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Inhibition of Acid Production in Dental Plaque Bacteria by Green Tea Catechins

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Key Words
Green tea catechins · Acid production in dental plaque · Lactate dehydrogenase

Abstract
The inhibition of acid production from dental plaque and mutans streptococci by epigallocatechin gallate (EGCg), one of the green tea catechins, was examined. The effect of EGCg solution on dental plaque pH was investigated. Subjects rinsed their mouths with 2 mg/ml EGCg solution and then, after 30-min interval, rinsed their mouths with 10% sucrose. Plaque samples were collected at appropriate times and the pH was measured. The pH values of plaque samples from 15 volunteers were significantly higher after treatment with catechin than after treatment with water. EGCg inhibited pH fall when cariogenic bacteria grown in medium with or without sucrose were incubated with sugar. In medium without sucrose, cultured cells were killed time-dependently by EGCg treatment. However, EGCg did not kill cells cultured in medium containing sucrose. Also, EGCg did not kill oral streptococci adhering to a saliva-coated hydroxyapatite disk. EGCg and epicatechin gallate inhibited lactate dehydrogenase activity much more efficiently than epigallocatechin, epicatechin, catechin or gallocatechin. These results suggest that EGCg is effective in reducing acid production in dental plaque and mutans streptococci.

Dental plaque, under which carious lesions may occur, contains acid-producing microorganisms, including the mutans streptococci. The acid production from these bacteria is the main cause of enamel demineralization [Kleinberg, 1970; Mühlemann, 1971]. The possibility of preventing dental caries by the elimination of different pathogenic factors has been reported [Ikeda et al., 1982; Hatta et al., 1997; Shouji et al., 2000; Michalek et al., 2001; Baehni and Takeuchi, 2003]. Catechins from green tea have been reported to have antibacterial [Elvin-Lewis et al., 1980; Sakanaka et al., 1989; Otake et al., 1991; Hamilton-Miller, 2001; Hirasawa et al., 2002], antifungal [Hirasawa and Takada, 2004], antiviral [Nakane and Ono, 1989; Nakayama et al., 1990] and protein-denaturing [Wu-Yuan et al., 1988; Otake et al., 1991; Makimura et al., 1993; Hirasawa et al., 2002] properties.

In this study, we examined the effects of epigallocatechin gallate (EGCg) on bactericidal activity and acid production from plaque bacteria and mutans streptococ-

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ci and further examined the inhibitory effect of individual tea catechins extracted from green tea on the activity of lactate dehydrogenase (LDH), which converts pyruvic acid to lactic acid.

Materials and Methods

Catechins

The catechins used in this study were (–)-EGCg, (–)-epicatechin gallate (ECg), (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (+)-catechin (C), and (+)-gallocatechin (GC). They were purchased from Funakoshi Co. (Tokyo, Japan). The catechin complex was well-purified Sunphenon® (Taiyo Kagaku, Yokkaichi, Mie, Japan) prepared from Japanese green tea [Sakanaka et al., 1989].

Microorganisms

Laboratory stock cultures of *Streptococcus mutans* NCTC10449, *Streptococcus sobrinus* ATCC27607, *Streptococcus sanguinis* ATCC10556, *Streptococcus oralis* ATCC10557, *Streptococcus gordonii* ATCC10558 and *Streptococcus mitis* NCTC3168 were used in this study. Bacteria were grown anaerobically at 37°C on brain-heart infusion (BHI; Difco Laboratories, Detroit, Mich., USA) agar supplemented with 5% (v/v) defibrinated horse blood and maintained at 4°C in a refrigerator.

In vivo Plaque pH Measurement

Plaque pH was measured according to previously reported methods [Rugg-Gunn et al., 1975; Neta et al., 2000]. Fifteen volunteers were chosen from among laboratory staff and dental students for in vivo experiments. Three subjects were caries free (male, age range 22–26) and 12 subjects were caries inactive with previous caries experience but no untreated cavities (6 males, 6 females, age range 24–55 years). Clinical specimens were collected from the buccal surfaces of molar teeth. Studies were performed in accordance with the ethical guidelines for human experiments at Nihon University School of Dentistry at Matsudo. All volunteers gave their informed consent before participation in this study.

