Genoprotection and genotoxicity of green tea (*Camellia sinensis*): Are they two sides of the same redox coin?

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Objectives: Regular intake of green tea associates with lower DNA damage and increased resistance of DNA to oxidant challenge. However, *in vitro* pro-oxidant effects of green tea have been reported. Both effects could be mediated by hydrogen peroxide (H_2O_2) which is generated by autooxidation of tea catechins. In large amounts, H_2O_2 is genotoxic, but low concentrations could activate the redox-sensitive antioxidant response element (ARE) via the Keap-1/Nrf2 redox switch, inducing genoprotective adaptations. Our objective was to test this hypothesis.

Methods: Peripheral lymphocytes from healthy volunteers were incubated for 30 minutes at 37°C in freshly prepared tea solutions (0.005, 0.01, 0.05%w/v (7, 14, 71 $\mu\text{mol/l}$ total catechins) in phosphate buffered saline (PBS), with PBS as control) in the presence and absence of catalase (CAT). H_2O_2 in tea was measured colorimetrically. Oxidation-induced DNA lesions were measured by the Fpg-assisted comet assay.

Results: H_2O_2 concentrations in 0.005, 0.01, and 0.05% green tea after 30 minutes at 37°C were, respectively, ~3, ~7, and ~52 $\mu\text{mol/l}$. Cells incubated in 0.005 and 0.01% tea showed less ($P < 0.001$) DNA damage compared to control cells. Cells treated with 0.05% green tea showed ~50% ($P < 0.001$) more DNA damage. The presence of CAT prevented this damage, but did not remove the genoprotective effects of low-dose tea. No significant changes in expression of ARE-associated genes (*HMOX1*, *NRF2*, *KEAP1*, *BACH1*, and *hOGG1*) were seen in cells treated with tea or tea + CAT.

Conclusion: Genoprotection by low-dose green tea could be due to direct antioxidant protection by green tea polyphenols, or to H_2O_2 -independent signalling pathways.

Keywords: Green tea, Hydrogen peroxide, Oxidative stress, Genoprotection, DNA damage, Pro-oxidant, ARE, Redox tone, HO-1

Introduction

Green tea is reported to have various health benefits, and these are thought to be due to its high content of polyphenolic antioxidants, the flavon-3-ols commonly referred to as catechins.^{1–3} The bioavailability of catechins is low, but small amounts are found in the circulating plasma soon after tea ingestion, and drinking green tea increases the antioxidant capacity of human plasma within 1 hour.^{4–6} The post-ingestion increases are short-lived, but regular tea ingestion could be expected to create small but regularly replenished increases in catechins that lead to improvements in biomarkers of oxidation-induced damage. This is supported by the findings of a placebo-controlled human trial that showed a significant (30%) decrease in oxidation-induced DNA damage in lymphocytes

after 4 weeks supplementation with green tea.⁷ However, green tea auto-oxidizes *in vitro*, generating hydrogen peroxide (H_2O_2), and *in vitro* experiments have revealed that green tea can induce DNA damage.^{8,9} Furthermore, very high doses of tea or polyphenol extracts, for example, >200 μM EGCG or >500 mg/kg EGCG, damaged liver and kidney tissues in mice and dogs.^{10–13}

The genotoxic effects of green tea are likely to be due to H_2O_2 , while its genoprotective effects could be due to direct antioxidant action of the tea polyphenols or to cellular adaptations to subtle changes in cellular redox balance induced by the low concentrations of catechins achieved *in vivo*. It is hypothesized that a mild shift induced by the pro-oxidant effect of green tea catechins at low concentrations causes activation of redox sensitive signalling pathway(s) or genes. Of interest, here is the antioxidant response element (ARE; also known as an electrophile-responsive

