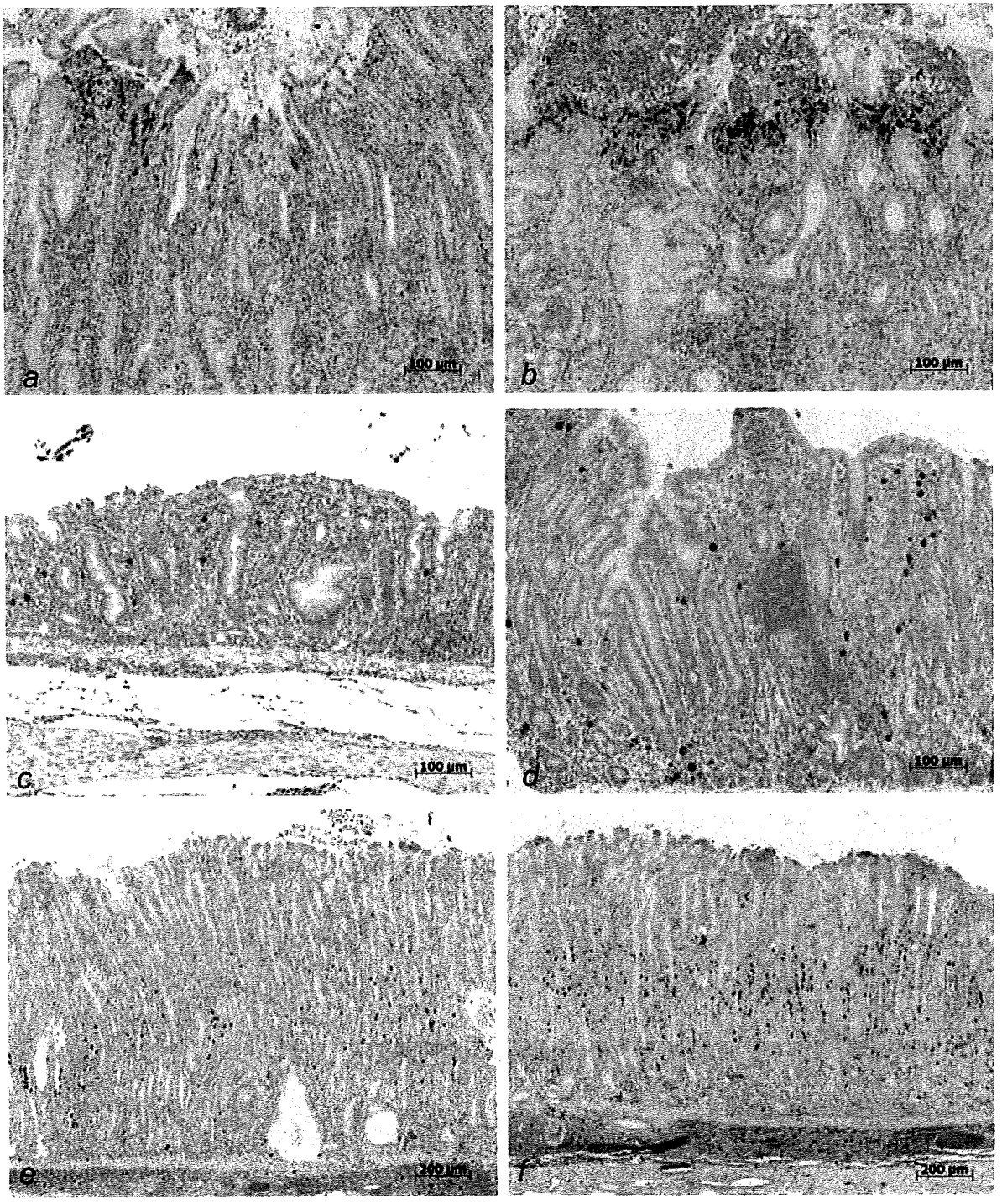

FIGURE 4 – Immunohistochemistry of gastric lesions. (*a* and *b*) COX-2. Original magnification, ×100. (*c* and *d*) iNOS. Original magnification, ×100. Note that the intensity of COX-2 and iNOS immunoreactivity in the canolol-treated gastric mucosa (*a* and *c*), is weaker than that in the control infection groups (*b* and *d*). (*e* and *f*) BrdU. BrdU positive cells are distributed more broadly in the control group (*f*) than the canolol-treated group (*e*). Original magnification, ×50.