To determine the conditions to be used in in vivo experiments, 2 preliminary experiments were performed using 4 volunteers (2 males, 2 females, age range 28–55 years). Experiment 1: The subjects rinsed their mouths for 5 min with 10 ml of 5 mg/ml EGCg solution or distilled water at least 2 h after eating or drinking. After 2, 30 or 120 min, they rinsed their mouths for 2 min with 10 ml of 10% sucrose. Plaque samples (approx. 1 mg) were then collected after 0, 3, 7, 11, 20 and 30 min, and dispersed in 30 μl of water, and pH was measured using a digital pH meter 90 s after the commencement of collection. Experiment 2: The subjects rinsed their mouths for 5 min with 10 ml of 1, 2 or 5 mg/ml EGCg solution or distilled water at least 2 h after eating or drinking. After 30 min, they rinsed their mouths for 2 min with 10 ml of 10% sucrose. The measurement of plaque pH was then performed as described for experiment 1. The 2 preliminary experiments were performed with a 1-week interval.

In vivo experiments using 15 subjects were performed with a 30-min interval between the EGCg and sucrose rinses and 2 mg/ml EGCg based on the results of experiments 1 and 2, respectively. The other methods were the same as those in preliminary experiment 1.

Inhibition of Acid Production

The inhibition of sucrose fermentation by EGCg was examined according to the method described previously [Neta et al., 2000] with some modifications. The bacteria were cultured at 37°C for 20 h in BHI or 5% sucrose added BHI (GF-BHI). The packed cells (approx. 1.3×10^{10} colony-forming units, CFUs) were preincubated with 0.4 ml of EGCg (2 mg/ml) in Stephan's buffer at 37°C for 15 min. Then 0.2 ml of 0.3 mol/l sucrose in Stephan's buffer was added to the reaction mixture and the mixture was incubated at 37°C in a water bath with shaking. Aliquots of 25 μl were collected at timed intervals, and pH was measured using a digital pH meter (Shindengen Co., Tokyo, Japan). Aliquots of 10 μl were also collected at timed intervals, then tenfold serial dilutions of each sample were prepared in 50 m*M* Tris-HCl buffer (TH, pH 7.2) and appropriate dilutions were spread in triplicate on mitis salivarius agar (Difco) plates. The plates were cultured anaerobically at 37°C for 2 days, and the CFU were counted.

Antibacterial Effect of EGCg on Cells Adhering to Saliva-Coated Hydroxyapatite

Synthetic hydroxyapatite ceramic (HA) disks (Asahi Kogaku Co., Ltd., Japan) were coated with human saliva at 37°C for 60 min (s-HA). Overnight bacterial cultures were centrifuged and washed with TH buffer. Centrifuged cell pellets (5×10^8 CFU) were suspended in 1 ml human saliva at 37°C for 60 min (s-cells). After centrifugation, s-cells were suspended in TH buffer, then a s-HA disk was put in a tube containing cell suspension and incubated at 37°C for 60 min. Thereafter, the s-HA disk was removed and gently washed with the buffer 3 times. Then the bacteria adhering to s-HA were incubated with or without 2 mg EGCg in buffer at 37°C for 30 min. The disk was gently washed with TH buffer 3 times and sonicated in 2 ml of buffer at 50 W for 20 s, then bacteria were diluted and plated on mitis salivarius agar and incubated at 37°C for 48 h. CFUs were counted.

