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Evaluation of Multiple Oxidation Products for Monitoring Effects of Antioxidants in Fenton Oxidation of 2′-Deoxyguanosine

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The objective of this study was to investigate the influence of the two antioxidants, ascorbic acid and (+)catechin, on the oxidation of 2′-deoxyguanosine (dG), using an iron-mediated Fenton reaction. The oxidation products 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8,5′-cyclo-2′-deoxyguanosine, together with the secondary oxidation products guanidinohydantoin and dehydroguanidinohydantoin, were identified and quantified through the use of an LC-MS/MS system. The results obtained showed that catechin inhibited the oxidation better than ascorbic acid did, indicating that the chelating ability of catechin rather than the radical scavenging mechanism alone is vital for the observed antioxidative efficiency. The correlation between the different oxidation products was found to be quite low, primarily because of the instability of 8-oxodG, making it prone to further oxidation. This led to apparent anti- and pro-oxidative results being obtained, emphasizing the potential problems in evaluating oxidative stress, by use of a single marker.

KEYWORDS: Ascorbic acid; catechin; Fenton reaction; 2′-deoxyguanosine; oxidation

INTRODUCTION

Oxidative damage caused by reactive oxygen species (ROS) is believed to be a major health issue due to its probable, although not proven, involvement in cancer development, cardiovascular disease, the process of aging, and neural decay in the brain (*1*). To survive, living organisms have developed various strategies for coping with the potentially dangerous ROS, such as by means of antioxidant enzymes and small-molecule antioxidants. Antioxidants acquired from the diet are an important part of this defense.

Although many studies concerning the health promoting properties of antioxidants have been undertaken, there still is a lack of knowledge about which antioxidants are most efficient in protecting important biomolecules. The most relevant information is gained from controlled feeding experiments, but these are very complex, and it is therefore difficult to isolate single effects of the antioxidants and their possible protection mechanisms. Thus, in some cases, more simple in vitro tests might be more convenient. In most model studies, artificial radicals are used to generate oxidative conditions. In our opinion, it might be better to use a more relevant radical, such as the hydroxyl radical (•OH) formed by the decomposition of the naturally occurring metabolite hydrogen peroxide (H_2O_2) by Ferrous iron (Fe^{2+}) (i.e., the iron-mediated Fenton reaction). In vivo, most iron is stored in proteins, such as ferritin, transferrin, and metallothionein. However, iron can be released if a reductant such as superoxide or ascorbate is present (*2*). Although Fenton-mediated oxidation is believed to be of great importance biologically, relatively few studies have used Fenton reagents to induce oxidative damage to DNA or its components. Nonetheless, Henle et al. and Frelon et al. have investigated adduct formation in dG and DNA, respectively, by use of the iron-mediated Fenton reaction (*3*, *4*).

Tests of antioxidant performance should preferably measure their ability to protect important biomolecules, such as DNA and its building blocks. The most popular marker for monitoring DNA oxidation is the guanine derived oxidation product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is readily formed in hydroxyl radical oxidation of 2′-deoxyguanosine (dG) (**Scheme 1**). It is well-known that guanine is the most easily oxidized nucleobase in DNA, but it has been shown that 8-oxodG is actually more susceptible to oxidation than dG itself (*5*, *6*). The formation of secondary oxidation products of 8-oxodG (**Scheme 2**) is highly dependent upon the oxidizing agent and conditions involved. It has been suggested that in one-electron oxidation, 5-hydroxy-8-oxodG is formed as an intermediate, and a spiroiminodihydantoin is formed if the pH is above 7, decarboxylation occurring otherwise, resulting then in the formation of two diastereomers of guanidinohydantoin (Gh) (*5*, *6*). Gh was found to be the main secondary oxidation product when dG was oxidized by the Fenton reaction (*3*, *7*). Gh itself may also oxidize further into dehydroguanidinohydantoin (Gh^{ox}). Another possibility is that Gh^{ox} is formed through decarboxylation of the peroxide 5-hydroperoxy-8-oxo-7,8 dihydro-2′-deoxyguanosine (5-OOH-8-oxodG) that has been reported to occur in singlet oxygen oxidation of 8-oxodG (*6*).