Nippon Gene, Tokyo, Japan). After DNase treatment, first strand cDNAs were synthesized using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Quantitative PCR of IL-1β, TNF-α, COX-2 and iNOS, was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using gerbil-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. The PCR was performed basically as described using a QuantiTect SYBR Green PCR (QIAGEN) kit with optimal Mg^{2+} concentration at 2.5 mM. The 5′- and 3′-primer sequences are listed in Table I. 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

TABLE III – INCIDENCE OF GASTRIC CARCINOMAS IN GERBILS

Experiments	Groups	No.	Treatments	Carcinoma		
				Dif.	Undif.	Incidence (%)
Experiment II	G	40	*Hp* + MNU->Canolol + BHT	5	1	6/40 (15.0)[1]
	H	33	*Hp* + MNU->BHT	11	2	13/33 (39.4)
	I	36	*Hp* + MNU	15	0	15/36 (41.7)
	J	5	Broth->Canolol + BHT	0	0	0/5 (0.0)

Dif., differentiated adenocarcinoma; Undif., undifferentiated adenocarcinoma. *Hp, H.pylori* (i.g.).
[1]$p = 0.031$ to Group H and $p = 0.011$ to Group I with Fisher's exact test.

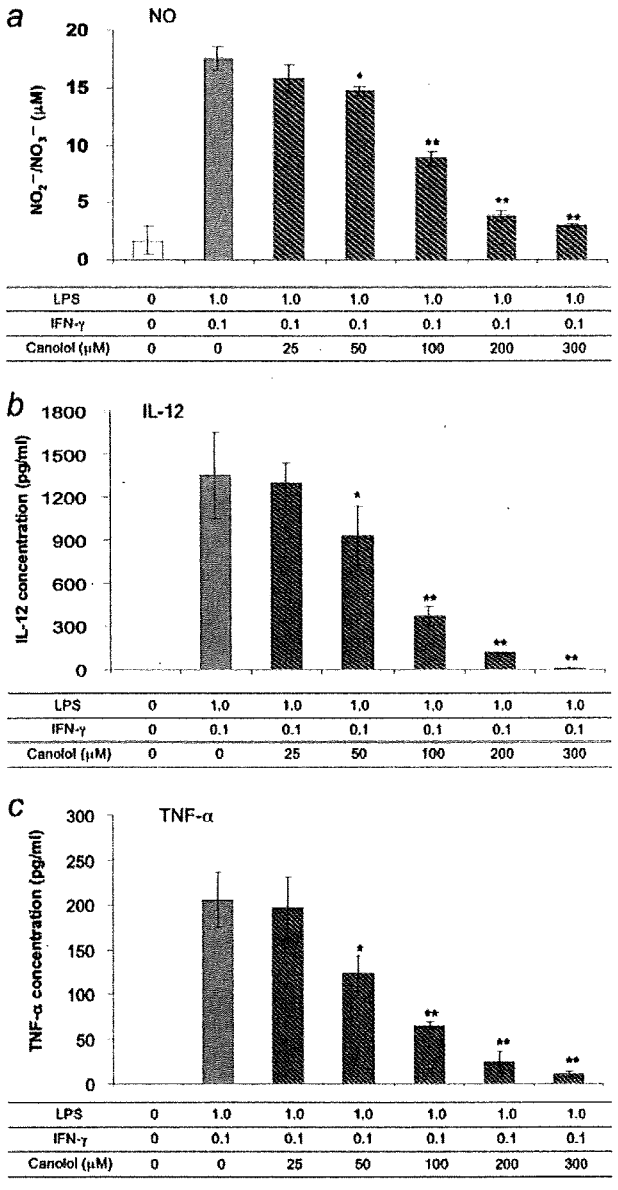

FIGURE 5 – Suppression of NO and inflammatory cytokine induction in mouse peritoneal macrophages *in vitro* by canolol. The concentrations of NO (*a*), IL-12 (*b*) and TNF-α (*c*), were significantly reduced by 12.5 μM canolol (means ± SD) ($n = 3$). *$p < 0.05$, **$p < 0.01$ *vs.* LPS plus IFN-γ.