Inhibition of LDH Activity by Various Catechins

LDH activity was measured using the LDH-UV Test Wako kit (Wako Pure Chemicals Co., Tokyo, Japan). Briefly, 500 μl of NADH substrate buffer containing 0.18 mmol/l β-NADH from yeast and 0.62 mmol/l lithium pyruvate in 50 mmol/l phosphate buffer, pH 7.5, was prewarmed at 35°C and then 10 μl of LDH from *Leuconostoc mesenteroides* (0.1 unit, Oriental Yeast Co., Ltd, Osaka, Japan) and 90 μl of various catechins (0.1 mg/ml) or distilled water were added, and the reduction of optical density at 340 nm was monitored for 5 min. In the other method, 10 μl of LDH and 90 μl of various catechins were preincubated for 15 min at 35°C and then mixed with 500 μl of prewarmed NADH substrate buffer, and the optical density was monitored at 340 nm for 5 min. The final concentration of EGCg in the assay was 9 μg. LDH activity was calculated from the exchange rate according to the manufacturer's instructions. The experiments were performed three times and the data were expressed as mean and standard deviation.

Statistical Analysis

Data shown are from three separate experiments and were expressed as means and standard deviations of the mean. Differences among experimental values were evaluated by Student's t test.

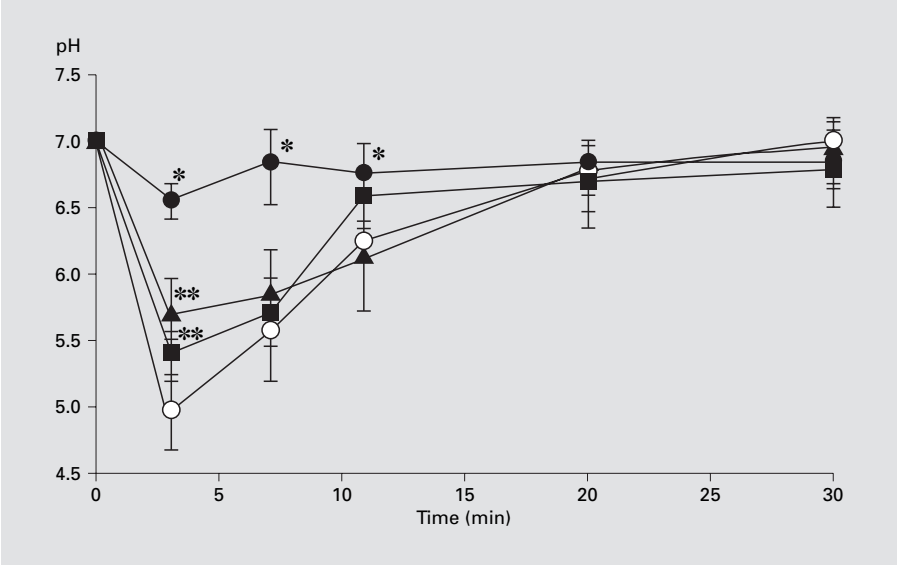

Fig. 1. Plaque pH time course following a 2-min mouth rinse with 10% sugar at various intervals after a 5-min mouth rinse with 5 mg/ml EGCg. ○ = No EGCg with 30-min interval; ▲ = 2-min interval; ● = 30-min interval; ■ = 120-min interval. * Significantly different at $p < 0.01$; ** significantly different at $p < 0.05$. Bars indicate standard deviations of the mean.

