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Salivary hydrogen peroxide produced by holding or chewing green tea in the oral cavity*

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Abstract

Tea (*Camellia sinensis*) catechins have been studied for disease prevention. These compounds undergo oxidation and produce H_2O_2. We have previously shown that holding tea solution or chewing tea leaves generates high salivary catechin levels. Herein, we examined the generation of H_2O_2 in the oral cavity by green tea solution or leaves. Human volunteers holding green tea solution (0.1–0.6%) developed salivary H_2O_2 with $C_{max} = 2.9–9.6\,\mu M$ and $AUC_{0\rightarrow\infty} = 8.5–285.3\,\mu M\,min$. Chewing 2 g green tea leaves produced higher levels of H_2O_2 ($C_{max} = 31.2\,\mu M$, $AUC_{0\rightarrow\infty} = 1290.9\,\mu M\,min$). Salivary H_2O_2 correlated with catechin levels and with predicted levels of H_2O_2 ($C_{max(expected)} = 36\,\mu M$ vs $C_{max(determined)} = 31.2\,\mu M$). Salivary H_2O_2 and catechin concentrations were similar to those that are biologically active *in vitro.* Catechin-generated H_2O_2 may, therefore, have a role in disease prevention by green tea.

Keywords: *Green tea, catechins, H_2O_2, cancer prevention, oral cavity*

Introduction

Green tea (*Camellia sinensis*, Theaceae) is a widely consumed beverage with worldwide popularity second only to water. Green tea (GT) and its major polyphenolic constituents, the catechins (Figure 1), have been extensively studied for their beneficial health effects, including the prevention of oral cancer and tooth decay [1–3]. In a number of animal experiments and human trials, GT has been shown to decrease plaque score and tooth decay [4–6]. Although epidemiological data is mixed regarding the oral cancer preventive effects of GT, an intervention study found that oral and topical application of a GT preparation reduced the size of lesions and the incidence of multinucleated oral mucosa cells in leukoplakia patients compared to controls [7,8].

We and others have shown that catechins are unstable at neutral and basic pH, undergoing oxidative polymerization with concomittant production of H_2O_2 and other reactive oxygen species (ROS) [9,10]. Cell culture studies have shown that some of the biological effects of (−)-epigallocatechin-3-gallate (EGCG) are dependent on these oxidative reactions [11]. For examples, our laboratory has shown that treatment of human esophageal cancer cells with EGCG results in dose-dependent decrease in the levels of phosphorylated and non-phosphorylated epidermal growth factor receptor. These effects are diminished by inclusion of superoxide dismutase

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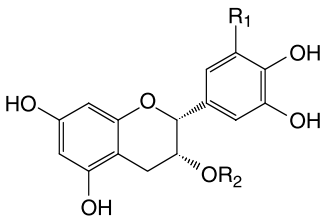

EC: $R_1 = R_2 = H$
EGC: $R_1 = OH$, $R_2 = H$
ECG: $R_1 = H$; R_2 = galloyl
EGCG: $R_1 = OH$; R_2 = galloyl

Figure 1. Structure of the major green tea catechins.

and catalase which stablizes EGCG, and apparently prevents oxidative damage of EGFR [12].

We have previously demonstrated that holding GT solution or chewing GT leaves are effective ways of delivering high levels of catechins (2.0–160 μg/ml) in the oral cavity of human volunteers [13]. Since the oral cavity is exposed to high oxygen partial pressure at elevated temperature (~37°C), we expect the catechins to be unstable in this environment and undergo oxidative polymerization with production of H_2O_2. Such H_2O_2 could play an important role in the biological effects of GT in the oral cavity. Herein, we report the results of our study.

Materials and methods

Reagents

Instant green tea powder and EGCG, EGC, EC, and ECG were provided by Mitsui Norin Co. Ltd. (Tokyo, Japan). Green tea leaves were purchased from Eden Food Co. (Clinton, MI). Standard solutions of EGCG, EGC, ECG and EC (10 μg/ml) were made in 0.2% ascorbic acid − 0.005% EDTA solution and stored at −80°C. All other reagents and HPLC solvents were of the highest grade available.

Treatment, saliva collection, and H_2O_2 determination

The protocol for human subjects in these studies was approved by the Institutional Review Board for the Protection of Human Subjects (Protocol no 92-034) at Rutgers University (Piscataway, NJ). All subjects ($n = 4$) were males aged 25–35. Subjects refrained from consuming tea, alcohol, or smoking for 2 days prior to the start of the experiment. After brushing teeth, volunteers held 200 ml of GT solution (0.1 or 0.6%) in their mouths for 2 min. The solution was voided and the subjects rinsed their mouths 10 times with 50 ml deionized water for 2 min. Unstimulated saliva was collected prior to holding tea and 0–90 min thereafter. An equal volume of saliva was combined with sodium phosphate buffer (0.1 M, pH 5.8) and stored at −80°C until analysis.

