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Anticaries Effects of Polyphenolic Compounds from Japanese Green Tea

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Abstract. The dental caries inhibiting effect of the extract from Japanese green tea, one of the most popular drinks in Japan, was studied both in vitro and in vivo. The crude tea polyphenolic compounds (designated Sunphenon®) from the leaf of *Camellia sinensis* were found to effectively inhibit the attachment of *Streptococcus mutans* strain JC-2 (serotype *c*) to saliva-coated hydroxyapatide discs. Sunphenon was also inhibitory to water-insoluble glucan formation from sucrose by crude glucosyltransferase of *S. mutans* JC-2 *(c)*. Among the tea catechins tested, (−)-epigallocatechin gallate and (−)-epicatechin gallate showed the most potent inhibition of the glucosyltransferase activity. Finally, significantly lower caries scores were observed in specific pathogen free rats infected with *S. mutans* JC-2 *(c)* and fed a cariogenic diet and/or drinking water containing 0.05% Sunphenon as compared with control rats not receiving polyphenolic compounds.

Dental caries is a multifactorial disease, and the factors associated with demineralization of enamel are complex. Keyes [1969] identified the primary caries-inducing factors as the teeth, the microflora, and the substrates, explaining that dental caries would be induced when these major factors were all in an appropriate state. Many approaches have been adopted to prevent dental caries after taking these factors into consideration. These approaches include the elimination of cariogenic bacteria, increasing the resistance of the teeth, and modifying the diet [Hamada and Slade, 1980; Loesche, 1986]. Some of these have been put to practical uses, while others remain at the level of research.

Tannin, which is distributed widely in the plant world, has been shown to possess anticariogenic potential [Strålfors, 1967; Shyu et al., 1977; Elvin-Lewis et al., 1980; Elvin-Lewis and Steelman, 1986]. It can reduce caries formation in experimental animals [Strålfors, 1967; Shyu et al., 1977; Rosen et al., 1984] as well as inhibit glucosyltransferase (GTase) activity [Paolino et al., 1980; Kakiuchi et al., 1986; Wu-Yuan et al., 1988] and adsorption of *Streptococcus mutans* to hydroxyapatite [Wolinsky and Sote, 1984; Kashket et

al., 1985]. Consequently, its application to the prevention of caries is being considered. However, the substance termed 'tannin' is a generic name for polyphenols with the property of binding to protein. As it was found that a component extracted from Japanese green tea, which is in daily use in Japan, has a bactericidal effect on *S. mutans* [Sakanaka et al., 1989], we have studied the effects of an extract from Japanese green tea on the synthesis of water-insoluble glucan, adsorption to hydroxyapatite, and on experimental caries in rats caused by a strain of *S. mutans*.

Materials and Methods

Polyphenolic Compounds

The well-characterized Sunphenon® (Taiyo Kagaku, Yokkaichi, Mie, Japan) was used which, using high-performance liquid chromatography, was found to contain six major polyphenolic compounds: (+)-catechin (C; 3.5%), (–)-epicatechin (EC; 7.0%), (+)-gallo-catechin (GC; 14.8%), (–)-epigallocatechin (EGC; 15.0%), (–)-epicatechin gallate (ECg; 4.6%), and (–)-epigallocatechin gallate (EGCg; 18.0%) [Maeda and Nakagawa, 1977; Sakanaka et al., 1989]. Briefly, Sunphenon was originally isolated from the leaf of *Camellia sinensis*, known as Japanese green tea, by extraction using ethyl acetate [Sakanaka et al., 1989]. Individual polyphenolic compounds were further fractionated from Sunphenon by silica gel column using methanochloroform (10:1) as elution solvent. Each fraction was purified further by recycled high-performance liquid chromatography (Japan Analytical Industry Co., Tokyo, Japan) using a PVA HP-GPC column (JAIGEL GS-320). Details of the purification and chemical characterization of individual compounds have been described elsewhere [Sakanaka et al., 1989]. The purified compounds are referred to in the text as polyphenolic compounds.

Bacteria

A laboratory stock culture of streptomycin-resistant (1.0 mg/ml) *S. mutans* JC-2 (serotype *c*) was used.