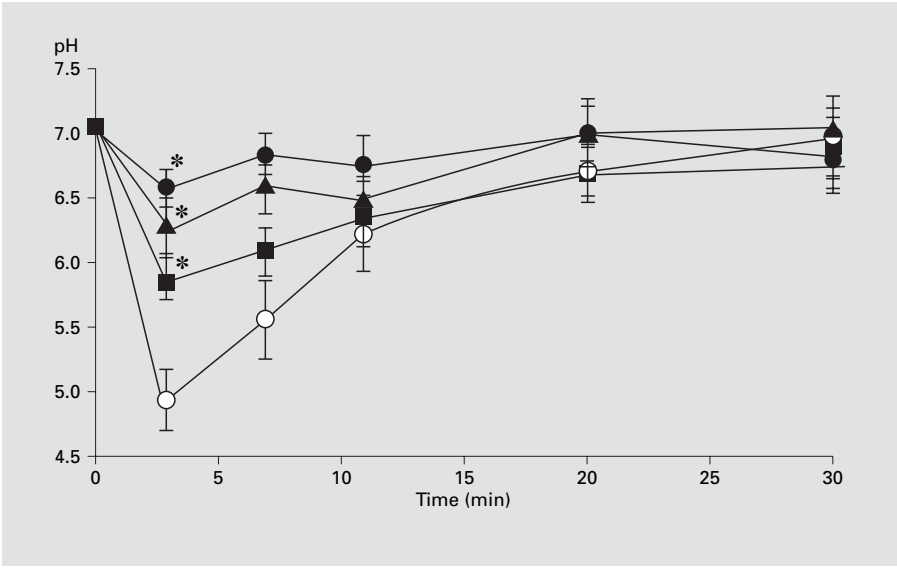

Fig. 2. Plaque pH time course following a 2-min mouth-rinse with 10% sugar 30 min after a 5-min mouth rinse with various concentrations of EGCg. ○ = No EGCg; ■ = 1 mg/ml EGCg; ▲ = 2 mg/ml EGCg; ● = 5 mg/ml EGCg. * Significantly different at $p < 0.01$. Bars indicate standard deviations of the mean.

Results

Effect of EGCg on Acid Production in Dental Plaque

Figure 1 shows the effect of the interval between rinsing with 5 mg/ml EGCg and rinsing with 10% sugar on plaque pH at various assay times in vivo. More inhibition was observed after a 30-min interval between rinses than after 2-min or 120-min intervals. The minimum pH after the 30-min interval was 6.5, compared to 4.8 in the water rinse control. Significant differences ($p < 0.01$) were seen between the pH in the control and 30-min interval groups at 3-, 7- and 11-min assay times.

The most effective concentration of EGCg was 5 mg/ml (fig. 2), but 2 mg/ml EGCg yielded a similar pH time course to 5 mg/ml. Significant differences ($p < 0.01$) were seen between the pH in the control and the 1, 2 and 5 mg/ml EGCg groups at 3-min assay time. However, there was no significant difference between the 2 and 5 mg/ml EGCg groups at 3-min assay time. As the 5-mg/ml solution of EGCg was too bitter for daily use, 2-mg/ml concentration was used for further experiments.

The plaque pH curves for a 2-min 10% sugar challenge after a 5-min 2 mg/ml EGCg rinse and a 30-min interval in 15 subjects are summarized in figure 3. The mean min-

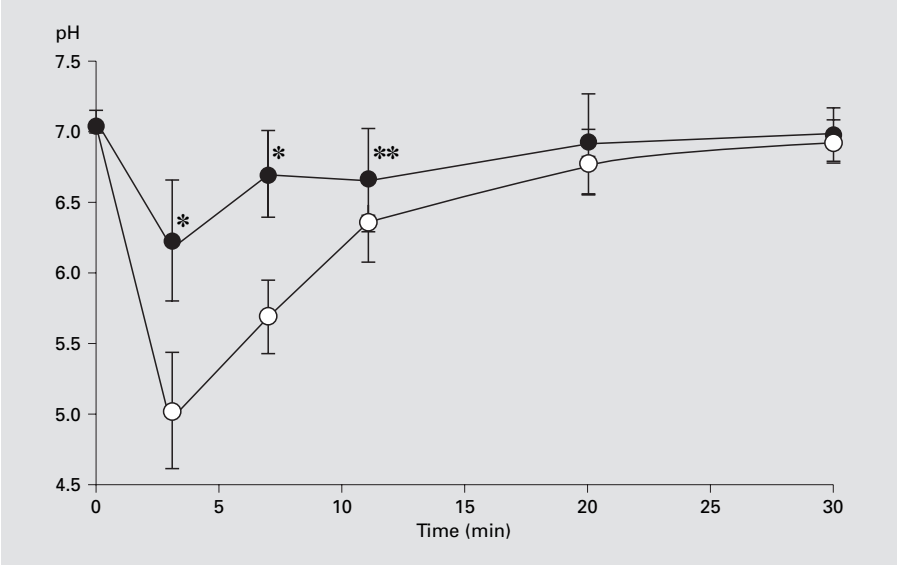

Fig. 3. Mean plaque pH time course in 15 volunteers following a 2-min mouth rinse with 10% sugar 30 min after a 5-min mouth rinse with 2 mg/ml EGCg. ○ = Control; ● = test. * Significantly different at $p<0.01$; ** significantly different at $p < 0.05$. Bars indicate standard deviations of the mean.