A similar experimental design was used to determine H_2O_2 generated after chewing GT leaves. In this instance, subjects chewed 2 g of GT leaves for 2 min, voided the leaves, and then rinsed their mouths as above. Saliva samples were collected and stored as above.

H_2O_2 levels were determined using the Amplex Red Hydrogen Peroxide assay kit with slight modifications (Molecular Probes, Eugene, OR). This assay is based on the peroxidase-mediated oxidation of the Amplex reagent to a fluorescent resorufin that can be monitored spectrophotometrically ($\lambda_{ex} = 550$ nm, $\lambda_{em} = 590$). In order to stabilize the catechins and prevent the formation of additional H_2O_2 during the analysis, the pH of the reaction buffer was adjusted to pH 6. Each sample was determined in duplicate.

HPLC quantification of catechins

To determine the level of catechins in saliva samples, 500 μl aliquot of saliva was mixed with an equal volume of 60% acetonitrile. A 50 μl aliquot of each sample was analyzed on an HPLC system composed of an ESA Model 465 refrigerated autosampler, two ESA Model 580 dual piston pumps, and an ESA 5500 coulochem electrode array system (CEAS). A Supelcosil C18 reversed-phase column (150 × 4.6 mm I.D.; Supelco Co., Bellefonte, PA) was used for all applications. The column and CEAS detector were housed in a temperature-regulated compartment maintained at 35°C. The autosampler was maintained at 6°C. For binary gradient elution, mobile phase A consisted of 1.75% acetonitrile and 0.12% tetrahydrofuran in 30 mM NaH_2PO_4 (pH = 3.35) whereas mobile phase B consisted of 58.5% acetonitrile and 12.5% tetrahydrofuran in 15 mM NaH_2PO_4 (pH = 3.45). The flow rate was maintained at 1 ml/min and the eluent was monitored by CEAS with potential settings at −100, 100, 300, and 500 mV. Gradient conditions were the same as those previously reported [14].

Results and discussion

We have previously shown that holding GT solution or chewing tea leaves is an effective way to deliver high concentrations of catechins in the oral cavity [13]. We and others have reported that catechins are unstable in aerobic environments: EGCG undergoes oxidative polymerization with concomitant production of H_2O_2 [15,16].

In the present study, we found that holding tea solution in the mouth for 2 min resulted in dose-dependent H_2O_2 levels in the saliva (Figure 2). These levels were maximal between 2 and 10 min after washout and decreased as a function of time. The C_{max}

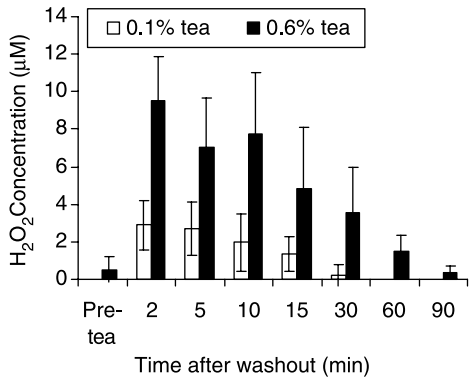

Figure 2. Salivary levels of H_2O_2 generated after holding GT solution in the oral cavity. Subjects held 200 ml of 0.1 or 0.6% GT solution in the mouth for 2 min, voided the solution, and rinsed the mouth with distilled water. $N = 4$, error bars represent the standard error of the mean.

and $AUC_{0\rightarrow\infty}$ for H_2O_2 were 2.9 μM and 45.8 μM min, respectively for 0.1% GT solution, and 9.6 μM and 285.3 μM min, respectively for 0.6% GT solution. Our previous studies have shown that the concentrations of catechins achieved in saliva following holding tea solution (0.7% GT results in 6.7–120.0 μM catechins) were very similar to those achieved following drinking tea solution (0.6% GT results in 1.8–143.5 μM catechins) of a similar concentration [13,17]. Based on this, we expect that drinking 0.6% GT solution will produce a similar amount of H_2O_2 compared to what is observed in the present study.