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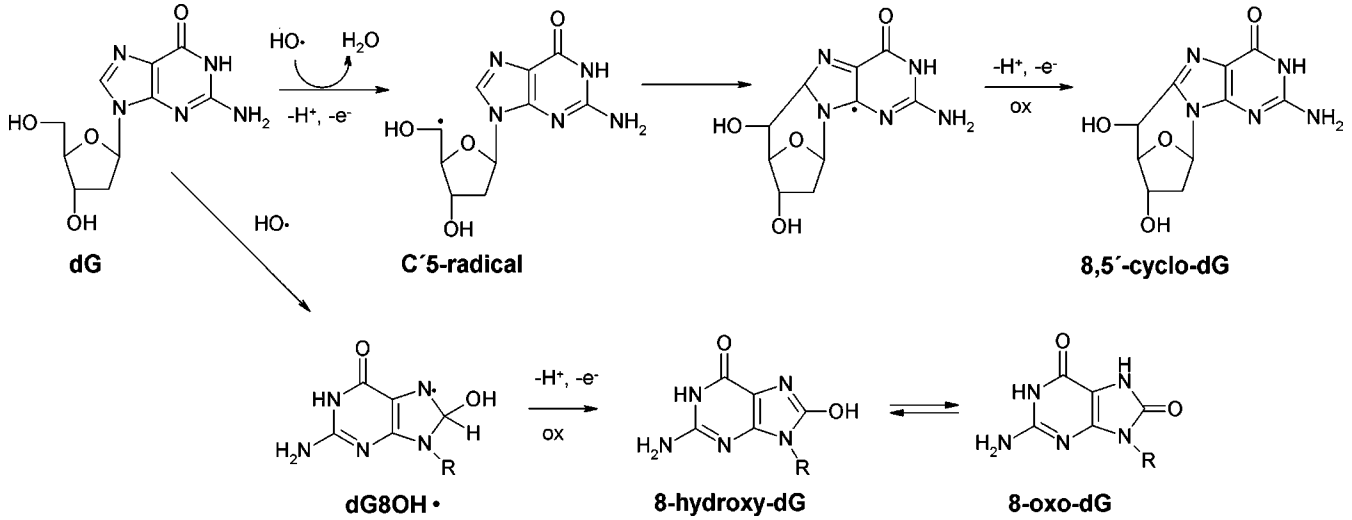
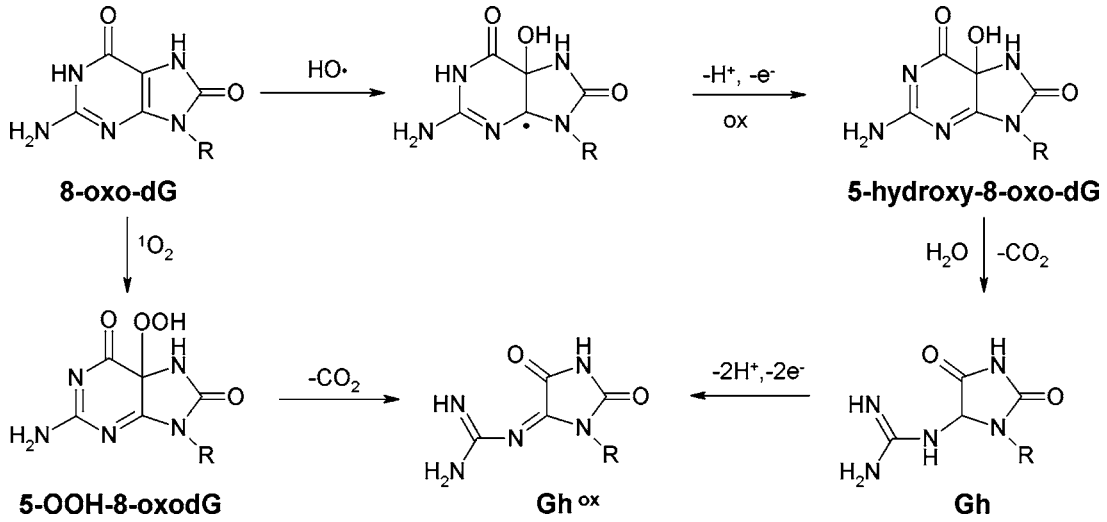
Scheme 1