Table 1. Times (min) taken to reach critical pH and pH 4 when mutans streptococci and sugar were reacted after preincubation with EGCg

	BHI (no sucrose)		GF-BHI (5% sucrose)	
	critical pH	below pH4	critical pH	below pH4
S. mutans				
No EGCg	11.8 ± 2.0	21.4 ± 2.5	9.0 ± 1.5	16.2 ± 2.4
EGCg	24.3 ± 2.1	41.5 ± 4.8	20.7 ± 2.6	36.2 ± 3.4
S. sobrinus				
No EGCg	9.5 ± 0.7	17.3 ± 1.7	9.0 ± 0.5	15.2 ± 3.8
EGCg	20.2 ± 1.8	36.1 ± 2.9	16.0 ± 2.8	29.8 ± 2.8

Values are expressed as means ± SD.

imum pH values were significantly higher for the EGCg rinse than for the water control rinse. The minimum pH was 6.23 at 3 min after the EGCg rinse and 5.04 at 3 min after the control rinse.

Inhibition of Acid Production from Mutans Streptococci by EGCg

EGCg as shown in table 1 inhibited the pH fall that was observed when both BHI and GF-BHI cultures of mutans streptococci were incubated with sucrose. The interval until reaching the critical pH when the bacteria had been preincubated with EGCg (5.4) [Rugg-Gunn et al., 1975] was two to three times longer than that without catechin pretreatment for both *S. mutans* and *S. sobrinus.* There were no differences between BHI- and GF-BHI-cultured mutans streptococci.

Effect of EGCg on Survival of Mutans Streptococci

The numbers of surviving cells when mutans streptococci and sucrose were reacted after preincubation with EGCg are shown in table 2. Both *S. mutans* and *S. sobrinus* cultured in medium without sucrose were killed with time passage by EGCg treatment. However, numbers of *S. mutans* and *S. sobrinus* cultured in medium containing sucrose were not reduced by EGCg treatment.

Antibacterial Effect of EGCg on Cells Adhering to Saliva-Coated Hydroxyapatite

Table 3 shows the percentage of surviving oral streptococci adhering to s-HA disk treated with EGCg compared with the percentage without EGCg treatment. EGCg did not kill these experimental oral streptococci. The average percent of surviving cells was 90.4%.

Inhibition of LDH Activity by Various Catechins

Table 4 shows the inhibition of LDH activity by various catechins in vitro. The catechin complex from Japanese green tea caused 61% inhibition. Catechol and pyrogallol compounds containing the galloyl radical (ECg and EGCg) caused greater inhibition than compounds without this radical. Inhibition by catechin complex and galloyl radical-containing compounds but not by compounds without the galloyl radical increased when LDH

Table 2. Proportions of surviving cells (%) when mutans streptococci and sugar were reacted after preincubation with EGCg

Time, min	*S. mutans*				*S. sobrinus*			
	BHI (no sucrose)		GF-BHI (5% sucrose)		BHI (no sucrose)		GF-BHI (5% sucrose)	
	Control	EGCg	Control	EGCg	Control	EGCg	Control	EGCg
Preincubate	100		100		100		100	
With sugar								
0	93.3 ± 19.3	32.9 ± 14.7	100	88.6 ± 14.5	93.2 ± 6.0	46.0 ± 40.3	95.7 ± 27.3	106.7 ± 68.3
10	–[a]	10.3 ± 0.5	–	71.6 ± 30.5	–	15.6 ± 5.3	–	76.0 ± 19.8
20	88.2 ± 19.2	6.8 ± 5.7	103.5 ± 37.0	91.5 ± 8.6	87.2 ± 12.6	3.1 ± 1.2	84.4 ± 12.1	91.7 ± 20.3
30	–	4.3 ± 2.4	–	82.2 ± 10.2	–	3.3 ± 3.5	–	81.4 ± 12.2

Values are expressed as means ± SD.
[a] Not done.