Hydroxyapatite Adsorption Assay

S. mutans JC-2 *(c)* was grown for 20 h at 37 °C in the partially defined medium M4 [Fukushima et al., 1981]. The cells were collected, washed three times with 0.01 *M* potassium phosphate buffer (pH 7.0), and resuspended in the same buffer to give approximately 10^7 colony-forming units (CFU) per milliliter. Hydroxyapatite discs (Apaceram®, diameter 10 mm, width 1 mm; Asahi Kagaku, Japan) were coated with clarified human saliva [Hay et al., 1971] by rotation (RT-50 rotor; Taiyo Kagaku Kogyo, Japan) for 1 h at room temperature. The saliva-coated hydroxyapatite discs (S-HA) were washed three times with distilled water and immersed in bacterial suspensions or suspensions pretreated with Sunphenon. After incubation by gentle agitation of the S-HA with bacteria for 90 min at 25 °C, the discs were washed and transferred to a tube containing phosphate buffer. Bacteria adsorbed on the S-HA discs were dispersed using a sonicator (5202, Otake, Japan; 50 W, 30 s), diluted, and spread on mitis salivarius agar (Difco, Detroit, Mich., USA) containing streptomycin (500 µg/ml). After incubation, the number of CFU on mitis salivarius agar was determined.

Effect of Sunphenon on Adsorption of S. mutans to S-HA

Bacterial suspensions were incubated with Sunphenon (10, 25, 50, and 100 µg/ml, final concentration) with gentle agitation for 90 min at 25 °C and washed with phosphate buffer and resuspended in the same buffer. Binding of Sunphenon-treated bacteria to S-HA was then performed using the assay described above. Alternatively, S-HA was pretreated with different concentrations of Sunphenon (10, 25, 50, and 100 µg/ml, final concentration). Binding of *S. mutans* to Sunphenon-pretreated S-HA was examined by the same assay. In these experiments, non-treated *S. mutans* and S-HA were used as a positive control.

GTase Preparation and Assay

The assay and preparation of crude GTase were based on the method of Fukushima et al. [1981]. *S. mutans* JC-2 *(c)* was grown in M4 medium at 37 °C for 20 h, and after centrifugation, cold absolute ethanol (–80 °C) was added to the cell-free supernatant to give a final concentration of 40%. The precipitate was collected after centrifugation (10,000 *g*, 30 min), resuspended in 5 m*M* triethanolamine, and dialyzed against the same solution. The dialyzed preparation containing crude GTase was clarified by centrifugation (13,000 *g*, 10 min) and stored at –80 °C until required.

In the assay of GTase activity, the reaction mixture (total volume 0.3 ml) consisted of 100 µl of crude GTase preparation (with an activity of approxymately 1.6 µmol glucose/min/ml), 100 µl of 0.3 *M* acetate buffer (pH 5.5) containing 0.15 *M* sucrose and 100 µl of distilled water (control) or Sunphenon (33.3, 166.7, and 333.3 µg/ml, final concentration), or purified polyphenolic compounds (166.7 µg/ml, final concentration). The mixture was incubated at 25 °C in a microcuvette. The increase of absorbance at 340 nm due to water-insoluble glucan production was recorded using a spectrophotometer (Hitachi model 100-10) [Fukushima et al., 1981]. The activity was determined from the slope of the linear section of the time course curve. Individual reaction mixtures which fitted within the linear section were heat inactivated, and the turbid materials were then precipitated by centrifugation (15,600 *g*, 20 min). These precipitates were extensively washed with distilled water [Fukushima et al., 1981]. Total amounts of water-insoluble glucan (WIG) were measured by the phenol-sulphuric acid method [Dubois et al., 1956] and expressed as the amount equivalent to glucose (µmol glucan/min). The percent inhibition by tea extracts was calculated from the following formula:

$$\frac{\text{WIG in tea extract treated sample (µmol glucan/min)}}{\text{WIG in control (µmol glucan/min)}} \times 100.$$

Rat Caries Study

Specific pathogen free Sprague-Dawley rats (19 days of age; Japan Clea Laboratory, Tokyo) were treated with ampicillin, carbenicillin, and chloramphenicol [Michalek and McGhee, 1977] for 3 days to eliminate the microbial flora. To this end, rat chow (Oriental Yeast, Tokyo) was pulverized and mixed with 1 g of each individual antibiotic per kilogram of diet. Oral swabs from individual rats were plated on brain-heart infusion (Difco) and mitis sali-