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element), a redox-sensitive gene promoter region that regulates expression of genes encoding for several cytoprotective products, including haem oxygenase 1 (HO-1) and the DNA repair enzyme hOGG1.^{14–16} A pro-oxidant shift in redox balance facilitates the release, nuclear translocation and binding to the ARE of the transcription factor, NF-E2-related factor 2 (Nrf2), with subsequent ARE activation. This has been clearly demonstrated, and indeed increased HO-1 expression is accepted as a sign of oxidative stress.^{14–16} Therefore, the genoprotective and the genotoxic effects of green tea could both be a consequence of H₂O₂ generated from catechins, the overall effect (damage or protection) being determined by the dose of tea-generated H₂O₂ and the ARE response to this. The objectives of this *in vitro* study were to determine whether H₂O₂ generated in green tea is responsible for the genoprotective as well as the genotoxic effects of green tea, and if the protective effects are mediated via H₂O₂-induced ARE activation.

Methods

Experimental design

Cryopreserved, pooled peripheral lymphocytes from five healthy subjects were used for this *in vitro* study. Approval for the study was obtained from the Human Subjects Ethics Sub-committee of The Hong Kong Polytechnic University, and all volunteers gave their written consent. Lymphocytes were harvested, washed, pooled, aliquoted, and cryopreserved as described previously.¹⁷ Lymphocyte aliquots were thawed (once only, and within 4 weeks of collection), washed in phosphate buffered saline (PBS), and incubated in freshly prepared green tea solutions (0.005, 0.01, and 0.05% w/v in PBS, and PBS as control). These tea solutions were made by dilution of a freshly prepared 1% w/v green tea infusion in distilled water. A matching set of PBS-diluted tea solutions containing 2.4 U/ml of catalase (CAT; Sigma Aldrich, St Louis, MO, USA) was prepared. The CAT was added to the tea 5 minutes before it was added to cells. Our preliminary experiments showed that this amount of CAT added to 1 ml of tea was sufficient to remove H₂O₂ at up to 160 µmol/l concentration in 5 minutes. The green tea used was pre-rain Loong-cheng tea, kindly provided by Ying Kee Tea House, Hong Kong. In three independent experiments, cells were incubated (in parallel) in the tea and tea + CAT solutions for 30 minutes at 37°C. The tea was removed, and cells were washed twice with cold PBS. Some cells from each treatment were used immediately after washing for measurement of oxidation-induced DNA damage (as strand breaks) using the Fpg-assisted version of the comet assay,^{7,18} and some were used immediately for RNA extraction in order to investigate changes in expression of

ARE-related genes using accepted protocols: genes of interest were *HMOX1*, *NRF2*, *KEAP1*, and *hOGG1*. Expression of *BACH1*, an inhibitor of ARE activation, was also investigated. Expression of these genes was normalized to two lymphocyte-specific reference genes, *CD3ε* and *CD8β*.¹⁹ Identity of gene products was confirmed by gene sequencing. In the Fpg-assisted comet assay, two gels for each treatment were prepared in each experiment, and DNA damage was scored (as %DNA in comet tail) in 50 nucleoids per gel. The amount of H₂O₂ generated in the different solutions of tea (freshly prepared and after 30 minutes at 37°C, but without cells added) was measured following a published protocol.^{20,21} The catechins concentrations of a freshly prepared tea solution were measured by liquid chromatography with tandem mass spectrometry (LC-MS/MS) as previously described.⁶ Results were used to estimate the catechin concentrations in the 0.005, 0.01, and 0.05% w/v tea solutions used for the *in vitro* incubation of cells.

Statistical analysis

The data on H₂O₂ in tea and for the Fpg-assisted comet assay are expressed as mean (SD). One-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was conducted on the H₂O₂ concentrations in the different concentrations of tea before and after 30 minutes incubation. Repeated-measures ANOVA with Bonferroni's *post hoc* test was performed to examine changes in oxidation-induced lesions in lymphocytic DNA with tea and tea + CAT pre-treatment. A *P* value of <0.05 was considered to be statistically significant. An open access gene analysis software tool, Relative Expression Software Tool (REST[®] 2009; Qiagen, Valencia, CA, USA) was used to analyse data obtained from real-time polymerase chain reaction (PCR).²² This software takes into account the PCR efficiencies of genes of interest and reference genes, and is able to use more than one reference gene for normalization to increase the reliability of the results. The statistical software uses randomization and bootstrapping methods to test for the statistical significance of the expression ratios determined. The data for gene expression are presented in box and whisker plots. A fold change of >2, was considered as a biologically meaningful change in relation to analysing the gene expression data.^{23–25}