were electrophoresed in 3% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Quantification was performed as earlier established using an internal control without any necessity for external standards. The levels of cytokine mRNAs were expressed relative to 1.0 in the control groups (Groups F and J).[20]

*Elevation of 8-OHdG, anti-*H. pylori *IgG and gastrin in* H. pylori *infected gerbil plasma*

Before the removal of stomachs, blood samples were collected from the inferior vena cava after laparotomy. Sera were separated from blood and their anti-*H. pylori* IgG antibody titers were measured with an ELISA (GAP-IgG; Biomerica, Newport Beach, CA) and values expressed as an arbitrary index (AI). AI values of more than 1.5 indicated *H. pylori* infection. Gastrin levels were measured using a radioimmunoassay kit (SRL, Tokyo, Japan). Serum samples were also centrifuged (4°C, 10,000*g* for 30 min) through centrifugal filter devices (Microcon YM-10, Millipore, Bedford, MA) and measured for 8-OHdG levels (ELISA; high sensitive 8-OHdG check; Japan Institute for Control of Aging, Shizuoka, Japan).[21]

Statistical analyses

Quantitative values were expressed as means ± SD, and differences between means were evaluated by the Bonferroni multiple-comparison test. *p* values of less than 0.05 were considered significant.

Results

Canolol intake and bacterial colonization

The survival rates of all groups were >95%, with no differences among groups. In the shorter term experiment (Experiment I), total canolol intakes in Groups A and D were 0.45 ± 0.01 and 0.47 ± 0.01 (g/gerbil), respectively. There were no significant differences between Groups A and D. At 12 weeks postinfection, the numbers of *H. pylori* colonies were 3.17 ± 1.51, 3.52 ± 0.67 and 3.59 ± 0.85 ($\times 10^4$ colony/half stomach) in Groups A, B and C, respectively. No significant inhibitory effect of canolol against bacterial growth was detected ($p = 0.64$). In the longer term experiment (Experiment II), total canolol intakes in Groups G and J were 1.84 ± 0.02 and 1.84 ± 0.01 (g/gerbil), respectively, again with no significance. There was also no significant variation in body weights (Supplementary Fig. 1) among long-term experiment groups G to J, confirming no apparent toxicity of canolol.

Effects of canolol against H. pylori*-induced gastritis and cell proliferation*

All gastric mucosal specimens from uninfected gerbils had normal histomorphology. The histological findings for gastric mucosal specimens in *H. pylori*-infected gerbils are shown in Table II. At 12 weeks (Experiment I), neutrophils and lymphoplasmocytic cell infiltration in the antral mucosa of the canolol-treated group (Group A) were significantly suppressed, compared to the *H. pylori*-infected control groups (Groups B and C) (Table II; Figs. 3*a* and 3*b*). Both antral and corpus BrdU labeling indices in the canolol-treated gerbils (Group A) were significantly reduced as compared to values for control groups B and C ($p < 0.05$) (Table II). The BrdU LIs in Group A were reduced to 62% in the antrum and 71% in the corpus of the Group B values. During the 52 weeks (Experiment II) there was a change over time in topography of the gastritis, with a shift from predominantly antral gastritis to pangastritis in *H. pylori*-infected gerbils. Infiltration of inflammatory