Table 1. Adsorption of Sunphenon-pretreated cells of *S. mutans* to S-HA

Pretreatment concentration of Sunphenon μg/ml	Adsorption to S-HA CFU/surface $\times 10^4$ (mean ± SE)	Percent inhibition
None	5.9 ± 0.3	0.0
10	5.9 ± 0.3	0.0
25	4.5 ± 0.4	23.7
50	3.2 ± 0.1	45.8
100	1.0 ± 0.1	83.1

Table 2. Adsorption of *S. mutans* to Sunphenon-treated S-HA

Pretreatment concentration of Sunphenon μg/ml	Adsorption to S-HA CFU/surface $\times 10^4$ (mean ± SE)	Percent inhibition
None	6.3 ± 0.1	0.0
10	6.2 ± 0.4	1.6
25	5.2 ± 0.4	11.9
50	4.4 ± 0.1	25.4
100	3.8 ± 0.1	35.6

Table 3. Inhibition of water-insoluble glucan synthesis from sucrose by *S. mutans* GTase in the presence of Sunphenon

Concentration of Sunphenon, μg/ml	Glucan synthesis μmol/min $\times 10^{-2}$	Percent inhibition
Control	5.23 ± 0.03	0.0
33.3	4.48 ± 0.03	14.5
166.7	2.77 ± 0.06	47.0
333.3	1.70 ± 0.05	67.5

Table 4. Water-insoluble glucan synthesis by *S. mutans* in the presence of purified polyphenolic compounds

Polyphenolyc compounds (166.7 μg/ml)	Glucan synthesis μmol/min $\times 10^{-2}$	Percent inhibition
Control	5.23 ± 0.03	0.0
C	5.23 ± 0.04	0.0
EC	3.65 ± 0.05	30.2
GC	3.40 ± 0.05	35.0
EGC	3.12 ± 0.03	40.3
ECg	0.97 ± 0.04	81.5
EGCg	0.49 ± 0.05	90.6

For explanation of abbreviations see text.

varius agars. *S. mutans* was not detected after antibiotic treatment. The rats were randomly separated into eight experimental groups, and each group contained 7 rats; all were fed diet 2000 [Keyes and Jordan, 1964]. The rats in group A were fed with diet 2000 and drinking water without any additives. Rats in groups B–D received diet 200 containing 0.025%, 0.05, or 0.1% of Sunphenon and normal drinking water. Normal diet 2000 and drinking water containing different concentration of Sunphenon (0.025%, 0.05, or 0.1%) were fed to rats in groups E–G. Finally, the rats in group H were fed with diet 2000 and drinking water, both of which contained 0.05% of Sunphenon.

The rats were infected with streptomycin-resistant (1 mg/ml) cells of *S. mutans* JC-2 *(c)* by pipette (50 μl of 1×10^{11} CFU/ml) at 23 days of age. Oral swabs were taken to confirm colonization by the inoculum. Feeding rats with diet 2000 and/or drinking water containing Sunphenon did not affect body weight, health, or growth of animals when compared with the control group. The rats were sacrificed at 76 days of age, 57 days after the start of the caries experiment. The caries scores of both sides of each mandible were measured by the method of Keyes [1958]. The caries scores were analyzed statistically by computing mean values and standard errors of the mean. Differences between the mean values of the experimental and control groups were evaluated by the Student *t* test. The p value was established by comparison of the mean values obtained from the individual experimental and the control group (A) only.

Results

Sunphenon Inhibition of Adsorption of S. mutans *to S-HA*

When bacterial cells were pretreated with Sunphenon at concentrations greater than 25 μg/ml, significant inhibition of bacterial attachment to S-HA ($p < 0.01$) was observed (table 1). Inhibition was enhanced with increased concentrations of Sunphenon. Alternatively, when S-HA was pretreated with Sunphenon (10, 25, 50, and 100 μg/ml, final concentration), little inhibition of adsorption of *S. mutans* to treated S-HA was observed at concentrations below 25 μg/ml (table 2). A significantly higher inhibition ($p < 0.05$) was observed at a concentration of 50 or 100 μg/ml (table 2).

