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Original Article

Effect of Black Tea Aqueous Non-Dialysate on *Helicobacter pylori* Infection in Mongolian Gerbils

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Abstract

Objectives: Recently, the appearance of *Helicobacter pylori* (*H. pylori*) resistant to antibiotics has been reported. The development of an antibiotic therapy which would not induce resistant strains of *H. pylori* is anticipated. In the present study, the antibiotic effect of black tea aqueous non-dialysate (BTND), the fraction different from tea catechins, on *H. pylori* was investigated using Mongolian gerbils infected with *H. pylori*.

Methods: BTND was extracted from black tea leaves. A 0.1 w/v% solution of BTND or green tea catechins (GTC) was provided as drinking water to male NGS/Sea Mongolian gerbils infected with *H. pylori* (ATCC43504) for two weeks. Their stomachs were then excised, the mucosal surfaces were macroscopically observed, and colony forming units (CFU) of *H. pylori* were counted. The data were compared between the BTND and GTC groups.

Results: The CFU of *H. pylori* were significantly decreased by intake of BTND. The body weight of the animals tended to be larger in the group supplied with BTND than in that supplied with GTC. Gastric mucosal injury tended to be smaller in the animals supplied with BTND than in those with GTC.

Conclusions: These results suggest that BTND may have an inhibitory effect on *H. pylori* infection.

Key words: *Helicobacter pylori*, tea aqueous non-dialysate, green tea catechins, gastric mucosal erosion, Mongolian gerbil

Introduction

Recently, antibiotic treatment combined with a proton pump inhibitor has been clinically employed for *Helicobacter pylori* (*H. pylori*) elimination (1). However, the appearance of *H. pylori* strains resistant to antibiotics has been reported, and the above combination treatment was also examined for the effect of secondary microorganism elimination (2). Therefore, development of a therapy which would prevent the occurrence of resistant *H. pylori* is anticipated. Various biological effects of the components of tea leaves (*Camellia sinensis*), particularly green tea catechins (GTC), have been reported (3–13). However, there are disadvantages in the clinical use of GTC, e.g., strong bitterness and cytotoxicity at high concentrations. Besides the catechins, there are also other effective substances in tea leaves. For example, tea aqueous non-dialysate (TND) is the generic name of the fraction of water-soluble polymer components with molecular weights over 12000 extracted from tea leaves. It has already been reported that black tea aqueous non-dialysate (BTND), which is well characterized among several kinds of TND, exhibits anti-microbial activity against a cariogenic bacterium (14). Therefore, the activity of BTND against *H. pylori* was investigated in the present study.

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Methods

The non-dialyzable fraction of black tea leaves (BTND) was prepared in our laboratory. Black tea leaves (50 g of commercially available Darjeeling tea leaves) were added to 500 ml of boiling water. After 10 min of infusion, the mixture was filtered and the filtrate was extracted sequentially with 400 ml each of chloroform, ethyl acetate, and *n*-butanol. The aqueous residue was dialyzed exhaustively against water with Seamless Cellulose Tubing (small size 18 from Wako Pure Chemical Ind., Ltd.) and then lyophilized. BTND is considered to be a complex mixture of tannins and polysaccharides (approx. 70% and 30%, respectively) containing polymers such as glucose, galactose, and rhamnose, flavonols, catechols, and

gallates. The BTND used in the present study contains practically no caffeine (0.018%). A 0.1% aqueous solution of BTND looks pale-brown and its taste is mild without any irritation (15). Polyphenon70S® (Tokyo Food Techno Co., Ltd., Tokyo, Japan) was used as green tea catechins (GTC). This preparation contains catechins extracted from green tea leaves in a total content of 73.4%, including epigallocatechin gallate (EGCg) (33.5%), epicatechin gallate (ECg) (10.1%), epigallocatechin (EGC) (17.4%), and epicatechin (EC) (8.6%).

Fifteen male NGS/Sea Mongolian gerbils (13 weeks old) infected with *H. pylori* (ATCC43504) were purchased from Seac Yoshitomi Co., Ltd. (Fukuoka, Japan). The infected animals were prepared according to the method of Hirayama et al. (16): Mongolian gerbils at 5 weeks of age were inoculated orally with 0.5 ml of *H. pylori* suspension, which contained about 2×10^8 CFU/dose, using a feeding needle. The antibody inspection was carried out at 12 weeks of age, and the positive animals showing an OD_{450} over 0.1 were used for the experiment. The animals were kept in cages in a temperature (23±2°C)- and humidity (55±5%)-controlled SPF room with a 12-h dark-light cycle throughout the experiment. Following a pre-feeding period of one week, the Mongolian gerbils were divided into three groups, designated as I, II, and III. Each group was given free access to distilled water, 0.1 w/v% of GTC solution, and 0.1 w/v% of BTND solution, respectively, for two weeks. All the animals were fed with Oriental MF® sterilized with γ-ray (Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the experiment. Since one animal in the control group was accidentally lost during the initial period, the study was conducted using the remaining 14 animals. After the two weeks of treatment, the animals were fasted for 24 hours and sacrificed. The stomachs were then extirpated and cut along the greater curvature to remove their contents. After macroscopic observation, each stomach was homogenized in 7 ml of saline. A 100-µl aliquot of each serial dilution of the homogenate was spread on a selective medium Nissui Helicobacter plate® (Nissui Co., Ltd., Tokyo, Japan). The plates were incubated at 37°C under 10% CO_2 atmosphere for 5 days, and the colonies were counted.