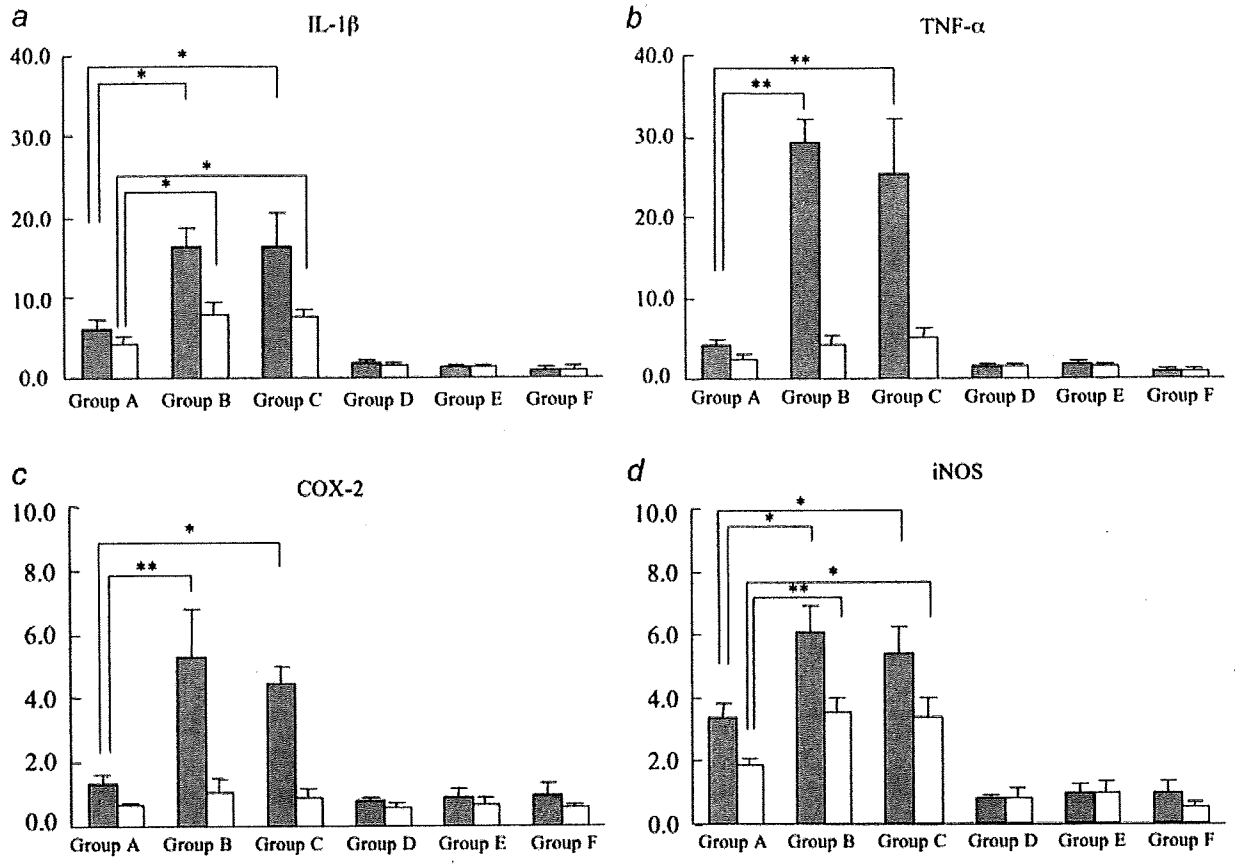

FIGURE 6 – Relative expression levels of IL-1β, TNF-α, COX-2, and iNOS mRNAs in glandular stomachs of gerbils at 12 weeks postinfection. (*a*) IL-1β; (*b*) TNF-α; (*c*) COX-2; (*d*) iNOS. Values are arbitrary unit values (mean ± SE) relative to 1.0 for controls. Note decrease in Group A (canolol group) as compared to Groups B and C (controls), especially in the antrum. ▨, Antrum; □, corpus. $*p < 0.05$ and $**p < 0.01$.

cells, hyperplasia and intestinal metaplasia lesions of gastric mucosa were markedly lower in the canolol-treated group (Group G) than in the *H. pylori*-infected control groups (Groups H and I). BrdU LIs in the canolol-treated gerbils (Group G) were again significantly lower both in antrum and corpus than in the control groups H and I, values being decreased to 56 and 64% of the Group H levels ($p < 0.05$) (Table II).

Immunohistochemistry of COX-2 and iNOS

Immunoreactivity against COX-2 and iNOS was evident in all *H. pylori*-infected gerbils. However, scores in the canolol-treated groups were significantly lower than in the canolol-untreated control groups (Fig. 4 and Table II).