Results

The estimated catechins concentrations in the 0.005, 0.01, and 0.05% w/v tea solutions are given in Table 1. H₂O₂ was generated in green tea in a dose-dependent manner (Table 2). No H₂O₂ was detected in tea solutions containing CAT (results not shown). With regard to the effect on DNA damage of

Table 1 Estimated catechins concentrations ($\mu\text{mol/l}$) of the tea solutions used in the *in vitro* study

Catechin	Catechins concentration of green tea at		
	0.005% w/v	0.01% w/v	0.05% w/v
Epigallocatechin gallate	3.6	7.1	35.7
Epigallocatechin	1.9	3.8	19.0
Epicatechin gallate	0.77	1.5	7.7
Epicatechin	0.73	1.4	7.3
Catechin	0.1	0.3	1.3
Total catechins	7.1	14.2	71

Table 2 Concentrations of H_2O_2 measured in freshly prepared tea infusions (in PBS) and after 30 minutes at 37°C ; data are mean (SD), $n = 3$

	Concentration of H_2O_2 (mol/l) in		
	0.005% Green tea	0.01% Green tea	0.05% Green tea
Freshly prepared solutions of tea	1.41 (0.10)	1.57 (0.07)	3.61 (0.58)
The same tea solutions after 30 minutes at 37°C	3.52 (1.18)	7.36 (1.64)*	51.73 (3.98)*

* $P < 0.05$ Compared to the fresh tea solution of the same concentration.

pre-treatment of cells with tea, results are presented in Table 3. Cells incubated in green tea at low concentration (0.005 and 0.01% w/v) showed less ($P < 0.001$) DNA damage compared to control (PBS-treated) cells, regardless of whether the tea solutions contained CAT or not. Cells treated with 0.05% w/v green tea showed significantly higher (50% more; $P < 0.001$) DNA damage, but CAT prevented this. No significant changes were observed in the expression ratio of any of the ARE-related genes of interest in lymphocytes treated with different concentrations of tea or tea + CAT (results not shown).

Discussion

Previous studies have shown that drinking green tea increases plasma antioxidant capacity rapidly, and that regular intake of green tea is associated with decreased DNA damage in lymphocytes of healthy subjects.^{5,7} However, it remains unclear if the

genoprotective effect of green tea on lymphocytic DNA is due to direct antioxidant protection, to an adaptive response to mild oxidative stress induced by H_2O_2 generated from the auto-oxidation of polyphenolic catechins in green tea, or to some other mechanism. In this *in vitro* study, H_2O_2 was shown to be generated in a dose-dependent manner in the green tea solutions. Significant genoprotective effects of green tea at low concentrations (0.005 and 0.01% w/v) were demonstrated, but 0.05% w/v green tea induced a marked increase in DNA damage. These findings are consistent with previously published findings of differential effects of green tea at low and high doses.^{7,13}

Tea infusions for drinking are generally of $\sim 1.5\%$ w/v, and *in vitro* studies of effects of green tea catechins have generally used these or higher concentrations. However, bioavailability of green tea catechins in humans is poor. In this study, we treated cells with low-dose green tea (0.005, 0.01, and 0.05% w/v) to try to emulate physiologically relevant concentrations. In the planning stages of this work we had no information on the actual catechin content of the green tea we planned to use, and we were guided by our previous work for selecting the concentrations used.⁷ As part of this study, we measured typical concentrations of catechins in freshly prepared infusions of the green tea used and so could estimate the catechin concentrations of the tea solutions used in this *in vitro* study. In addition, we have also studied acute post-ingestion changes in plasma catechins, and found that total catechins can reach and exceed $1 \mu\text{mol/l}$ in the 1–2 hours after ingestion of a modest dose (200 ml of 1% w/v) of the same type of green tea as was used here.⁶ Higher amounts ingested likely induce higher plasma catechins responses. Therefore, while no *in vitro* study can fully emulate the *in vivo* situation, the concentration of catechins used here approach the concentration range that could be achieved after ingestion of green tea.