All experiments were carried out in observance with the guidelines for animal experiments drawn up by the junior college of the researcher.

A one-way analysis of variance (ANOVA) test was used and $p<0.05$ was considered to be significant.

Results

The mean daily intake volume for the day per head of distilled water (Group I) and the two drug solutions (Group II and III) are shown in Table 1. The gerbils of Group III (BTND) had a tendency to consume a higher volume and those of Group II (GTC) a lower volume of the drug solution than the volume of water consumed by the control gerbils (Group I). Consequently, the intake of GTC (Group II) and BTND (Group III) were 6.3 mg/head/day and 12.7 mg/head/day, respectively.

The mean body weights on the first and 14th days are shown in Table 2. The body weight of the BTND group (Group III) had a tendency to increase at a higher rate compared with that of the distilled water group (Group I).

Table 1 The mean intake volumes of drinking water (ml/head/day) consumed by gerbils during the 2-week experiment

	Experimental group and drinking water supplied	Intake of solution[a] (ml/head/day)
I	Distilled water (n=4)	7.6±1.58
II	0.1 w/v% GTC solution (n=5)	6.3±0.90
III	0.1 w/v% BTND solution (n=5)	12.7±4.38

[a]: The data are expressed in means±SD.

Table 2 Body weights on the first and 14th days of each gerbil group

	Experimental group and drinking water supplied	Body weight[a] (g)	
		The first day	The 14th day (Ratio compared to the first day)
I	Distilled water (n=4)	66.6±2.55 (100)	70.3±3.25 (105.6)
II	0.1 w/v% GTC solution (n=5)	65.3±3.68 (100)	71.6±6.43 (109.6)
III	0.1 w/v% BTND solution (n=5)	66.0±4.03 (100)	77.0±3.67 (116.6)

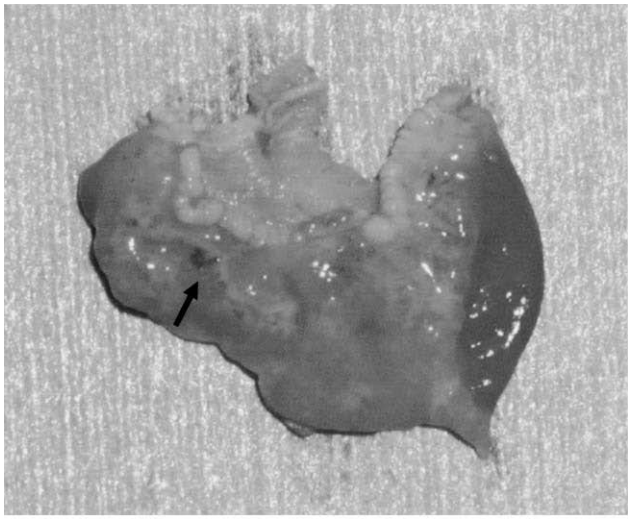
[a]: The data are expressed in means±SD and a one-way analysis of variance (ANOVA) test was performed.

Table 3 The mean scores of hemorrhage and gastric mucosal erosion in gerbils infected with *H. pylori*

	Experimental group and drinking water supplied	Mean score[a] ±SD	
		Hemorrhage[b]	Gastric mucosal erosion[c]
I	Distilled water (n=4)	3.5±0.58	3.4±0.75
II	0.1 w/v% GTC solution (n=5)	3.0±1.73	4.0±0.00
III	0.1 w/v% BTND solution (n=5)	2.4±0.82	3.2±0.84

[a]: The data are expressed in mean±SD and a one-way analysis of variance (ANOVA) test was performed.
[b]: Hemorrhage of the gastric mucosa was scored as follows: 0, no bleeding; 1, one small bleeding spot; 2, multiple small bleeding spots; 3, one bleeding area; 4, multiple bleeding areas.
[c]: Gastric mucosal erosion was scored as follows: 0, normal; 1, edematous; 2, erosion; 3, multiple erosions; 4, hemorrhage erosions and/or ulcers larger than 1 mm in diameter.