Table 3. Effect of EGCg on various streptococci that adhered to hydroxyapatite

Strain	Surviving cells, %
S. mutans	103.3 ± 18.7
S. sobrinus	95.1 ± 15.3
S. sanguinis	79.9 ± 22.6
S. oralis	93.7 ± 19.4
S. gordonii	96.6 ± 16.2
S. mitis	73.8 ± 24.8

Values are expressed as means ± SD.

Table 4. Percent inhibition of lactate dehydrogenase activity by various catechins

Catechin	No preincubation	15-min preincubation
Control[a]	0	0
Catechin complex	61.3 ± 4.9	80.7 ± 5.3
C	7.8 ± 3.9	10.1 ± 1.1
GC	1.4 ± 1.4	0.4 ± 0.3
EC	5.4 ± 0.7	5.3 ± 2.7
ECg	69.1 ± 5.6	92.8 ± 2.0
EGC	4.0 ± 2.3	9.9 ± 2.0
EGCg	51.3 ± 2.5	99.4 ± 0.9

Values are expressed as means ± SD.
[a] The experiment was performed using distilled water in place of catechin.

and the catechins were preincubated for 15 min before the reaction with the substrate. The percent inhibition of LDH activity caused by EGCg, ECg and catechin complex was 99, 92 and 80%, respectively.

Discussion

GC, EGC and EGCg possess strong bactericidal as well as antibacterial activity [Sakanaka et al., 1989]. A common characteristic of these components is the presence of a galloyl radical (pyrogallol). ECg and EGCg also inhibit glucosyltransferase from mutans streptococci [Wu-Yuan et al., 1988; Otake et al., 1991] and collagenase from *Porphyromonas gingivalis*, which are periodontopathic bacteria [Makimura et al., 1993]. The galloyl radical in catechins is responsible for the inhibition of enzyme activity. In this study, we demonstrated similar results showing that catechins containing the galloyl radical are able to directly inhibit LDH activity (table 4), perhaps through binding to the enzyme.

The inhibition of plaque pH fall by EGCg after too short (2 min) or too long (120 min) an interval was not satisfactory. This finding suggests that the reaction between EGCg and dental plaque needs a suitable interval for penetration of EGCg into the dental plaque. To be effective, more than 2 mg/ml of EGCg is required for mouth-rinsing. Acid production from mutans streptococci cultured with or without sucrose in the medium was inhibited by EGCg (table 1). As the bacteria are killed by prolonged exposure to EGCg, inhibition of acid production from mutans streptococci cultured in medium with-

out sucrose was increased by EGCg (tables 1 and 2). However, mutans streptococci cultured in medium containing sucrose were not killed by EGCg treatment (table 2). Also, bacteria, which adhered to HA disk, were not killed by EGCg treatment (table 3). These results suggest that the inhibition of acid production from bacteria that are glucan coated or adherent to hard surfaces, such as dental plaque bacteria, by EGCg treatment is another mechanism involved in bactericidal activity. EGCg may inhibit sugar transport and acid secretion by interfering with membrane-bound enzymes, and/or acid-producing enzymes such as LDH. EGCg solutions stronger than 5 mg/ml tasted bitter and thus would not be appropriate for daily use. The major catechin in Japanese green tea is EGCg and typical preparations of green tea contain approximately 0.5–1.0 mg/ml catechins [Otake et al., 1991]. Thus, regular drinking of green tea might effectively inhibit these bacteria. The application of catechins on a daily basis may be a useful method of preventing dental caries.

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