Interestingly, these new data show that while the presence of CAT prevented the damage inflicted by the higher dose of tea, it did not prevent the protective effect seen with low dose tea. Furthermore, no changes in expression of genes that are expected to be affected by redox-driven ARE activation were seen. Therefore,

Table 3 DNA damage in cells treated with green tea or green tea + catalase for 30 minutes at 37°C

	Green tea (% w/v in PBS)				Green tea (% w/v in PBS) + catalase			
	0% (PBS)	0.005% Tea	0.01% Tea	0.05% Tea	0% (PBS)	0.005% Tea	0.01% Tea	0.05% Tea
%DNA in tail	12.99 (1.14)	8.52* (0.25)	9.57* (0.21)	19.72* [#] (1.41)	13.24 (0.41)	9.25* (0.99)	9.02* (0.99)	11.24 (1.28)

Data are mean (SD) %DNA in tail, $n = 3$ (Fpg-assisted comet assay used).

* $P < 0.001$ Compared to PBS-treated (no tea) cells in same set; [#] $P < 0.001$ compared to lower concentrations of green tea in same set.

results indicate that H_2O_2 is the cause of the DNA damage induced by a relatively high dose of green tea, but this does not trigger detectable redox-linked cellular adaptations leading to the genoprotection induced by exposure to low doses of green tea, as has been hypothesized. Furthermore, genoprotection by low-dose green tea is not mediated by the small amounts of H_2O_2 generated.

It is possible that 30 minutes incubation is insufficient time for ARE-related cytoprotective genes to be expressed at measurably greater levels. Several studies have examined the effects of time-dependent change in the expression of ARE-related genes in different cells and with various inducers, including cigarette smoke in mouse fibroblasts, sulphoraphane in human astrocytes, and arsenite in human keratinocytes.^{26–28} A change in *HMOX1* gene expression with cigarette smoke was observed only after 1 hour of stimulation.²⁶ Further study is needed to investigate whether a longer exposure time to tea is needed to reveal changes in expression of ARE-regulated genes, even though the results presented here show that 30 minutes treatment with tea is long enough to lower DNA damage. Direct antioxidant effects of green tea polyphenols could explain the protection. It is also possible to speculate that green tea increases the efficacy (rather than the amount) of the DNA repair enzyme hOGG1 by blocking the actions of inhibitors, such as proteases. This remains to be confirmed. The enzyme used in the Fpg-assisted comet assay is specific for oxidized purines, including 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FaPyGua), and 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines. The enzyme is a microbial analogue of hOGG1, which catalyses the first step in base excision repair.²⁹ It is possible also that other DNA repair enzymes responsible for the elimination of oxidation-induced DNA damage, e.g. MYH encoding A/G-specific adenine DNA glycosylase, NTH1 encoding for endonuclease III, and MTH1 encoding 8-oxo-dGTPase, may be affected by green tea.^{30,31} Besides, flavonoids, the polyphenolic family to which green tea catechins belong, have been suggested to directly trigger cytoprotection, as opposed (or in addition) to cytoprotective effects being mediated via tea-generated H_2O_2 .³² If this is true, the effect may be via a different set of cytoprotective genes than the ones studied here. These speculations require further study.

To conclude, green tea at 0.05% w/v generated quite large amounts of H_2O_2 , and this was responsible for induction of DNA damage. Low-dose (0.005 and 0.01% w/v) green tea, containing physiologically relevant catechins concentrations, was shown to be genoprotective whether or not CAT was present. The protective mechanism may be direct antioxidant

action. More complex molecular mechanisms may exist, but require further probing.

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