(a)



(b)

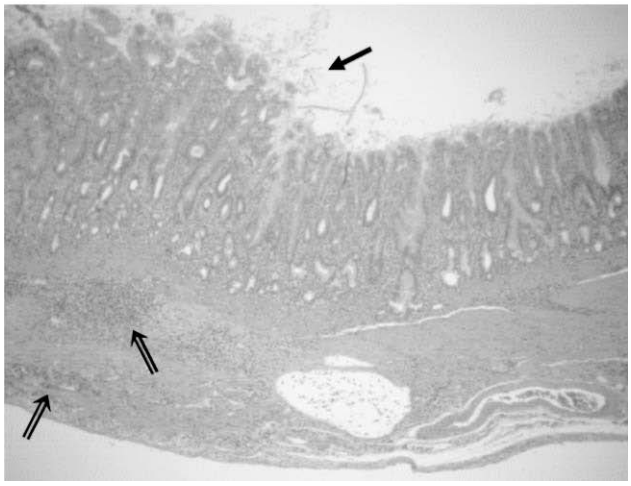


Fig. 1 A stomach of the control group. Severe edema was observed from the stomach body over the pylorus division, and a hemorrhage area (arrow) and spots were observed (Fig. 1a). Moreover, the structure of the gastric gland was deranged (black arrow), and the invasion of leukocytes (arrows) was observed (Fig. 1b).

Macroscopic findings on the gastric mucosa of the *H. pylori*-infected gerbils are shown in Table 3. The changes in the antral and fundic mucosa, such as severe edema and hemorrhages derived from gastric erosion or ulcer, were observed in all control animals (Group I). A typical example of a stomach from a control animal is shown in Fig. 1. Edema was observed in almost the whole gastric mucosa and hemorrhagic lesions were also observed (Fig. 1a). Moreover, the structure of the gastric gland was deranged, and the invasion of leukocytes was observed (Fig. 1b). The stomach of an animal from the GTC group (Group II) is shown in Fig. 2. Severe edema and hemorrhage lesion areas were also observed. The stomach of an animal from the BTND group (Group III) is shown in Fig. 3. While edema was observed in all animals of this group, it appeared milder than that in both the control and GTC groups, and only a few small hemorrhagic spots were observed.

The colony forming units (CFU) are shown in Table 4. While the CFU of the GTC (Group II) and BTND (Group III) groups were decreased from those of the distilled water group (Group I), a significant difference was observed only between the BTND (Group III) and distilled water (Group I) groups ($p<0.05$).

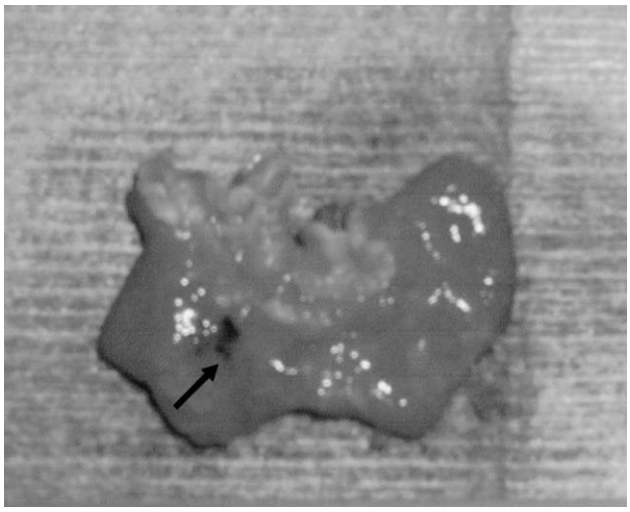


Fig. 2 A stomach of Group II, supplied with 0.1 w/v% of GTC aqueous solution as drinking water for 2 weeks. Severe edema and a hemorrhage area (arrow) derived from gastric erosion were observed.

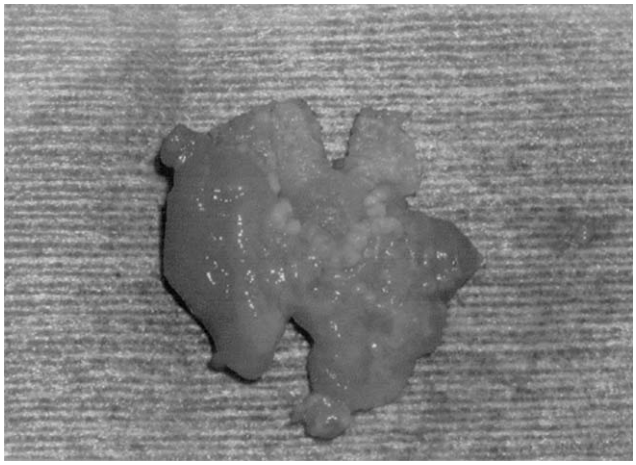


Fig. 3 A stomach of Group III, supplied with 0.1 w/v% of BTND aqueous solution as drinking water for 2 weeks. While edema was observed in all animals of this group, it appeared milder than both the control and GTC groups, and only small hemorrhage spots were observed.