Canolol suppression of gastric carcinogenesis

In Experiment II, the incidence of glandular stomach tumors overall was significantly lower in Group G (*H. pylori* + MNU + canolol + BHT) compared to Groups H (*H. pylori* + MNU + BHT) [15.0% (6/40) *vs.* 39.4% (13/33), $p = 0.031$] and I (*H. pylori* + MNU) [15.0% (6/40) *vs.* 41.7% (15/36), $p = 0.011$] at 52 weeks postinfection (Table III; Figs. 3*c* and 3*d*). There was no difference in the incidence of gastric adenocarcinomas between Groups H and I [39.4% (13/33) *vs.* 41.7% (15/36), $p = 1.00$]. In the control group J and Experiment I, no tumors developed in the glandular stomach.

Suppression of NO and inflammatory cytokines by canolol in mouse peritoneal macrophages in vitro

LPS and IFN-γ induction of NO, IL-12 and TNF-α was significantly inhibited by 50 μM canolol or above *in vitro* (Figs. 5*a*–5*c*).

Oral administration of canolol and mRNA expression of IL-1β, TNF-α, COX-2 and iNOS

Gastric IL-1β, TNF-α, COX-2 and iNOS were found to be expressed at very low levels in the uninfected control gerbils. However, in the *H. pylori*-infected groups, the levels of these cytokines and enzymes were markedly elevated in the antrum and corpus already 12 weeks after infection (Experiment I, Fig. 6). Relative expression of IL-1β (Fig. 6*a*) in Groups B and C was upregulated 16 ± 2 and 16 ± 4 times, respectively, compared to the uninfected group F (control, its value set at 1.0 ± 0.2). Canolol treatment at 0.1% in the diet (Group A) significantly attenuated the increase of mRNA expression to 6.2 ± 1.1 times in the antrum ($p < 0.05$). For the corpus, it was elevated to 8.0 ± 1.3 (Group B) and 7.6 ± 0.7 (Group C) times with *H. pylori* infection and decreased to 4.3 ± 0.9 times with canolol treatment (Group A) ($p < 0.05$) compared to Group F (0.86 ± 0.15 times to corpus of Group F). The figures in Groups D, E and F were comparable. Regarding the expression of TNF-α (Fig. 6*b*), transcriptional upregulation 29 ± 3 and 25 ± 7 fold in *H. pylori* infected groups B and C, respectively, was alleviated to 4.0 ± 0.6 times in Group A ($p < 0.01$). Concerning Cox-2 expression (Fig. 6*c*), the figures were elevated to 5.3 ± 1.5 ($p < 0.01$) and 4.5 ± 0.5 ($p < 0.05$) times in Groups B and C, respectively, and decreased to 1.4 ± 0.7 times in Group A. For iNOS (Fig. 6*d*), the values were 6.1 ± 0.8 (Group B) and 5.4 ± 0.9 (Group C) times and lowered to 3.4 ± 0.4 times (Group A) in the antrum and 3.6 ± 0.4, 3.4 ± 0.6 and 1.8 ± 0.2 times in the corpus, respectively.

In Experiment II (Fig. 7), transcription of the inflammatory cytokines reached much higher levels than with the shorter experimental period. IL-1β mRNA was strongly upregulated 116 ± 17 (Group H) and 119 ± 23 (Group I) fold with long term *H. pylori*