Chewing 2 g of GT leaves resulted in significantly higher levels of salivary H_2O_2 (Figure 3) than holding GT solution. H_2O_2 levels increased to a C_{max} of 31.2 μM at 5 min and then declined as a function of time. The $AUC_{0\rightarrow\infty}$ of H_2O_2 following chewing GT leaves was 1290.9 μM min. The higher levels H_2O_2 achieved by chewing GT leaves are likely due to the

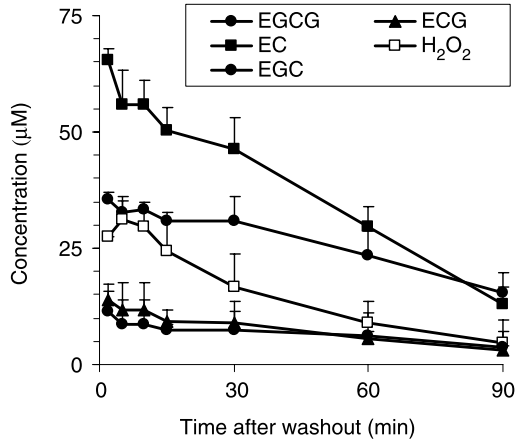

Figure 3. Salivary levels of H_2O_2 and catechins generated after chewing GT leaves. Subjects chewed 2 g of GT leaves for 2 min, voided the leaves, and rinsed the mouth with distilled water. $N = 4$, error bars represent the standard error of the mean.

higher catechin levels achieved by this dosage form [13,17].

We compared the levels of H_2O_2 formed with the salivary catechin levels achieved by chewing GT leaves (Figure 3). EC and EGCG were the most abundant catechins ($C_{max} = 65.6$ and 35. 6 μM, respectively) followed by ECG and EGC ($C_{max} = 13.8$ and 11.4 μM, respectively). Based on these maximal concentrations, and previous research by our laboratory and others on H_2O_2 production by catechins, we expected to observe a maximal H_2O_2 concentration of approximately 36 μM [10,12,15]. This value is in good agreement with the observed H_2O_2 C_{max} of 31.2 μM (Figure 3). Human saliva has been reported to contain at least two peroxidase enzymes, human myeloperoxidase and human salivary peroxidase, as well as bacteria-derived catalase all of which function to eliminate ROS such as H_2O_2 from the saliva [18]. It is possible that these enzymes are causing an underestimation of the $AUC_{0\rightarrow\infty}$ of salivary H_2O_2 by enhancing its elimination. Based on the strong agreement between the predicted levels of H_2O_2 and the measured levels of after chewing tea leaves (as shown above), we believe it is unlikely that these enzymes are causing underestimation of the peak H_2O_2 concentrations.

An increasing number of reports indicate that the pro-oxidant activity of the tea catechins may play an important role in the biological activities of these compounds. We have previously shown that inclusion of catalase reduces the induction of apoptosis in H661 human lung cancer cells and 21BES human transformed human bronchial cell lines [19,20]. Weisburg et al. found that oral cancer cells were more susceptible than normal oral fibroblasts to the cytotoxic effects of EGCG ($IC_{50} = 45.8$ vs. 246 μM), and that this cytotoxicity was H_2O_2 dependent [21]. Treatment of oral cancer cells with EGCG resulted in depletion of intracellular reduced glutathione, further suggesting a role for the pro-oxidant activity of EGCG. The effective concentrations of EGCG (20–50 μM) and H_2O_2 (10–25 μM) in these cell lines studies are of the same order of magnitude as those that we presently observe in the saliva after chewing GT leaves.

Studies in animal models and clinical trials in humans have indicated that concentrations of H_2O_2 less than 3% or 88 μM have not been associated with tissue damage, carcinogenicity, or tumor promotion in combination with carcinogens [22]. In contrast, 44 μM H_2O_2 used as a daily rinse by orthodontic patients was found to be more effective at maintaining periodontal health than toothbrushing alone [23]. Such data suggest that the levels of H_2O_2 generated by drinking or holding GT solution or chewing GT leaves is not strong enough to increase cancer risk or cause oral pathology, but my be beneficial in maintaining oral health including prevention of oral cancer.

Further studies in animal models and human volunteers are needed to further assess what role if any GT-mediated formation of H_2O_2 plays in the disease preventive effects of GT.

In summary, we have observed that holding GT solution in the oral cavity or chewing GT leaves results in dose-dependent production of H_2O_2 in the mouth. The levels H_2O_2 correlate well with the levels of salivary tea catechins, suggesting that these polyphenolic compounds are the source of H_2O_2. The concentrations of H_2O_2 and catechins found in the saliva are similar to or lower than clinically used as mouth rinse. We suggest that during consumption of tea, the catechin-generated H_2O_2 is not cytotoxic and may play a role in the oral cancer and dental caries.

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