Scheme 2



Another major group of lesions formed by oxidation of DNA by the hydroxyl radical is 8,5′-cyclo purine-2′-deoxynucleosides. They are formed by hydrogen abstraction of the deoxyribose at the 5′-postion followed by intramolecular cyclization by the purine (**Scheme 1**) (*2*, *8*). It has been shown that 8,5′cyclo-2′-deoxyguanosine (8,5′-cyclodG) is much more resistant to further oxidation than 8-oxodG, even under harsh conditions when the Fenton reaction is employed (*7*, *9*).

The aim of the present study was to investigate the specific products formed when dG is oxidized by the iron-mediated Fenton reaction in the presence of either ascorbic acid (vitamin C) or (+)catechin. Although reports of antioxidants inhibiting the oxidation of DNA by use of the Fenton reaction are rather common, most of the studies have been concerned with measuring the inhibition of 8-oxodG alone (*10*−*12*). Ascorbic acid is widely present in fruits and vegetables. It is regarded as an excellent water-soluble radical scavenger because of its reducing capabilities and its fairly unreactive ascorbyl radical, which is formed by a one-electron reduction (*13*, *14*). The catechins, which consist of various polyphenols closely related to one another, are a group of potent versatile antioxidants having both radical scavenging and metal chelating abilities (*15*−*17*). They are found in large amounts in beverages such as tea and red wine and also in some fruits such as apples (*18*). The thought behind the choice of antioxidants was to use a good scavenger (ascorbic acid) and compare it with an antioxidant known to also have iron-chelating properties (catechin). The products quantified were the two primary oxidation products 8-oxodG and 8,5′-cyclodG, together with the secondary oxidation products Gh and Gh^{ox}.

MATERIALS AND METHODS

Chemicals. The nucleosides 2′-deoxyguanosine (dG) and 8-oxo-7,8 dihydro-2′-deoxyguanosine (8-oxodG), ascorbic acid, and (+)-catechin were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (30%) and acetonitrile were obtained from Merck (Darmstadt, Germany). Acetic acid and ammonium acetate were obtained from Fluka (Buchs, Swizerland). Iron(II) sulfate ($FeSO_4$) was purchased from Aldrich (Milwaukee, WI). All water used was of Millipore quality (Millipore Corp.; Bedford, MA).

Fenton Oxidation of dG. Stock solutions of dG, $FeSO_4$, H_2O_2, 8-oxodG, ascorbic acid, and catechin were prepared, typically in concentrations 10 times as high as the final concentration. All solutions were freshly prepared just prior to the experiments. To avoid too high a concentration of the Fenton reactants, the samples were mixed in the order water, dG, antioxidant, $FeSO_4$, and H_2O_2 to a final volume of 1 mL. The samples were shaken and incubated at room temperature for 1 min. They were then filtrated immediately and injected into the HPLC. Standard samples of dG and 8-oxodG, ascorbic acid, and catechin were analyzed the same day to obtain an accurate standard curve.

LC/MS Analysis. The compounds in the oxidation mixture were separated on a PE series 200 HPLC (Perkin-Elmer Instruments LLC,

Shelton) with a Supelco C_{18} reversed-phase column (Supelcosil LC-18S, 250 mm × 2.1 mm and 5 μm particle size). The mobile phase consisted of 5% acetonitrile in water with a 10 mM ammonium acetate buffer at pH 4.8 (solvent A). A constant flow of 200 μL/min under isocratic conditions was employed in all the samples, except for those containing catechin, the first 8 min in that case being run isocratically as described previously, then the amount of acetonitrile (solvent B) was increased up to 80% during a 4 min period, and this level was held constant then for another 4 min.