Inhibition of GTase Activity by Sunphenon and Polyphenolic Compounds

The inhibition of water-insoluble glucan formation by Sunphenon (33.3, 166.7, and 333.3 μg/ml, final concentration) is shown in table 3. Inhibition increased with increasing Sunphenon concentration. For example, the GTase activity was inhibited by 67.5% by a concentration of 333.3 μg/ml. The inhibitory effects of polyphenolic compounds such as EGCg and ECg

Table 5. Mandibular mean caries scores of JCL-SD rats infected with *S. mutans* JC-2 *(c)* and fed with diet 2000 and drinking water with or without Sunphenon

Group	Concentration of Sunphenon in diet 2000, %	Concentration of Sunphenon in drinking water, %	Mean (±SE) caries score[a]			
			sulcal	buccal	approximal	total
A	none[b]	none	84.1 ± 4.1	15.1 ± 3.0	10.0 ± 1.8	109.3 ± 8.6
B	0.025[c]	none	66.0 ± 3.7[d]	18.7 ± 2.7	9.3 ± 1.3	94.0 ± 7.3
C	0.05	none	55.1 ± 3.7[d]	6.3 ± 2.5[e]	3.1 ± 1.2[e]	64.6 ± 6.8[d]
D	0.1	none	54.7 ± 3.9[d]	6.4 ± 1.0[e]	3.1 ± 0.7[d]	64.3 ± 4.8[d]
E	none	0.025	67.7 ± 2.9[d]	18.4 ± 5.7	9.9 ± 1.0	96.0 ± 8.1
F	none	0.05	54.3 ± 5.9[d]	8.9 ± 3.7	3.0 ± 1.3[d]	66.1 ± 9.2[d]
G	none	0.1	55.4 ± 3.6[d]	8.6 ± 2.5	3.9 ± 0.7[e]	67.9 ± 6.1[d]
H	0.05	0.05	52.6 ± 3.7[d]	10.6 ± 2.8	3.7 ± 0.9[e]	66.9 ± 7.0[d]

[a] Caries scores were determined in rats aged 78 days by the method of Keyes [1958]. Statistical analyses (*t* test) were carried out between group A and the other groups.
[b] No Sunphenon in diet or drinking water.
[c] This ratio is diet per 100 g and/or drinking water per 100 ml.
[d] $p < 0.01$.
[e] $p < 0.05$.

were markedly greater as compared with other polyphenolic compounds; for example, 166.7 μg EGCg/ml inhibited the activity by 90.6% (table 4).

Influence of Sunphenon on Experimental Caries in Rats

When rats infected with *S. mutans* JC-2 *(c)* were fed a diet containing more than 0.05% Sunphenon, the mean caries scores were significantly lower (by at least 40%; $p < 0.01$) than that of rats receiving no Sunphenon (table 5). Sunphenon reduced caries on sulcal, buccal, and approximal surfaces. Similar findings were also observed after addition of Sunphenon to the drinking water (table 5). A significant reduction in caries scores was found in rats receiving drinking water containing 0.05 or 0.1% of Sunphenon. Further, when equal concentrations of Sunphenon (0.05%) were added to both diet and drinking water, synergetic effects were not seen.

Discussion

No method for achieving the complete prevention of dental caries has been established yet. This is because many interrelated factors are associated with caries initiation and progression. Therefore, methods which provide individuals with multiple preventive measures against the respective factors may be the most effective. These may include substances which exert inhibitory activity against *S. mutans*, which is the major causal bacterium for caries, and its cariogenic factors [Hamada and Slade, 1980; Loesche, 1986]. The present study investigated the effect of Sunphenon, which contains tannin-like compounds extracted from Japanese green tea, on preventing caries in a rat model. Tannins exist naturally in plants, and they possess potentially valuable anticariogenic activities, including inhibition of bacterial growth [Elvin-Lewis et al., 1980; Wu-Yuan et al., 1988; Sakanaka et al., 1989], aggregation [Elvin-Lewis et al., 1980; Wu-Yuan et al., 1988], and glucan synthesis [Paolino et al., 1980; Kashket et al., 1985; Kakiuchi et al., 1986], and they can reduce caries development in animals [Strålfors, 1967; Shyu et al., 1977; Rosen et al., 1984]. In this study we have provided evidence that Sunphenon is a strong anticariogenic compound. Although it is difficult to compare the exact level of the anticariogenic effect of Sunphenon with other tannins, our findings are in agreement with other studies [Elvin-Lewis and Steelman, 1986].