Table 4 Colony forming units (CFU) of *H. pylori* in each gerbil group

	Experimental group and drinking water supplied	CFU[a] ($\times 10^4$/stomach)
I	Distilled water (n=4)	3.30±2.17
II	0.1 w/v% GTC solution (n=5)	1.32±1.16
III	0.1 w/v% BTND solution (n=5)	0.65±0.2*

[a]: The data are expressed in means±SD and a one-way analysis of variance (ANOVA) test was performed.
* Significant as compared with Group I ($p<0.05$).

Discussion

The gastric mucosal erosion model of the Mongolian gerbil used in this study was created by the method of Hirayama et al. and it is used widely as a human *H. pylori* infection model. In this model, edema of the mucosa, hemorrhage spots, invasion of the inflammatory cells and disturbance of the covering epithelial cells of the stomach are observed 6 weeks after infection (16). In the present study, though the experiment ended 11 weeks after infection, edema, erosion, and hemorrhage of the gastric mucosa were still observed in the stomachs of the control gerbils, but ulcers were not observed (Fig. 1).

BTND is a complex mixture of hydrolyzable/condensed tannins containing polyphenols and sugars, and the yield of BTND is about 2% of dried black tea leaves (15). There have been reports that BTND exhibited an anti-tumor-promoting effect in mouse epidermal JB6 cell lines, an inhibitory effect on duodenal carcinogenesis in mice (15, 17), induction of apoptotic cell death in cancer cells (18), and an antibacterial action (14). Moreover, it has been considered that the active ingredients of BTND are polyphenolic compounds of flavonols and gallates, quinic acid and carbohydrates of D-glucose and D-galactose units (15). The same activities have already been found in GTC. However, there are disadvantages in the clinical use of GTC, such as strong bitterness and cytotoxicity at high concentrations. The LD_{50} of EGCG, which is the main ingredient of GTC, is 8.4 μg/ml, and that of BTND is 68 μg/ml (19). The less cytotoxic and less bitter properties (15) of BTND seem to be favorable for clinical application, because BTND can be administered in larger doses. Though the 0.1 w/v% GTC aqueous solution used in the present experiment had a concentration slightly higher than that of green tea taken daily by the average Japanese, the intake of the GTC aqueous solution by gerbils showed a slight decline. However, the intake of the BTND aqueous solution by gerbils tended to increase (Table 1). The animal might like the taste, because the bitterness of the BTND aqueous solution is mild, and the residue has a subtle sweetness.

Though the GTC aqueous solution did not show efficacy in the gastric mucosa, the BTND aqueous solution indicated a tendency of improvement of the mucosal hemorrhage (Table 3). It has been clarified that *H. pylori* induces inflammation in the gastric mucosa, not only by clinical studies showing that the concentration of vitamin C in the gastric juice of the *H. pylori*-infected patient was low (20) and the amount of 8-oxodG, a biomarker of oxidative stress, of the gastric mucosa of the *H. pylori* infected patient was high (21), but also by animal data showing that *H. pylori* induced IL-8 production by gastric mucosal epithelial cells (22). Antioxidative substances have been expected to suppress inflammation, because the reactive oxygen species is related to the improvement of inflammation. GTC shows the antioxidative activity, because it has phenolic hydroxyl groups. For the same reason, the antioxidative activity can also be expected from BTND, because BTND contains some kinds of polyphenolic compounds (15). Mabe et al. have reported that the gastric mucosa injury score of Mongolian gerbils infected with *H. pylori* was reduced to almost 1/3 by GTC intake (12), but the animals could not take a higher amount of the drug because of its very bitter taste. It is likely that the higher intake of BTND contributed to the improvement in the gastric mucosal injury score in the present study.

Moreover, the BTND aqueous solution depressed the CFU significantly while the decrease of the CFU by the GTC aqueous solution was not significant (Table 4). Matsubara et al. reported that green tea extract inhibited *H. pylori* urease, an essential enzyme for *H. pylori*, *in vitro*, and reported that the active principle was the hydroxyl group at the 5(')-position of the catechins (23). BTND contains components with a similar structure, probably explaining its anti-*H. pylori* activity. It will be a future subject to clarify the action mechanism of BTND, because the composition and the principal components of BTND have not been well characterized (15). However, these results suggest the possibility of using BTND as an agent to inhibit *H. pylori* infections by itself or to reduce the dose of antibiotics if it is used as part of the combination treatment mode.

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