Each of the eluted compounds was detected using a quadrupole time-of-flight (Q-TOF) hybrid tandem mass spectrometer (API Qstar, MDS Sciex, Ontario, Canada) equipped with an electrospray ionization source (Turboion Spray) set in positive mode. The response was first optimized for dG dissolved in the mobile phase through direct injection using a Harvard model 22 syringe pump (Harvard Apparatus Inc, Holliston, MA). The ion source voltage was set to 5500 V, the drying gas having a temperature of 350 °C. The same settings were used in both the MS and in the MS/MS mode, except for the collision energy, which was set to 10 and 20 eV when MS/MS spectra were obtained. Argon was used as the collision gas.

The mass spectrometer was used for quantification. The peak area of a narrow mass range of the pseudo-molecular ion was integrated so as to obtain an optimal signal-to-noise ratio. Ascorbic acid, catechin, 8-oxodG, and dG were quantified using 12-point calibration curves (external calibration), whereas the other compounds were only compared relatively. Linear regression was employed since the response was reasonably linear within the concentration range investigated. Although the isotope-dilution quantification method is the method of choice due to its high stability and precision, the external standard method proved to be quite adequate. This was confirmed by comparison of the standard deviation of the standards in the calibration curve and repeated dG oxidation experiments, a larger portion of the standard error occurring during sample preparation than in the analysis.

Statistical Analysis. The standard error was estimated using triplicates of three representative samples. It was assumed that the relative standard deviation was constant within the range investigated. This assumption, tested by the use of a two-tailed *F*-test, could not be rejected at a $p = 0.05$ significance level for any of the products that were measured. The pooled relative standard deviation was calculated, serving to determine the 95% confidence interval with use of the Student *t*-distribution. Significance tests were also performed using the *t*-distrubution and assuming that the standard deviation of the samples was equal.

RESULTS

Preliminary experiments were performed to elucidate the identity and retention times of dG, the oxidation products, and antioxidants employed. Most of the compounds were identified by the protonated molecular ion $[M + H]^+$ and the fragmentation pattern in MS/MS. Most nucleosides fragment easily, the most intense fragment being the protonated nucleobase $[B + 2H]^+$ in positive ESI, which corresponds to the loss of the deoxyribose moiety (−116 amu) (*19*). The antioxidants ascorbic acid and catechin, and the nucleosides dG and 8-oxodG, were compared with the standards. Gh and Gh^{ox} were identified by the masses of the protonated molecular ion and the base fragment, $m/z = 274/158$ and 272/156, respectively. An additional mass of $m/z = 114$ was found in the Gh^{ox} MS/MS spectrum, reported to be characteristic of this compound (*20*, *21*). Since the two diastereomers of Gh could not be separated on HPLC entirely, the decision was made to quantify the diastereomers together. The cyclic adduct 8,5′-cyclodG was identified with its molecular mass of $m/z = 266$ and its characteristic fragment of $m/z = 180$ in MS/MS (*8*). Both the (5′R) and the (5′S) diastereomers were found, but only the more abundant (5′R) was quantified. It is important that one recognizes the importance of the reaction time, which strongly influences the amounts of the oxidation products produced. We have found that at 1 mM Fenton reagent concentrations, 8-oxodG is formed and consumed within a few seconds, whereas Gh and Gh^{ox} are formed more slowly, 8,5′-cyclodG also being formed quickly but being much more stable with increased incubation time (*7*). Accordingly, the reaction time was held constant in all the experiments, so as to be able to compare the results properly. Further details regarding the analysis, including chromatograms, retention times, and MS/MS spectra have been presented in a previous study (*7*).

Ascorbic Acid. The experimental design selected for the ascorbic acid experiments was a two-level full-factorial design regarding the Fenton reagent concentrations (0.1 and 4 mM). The ascorbic acid concentration was tested at four different levels, ranging from 0 to 10 mM for each combination of the Fenton reagent concentrations. Three replicates were prepared at the central level of 2 mM H_2O_2 and $FeSO_4$ for evaluating the standard error. An additional Fenton reagent concentration of 1 mM each was also tested. An initial dG concentration of 400 μM was employed in all the experiments, which is within the concentration range of phosphorylated nucleosides found in cells (*22*).