Among various polyphenolic compounds in Sunphenon, it has been shown that GC, EGC, and EGCg possess strong bactericidal as well as antibacterial activities [Sakanaka et al., 1989]. A common characteristic of these components is the presence of a gallo radical (pyrogallol) [Sakanaka et al., 1989]. Our current experiments are using these individual polyphe-

nolic compounds to determine the most useful anticariogenic properties to reduce dental caries in vivo.

In the present study, adsorption of *S. mutans* to S-HA was markedly inhibited either by pretreating S-HA or bacteria with Sunphenon. This result is in agreement with that reported by Wolinsky and Sote [1984] who studied extracts of plants of African origin. It is known that tannin forms a stable complex with proline-rich protein [Hagerman and Butler, 1981]. As whole saliva in humans contains relatively large amounts of proline-rich glycoprotein [Hay et al., 1971], which is involved in the adsorption of oral bacteria to pellicle, we have assumed that adsorption of *S. mutans* to S-HA was inhibited because the polyphenolic compounds in Sunphenon bound to these glycoproteins. Also, it is reported that the cell surface proteins of bacteria may act as receptors for the adsorption of bacteria to S-HA [Weerkamp et al., 1983]. Again, as tannin has the property of binding to protein, it may interfere with cell surface receptors involved in adhesion. Since purified tannin absorbs at a characteristic wavelength, it might be possible to study its binding to bacteria or S-HA using spectrophotometric techniques. However, it was impossible to determine whether Sunphenon directly bound to the surface of bacteria or S-HA, because Sunphenon contained various polyphenolic compounds and other contaminants which had a range of different wavelengths. Thus, we are currently testing the binding of polyphenolic compounds to bacteria or S-HA using purified materials (e.g. C, EC, GC, EGC, ECg and EGCg).

Sunphenon markedly inhibited GTase activity in the present study. The inhibitory effect was high with EGCg and ECg, which have galloyl radicals, suggesting that compounds which have this galloyl radical are responsible for the inhibition. The gallo radical (pyrogallol) was also important for the bacteriocidal activity [Sakanaka et al., 1989]. Kakiuchi et al. [1986] reported in their studies on GTase of *Streptococcus sobrinus* OMZ176 that the inhibition of synthesis of water-insoluble glucan by gallotannins depended on the number of galloyl residues and that penta- and hexagalloylglucose had the most potent inhibitory effect, with 1 m*M* inhibiting synthesis by 94%. Similar work which showed that gallotannins, isolated from *Melaphis chinensis*, inhibited the GTase activity was reported by Wu-Yuan et al. [1988]. The results we obtained support this concept, since the polyphenolic compounds containing galloyl radicals, EGCg and ECg, exhibited the strongest GTase inhibitory activity. Furthermore, our results suggest that different conformations, e.g., gallo and galloyl radicals, in individual compounds of Sunphenon may determine their anticariogenic effects, including antibacterial activity and inhibition of GTase activity.

Finally, Sunphenon inhibited caries formation in rats which is in agreement with other in vivo studies using different tannins [Strålfors, 1967; Shyu et al., 1977; Rosen et al., 1984]. We believe that the inhibition of caries formation in rats in the present study can be explained by the inhibition of both GTase activity and the adsorption of bacteria to the tooth surface as well as by the bactericidal activity of Sunphenon. The drinking water supplemented with Sunphenon also produced a significant reduction in caries in the present study. It should be noted that the concentration of tea extracts and Sunphenon, which possess anticariogenic activity, is physiologically relevant, since 100 ml of green tea contains approximately 50–100 mg of Sunphenon [Maeda and Nakagawa, 1977], and the daily consumption of green tea in Japan is 300–400 ml. In this respect, it has been shown that the use of *C. sinensis* tea (1–3 cups/day) resulted in a reduction of DMFT and plaque score in schoolchildren [Elvin-Lewis and Steelman, 1986]. Thus, the application of Sunphenon on a daily basis can be considered a useful and a practical method for the prevention of dental caries.

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