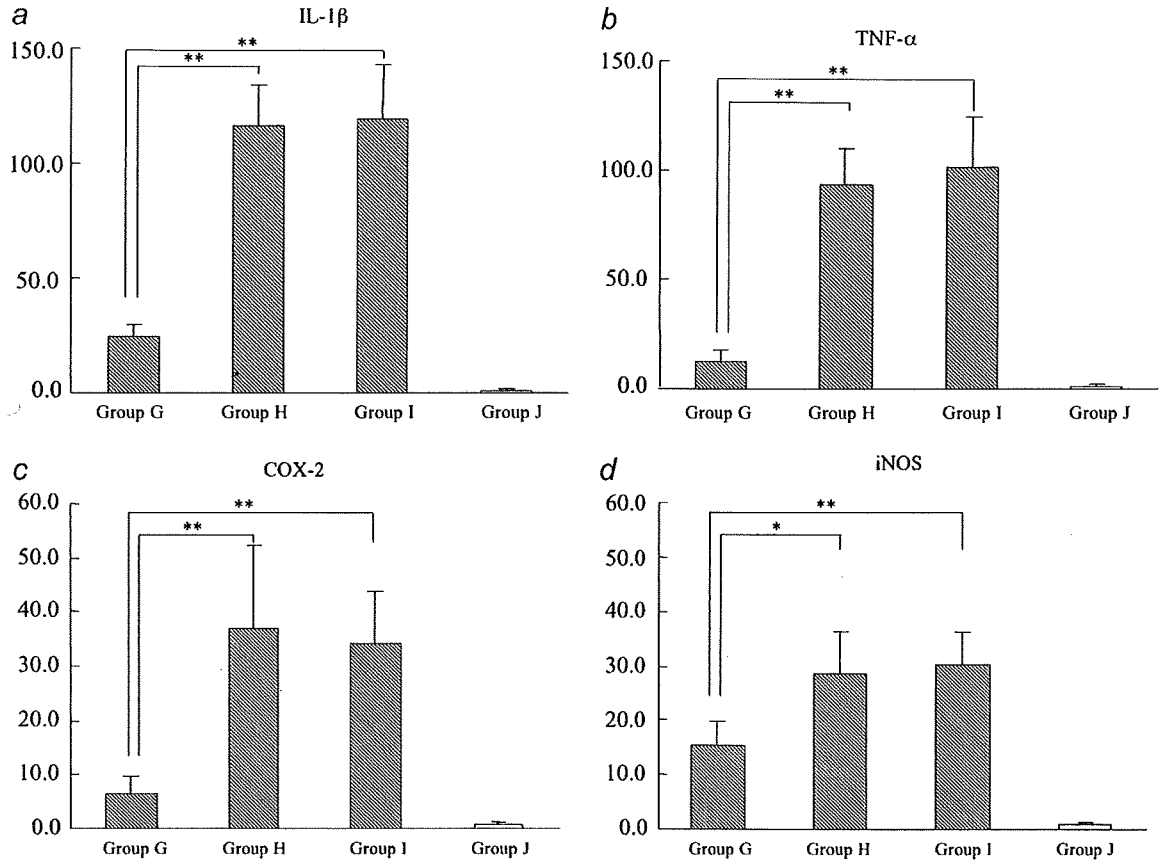

FIGURE 7 – Relative expression levels of IL-1β, TNF-α, COX-2 and iNOS mRNAs in glandular stomachs of gerbils at 52 weeks postinfection. (*a*) IL-1β; (*b*) TNF-α; (*c*) COX-2; (*d*) iNOS. Values are arbitrary unit values (mean ± SE) relative to 1.0 for controls. Note decrease in Group G as compared to Groups H and I. ▨, glandular stomach mucosa at the border between the antrum and corpus. $*p < 0.05$ and $**p < 0.01$.

TABLE IV – SERUM 8-OHDG, ANTI-*H.pylori* IGG TITERS AND GASTRIN LEVELS

Experiments	Groups	No.	Treatments	8-OHdG (ng/ml)	Antibody titers (A.I.)	Gastrin (pg/ml)
Experiment I	A	10	*Hp* -> Canolol + BHT	0.33 ± 0.05[1]	19.6 ± 5.5[2]	ND
	B	10	*Hp* -> BHT	0.48 ± 0.12	29.8 ± 7.6	ND
	C	10	*Hp*	0.51 ± 0.19	30.5 ± 8.2	ND
	D	10	Broth -> Canolol + BHT	0.30 ± 0.05	1.0 ± 0.4	ND
	E	10	Broth -> BHT	0.27 ± 0.05	1.4 ± 0.5	ND
	F	8	Broth	0.26 ± 0.07	1.1 ± 0.5	ND
Experiment II	G	40	*Hp* + MNU -> Canolol + BHT	0.41 ± 0.04[3]	186.4 ± 74.2[4]	634.0 ± 160.7[5]
	H	33	*Hp* + MNU -> BHT	0.57 ± 0.07	249.5 ± 98.5	780.7 ± 216.2
	I	36	*Hp* + MNU	0.63 ± 0.12	257.9 ± 95.1	764.1 ± 195.7
	J	5	Broth -> Canolol + BHT	0.29 ± 0.09	1.1 ± 0.3	202.2 ± 54.4