In **Figure 1A**, the amounts of dG that remained are shown, indicating the extent of the oxidation. In general, dG decreased with an increase in the concentrations of H_2O_2 and $FeSO_4$. The effect of ascorbic acid was most obvious at 10 mM when the oxidative conditions were harsh, at either H:2-F:2 or H:4-F:4 (2 or 4 mM each of H_2O_2 and $FeSO_4$).

At these conditions, the amount of dG remaining was much greater than when lower concentrations of ascorbic acid were applied. In contrast, at the lowest concentration of Fenton reagents applied (0.1 mM), ascorbic acid seemed to reduce the amount of remaining dG to some extent ($p = 0.23$). This was also the case at H:4-F:0.1 (4 mM H_2O_2 and 0.1 mM $FeSO_4$) and ascorbic acid concentrations above 1 mM ($p = 0.12$). At H:1-F:1, both trends could be observed: a decrease in the dG remaining at 0.1 mM ascorbic acid, followed by the status quo at 1 mM and an increase at 10 mM.

The formation of 8,5′-cyclodG is shown in **Figure 1B**. In all the samples with equimolar concentrations of the Fenton reagents, the influence of the ascorbic acid was similar; the amount of 8,5′-cyclodG increased with increasing concentrations of ascorbic acid up to 1 mM, but the effect was the opposite at 10 mM, a decrease in 8,5′-cyclodG occurring. When the iron concentration was low and H_2O_2 was in excess (H:4-F:0.1), the ascorbic acid increased the amount of 8,5′-cyclodG significantly ($p < 0.01$), especially at 1 and at 10 mM ascorbic acid concentrations. If only the effects of the $FeSO_4$ and H_2O_2 concentrations are considered, one can note that the formation of 8,5′-cyclodG clearly increased up to H:2-F:2 and then decreased somewhat at H4:-F:4, except in the case of an ascorbic acid concentration of 10 mM.

The amounts of 8-oxodG produced are shown in **Figure 1C**. Without any ascorbic acid being added, 8-oxodG increased with the addition of up to 2 mM both H_2O_2 and $FeSO_4$, followed by a large reduction at 4 mM. The $FeSO_4$ concentration appeared to affect the results more than the H_2O_2 concentration did. At 0.1 mM ascorbic acid and H:0.1-F:4, the ascorbic acid reduced the amount of 8-oxodG significantly ($p < 0.01$). The opposite effect, although less decisive, was seen at H:1-F:1 ($p = 0.36$). When the concentration of ascorbic acid was increased a magnitude further to 1 mM, the inhibition was strong at a H_2O_2 concentration of 0.1 mM, regardless of the iron concentration, but at H:1-F:1, there was only a slight inhibition if any, and at H:2-F:2 and H:4-F:4, there was a significant increase in 8-oxodG