8-OHdG, 8-hydroxy-2′-deoxyguanosine. ND, not determined. Values for results are expressed as means ± SD.
[1]$p < 0.05$ *vs.* Groups B and C.–[2]$p < 0.01$ *vs.* Groups B and C.–[3]$p < 0.01$ *vs.* Groups H and I.–[4]$p < 0.05$ *vs.* Groups H and I.–[5]$p < 0.05$ *vs.* Groups H and I.

infection and this was drastically attenuated to 25 ± 6 times with canolol treatment (Group G, $p < 0.01$ *vs.* Groups H and I). TNF-α transcription also increased 93 ± 17 and 101 ± 23 times in Groups H and I and reduced to 13 ± 5 times in Group G ($p < 0.01$). Regarding Cox-2, the figures of 37 ± 15 and 34 ± 10 times (Groups H and I) were decreased to 6.3 ± 3.2 times (Group G, $p < 0.01$). Finally, the values of iNOS (28 ± 8 and 30 ± 6 times in Groups H and I, $p < 0.05$ and $p < 0.01$, respectively) were again lowered to 15 ± 5 times (Group G).

*Effects of canolol on serum 8-OHdG, anti-*H. pylori *antibodies and gastrin levels*

Infection with *H. pylori* remarkably elevated the serum level of 8-OHdG and anti-*H. pylori* IgG titers in both Experiments I and II. Significant reduction was noted with canolol treatment (Table IV). After *H. pylori* infection, serum gastrin levels in Experiment II were elevated at 52 weeks, and this increase was alleviated in the canolol-treated group G (Table IV).

Discussion

Triple therapy consisting with a proton pump inhibitor and 2 antimicrobial agents, amoxicillin and clarithromycin, is usually recommended as the general therapy for *H. pylori* eradication in Japan.[22] However, frequent emergence of resistant strains to these antimicrobial agents, and persistence of gastric inflammation even after the eradication of *H. pylori*, has been observed by

physicians. Therefore we need to find means to attenuate gastric inflammation and provide cytoprotection against *H. pylori*-induced cytotoxicity.[23]

Our study showed *H. pylori*-associated chronic active gastritis and gastric carcinogenesis to be effectively suppressed by oral administration of canolol at 0.1% in the diet. In addition, iNOS, COX-2, and inflammatory cytokine IL-1β, IL-12 and TNF-α mRNA expression levels were substantially decreased after canolol administration *in vivo* and *in vitro*. It has been reported that a predominantly *H. pylori*-specific Th1 response, characterized by induction of high level of TNF-α, IL-1β, and IFN-γ is associated with *H. pylori*-infected gastritis.[24,25] COX-2 and iNOS are well known to play important roles in gastric cancer growth and progression. These results indicate that canolol inhibits the mRNA expression of COX-2, upregulated by *H. pylori*-infection, and might reduce release of prostaglandin E2 from the gastric mucosa.[26] Canolol also suppressed iNOS activity and presumably the NO endogenously produced by this family of enzymes. It is interesting to note that the numbers of *H. pylori* colonies in the glandular stomach at 12 weeks postinfection was not significantly reduced. Thus, suppression of IL-1β, TNF-α, COX-2 and iNOS, activated by *H. pylori*-infection, appears critical for the inhibition of gastric carcinogenesis. Crabtree *et al.*[27] showed an increase in the production of TNF-α in antral biopsy specimens from patients with *H. pylori* gastritis coinciding with neutrophil infiltration. Similarly, Harris *et al.*[28] described the number of mRNA molecules for IL-6 to be elevated to a greater extent in persistently infected rhesus monkeys (6 years) compared to the early phase (7 weeks after infection), whereas expression of IL-1β and TNF-α declined. However, gastric biopsies from persistently infected animals only showed weak gastritis. Yamaoka *et al.*[25] have reported natural history of *H. pylori* (ATCC43504) induced gastritis and associated gastric mucosal cytokine expression in Mongolian gerbils. In their results, polymorphonuclear and mononuclear cell infiltration was apparent relatively early (8 and 4 weeks after inoculation, respectively) and declined thereafter. Levels of cytokines, including IL-1β, INF-γ, IL-4, IL-6 and IL-10, appeared to be mostly parallel; the values for IFN-γ correlated particularly well with numbers of both polymorphonuclear and mononuclear cells. However, in our experiment, inflammation induced cytokine overexpression was greater in the long-term experiment compared to the short one (Fig. 7 *vs.* Fig. 6). In contrast to the data by Yamaoka *et al.*,[25] infiltration of the inflammatory cells progressively increased. Thus, in terms of the correlation of inflammatory cell infiltrate and cytokine expression level, data in our experiment and their results do not appear to be incompatible. *H. pylori* inoculated in gerbils might have been partially eradicated or their virulence could have become attenuated in their system.

Of note, at 12 and 52 weeks postinfection, BrdU-labeled cells in gastric mucosa decreased almost 50–70% in canolol-treated gerbils compared to those in *H. pylori*-infected control groups. Gonzalez *et al.* found a similar reduction of proliferating cells in ultraviolet B (UVB) exposed mouse epidermis with oral administration of antioxidants like lutein + zexanthin.[29] Kim *et al.* also observed lowering of BrdU LIs with carotenoids (lycopene, fucoxanthin and lutein) and curcumin and its derivative (tetrahydrocurcumin) in 1,2-dimethylhydrazine treated mouse colonic crypts, along with reduced aberrant crypt formation.[30] At the molecular level, reactive oxygen intermediates may alter the expression and function of Cox-2 and iNOS, which may influence the expression of proteins involved in regulation of cell cycle progression.[31] Similarly, anti-inflammatory and antioxidant agents could protect against such effects and also act on the expression and function of several cell cycle regulating proteins.[32]

Furthermore, the remarkable elevation of serum 8-OHdG by *H. pylori*-infection was alleviated by canolol. 8-OHdG has been proposed as a key biomarker of oxidative DNA damage relevant to carcinogenesis,[33] because of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2), singlet oxygen and hydroxyl radicals ($^{\bullet}OH$) as well as reactive nitrogen species (RNS) including $ONOO^-$, which is known to cleave DNA and also nitrate guanine to generate 8-nitroguanine. Mutation of *Salmonella sp.* (TA98) by $ONOO^-$ was earlier found to be effectively suppressed by canolol,[13] one of the most potent anti-$ROO^{\bullet}$ antioxidants.[13,34,35] Therefore, one of the underlying mechanisms is reduction of free radical scavenging oxidative damage.[36] In an *in vitro* system we could also show that canolol suppresses inflammation mediators (Fig. 5).

In conclusion, oral administration of canolol significantly reduced anti-*H. pylori* IgG antibody titers and gastrin levels in serum, without apparently suppressing *H. pylori* colonization. A lack of any direct correlation between anti-*H. pylori* IgG antibody titers and number of colonies was also reported by Murakami *et al.*[37] Canola oil is a traditional cooking oil in many countries. The canolol concentration in crude canola oil is estimated to be ~220–1,200 ppm, which could provide doses similar to that used in our study. It should be noted, however, that the concentration in refined canola oil is significantly lower[12] so that suggestions to the edible oil industry for alternative methods of refining might be warranted. Alternatively, synthesized or extracted canolol could be added to the refined oil and used as table oil or taken as a supplement. Taken together, these findings indicate that the antioxidative compound, canolol, can prevent *H. pylori*-induced gastritis and carcinogenesis in a gerbil model. Therefore, this dietary factor may have a potential role in controlling *H. pylori*-associated gastroduodenal diseases.

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