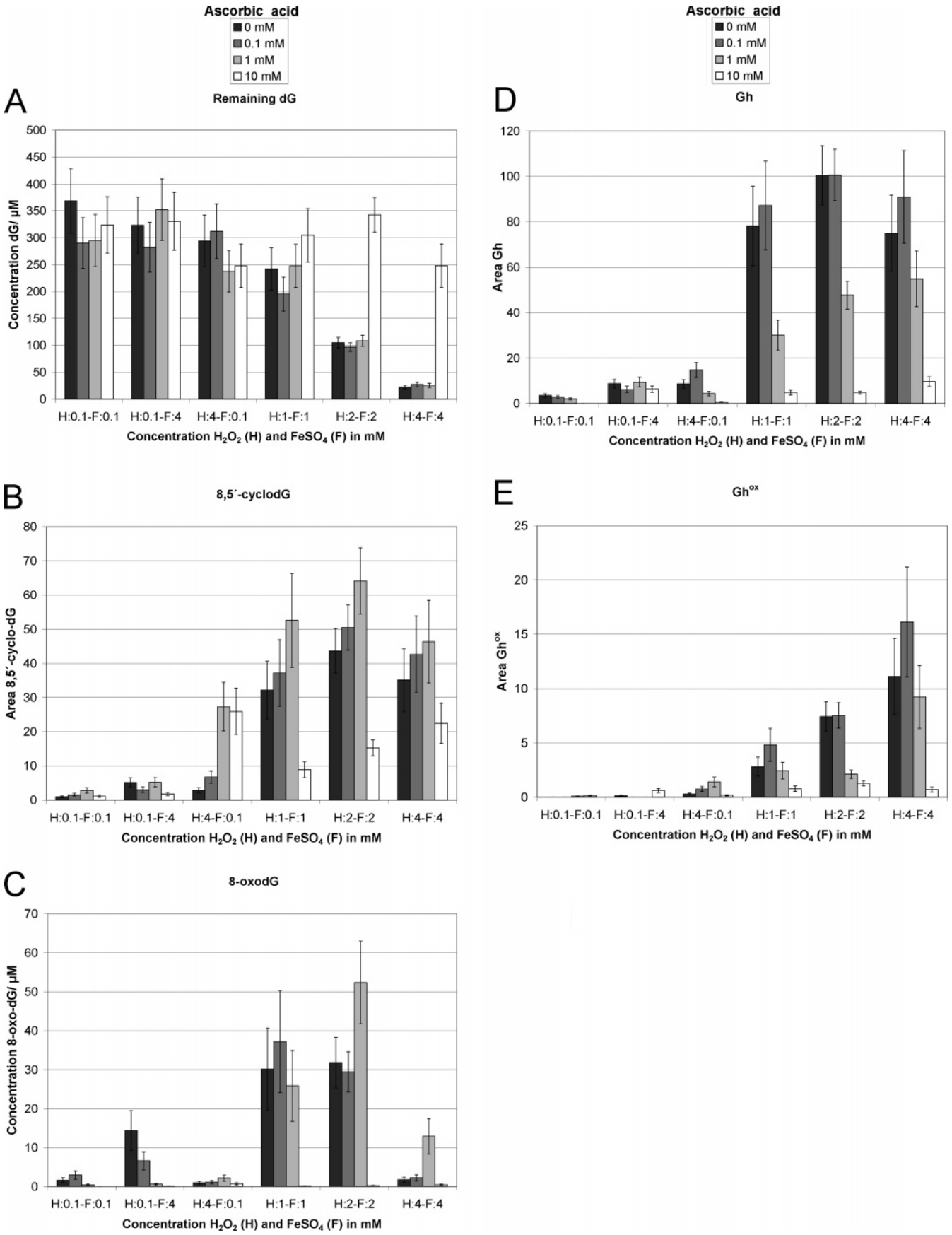


Figure 1. Effect of ascorbic acid on the amount of (**A**) remaining dG, (**B**) 8,5′-cyclodG, (**C**) 8-oxodG, (**D**) Gh, and (**E**) Gh^{ox}. The different concentrations (mM) of H_2O_2 (**H**) and $FeSO_4$ (**F**) are shown, and the concentration of ascorbic acid applied is indicated by the color in the legend. The error bars represent the calculated 95% confidential interval.

($p < 0.01$). At 10 mM, the highest concentration of ascorbic acid applied, there was almost no 8-oxodG detected at any Fenton reagent concentration.

The relative amounts of Gh, the secondary oxidation product, are shown in **Figure 1D**. Ascorbic acid resulted in basically the same effect for all samples containing more than 0.1 mM H_2O_2, that of a slight increase in Gh at 0.1 mM ascorbic acid, a significant decrease at 1 mM ($p < 0.05$), and a still greater decrease at 10 mM. The effect of $FeSO_4$ and H_2O_2 was similar to that for 8-oxodG, but the large decrease at 4 mM each was not as drastic, and an excess of iron (H:0.1-F:4) did not increase product formation as much.

The relative amounts of the further oxidized species, Gh^{ox}, are shown in **Figure 1E**. The results resemble those for Gh considerably, with two exceptions, that the amounts of Gh^{ox} increased with an increase in Fenton reagent concentrations up to 4 mM, the highest concentration applied, and that the observed amounts of Gh^{ox} at H:4-F:0.1 increased with an increase in the ascorbic acid concentration up to 1 mM ($p < 0.01$).

The concentration of the ascorbic acid that remained was found to be low in almost all the samples. The antioxidant was completely consumed, except when 10 mM of it was added, some remaining ascorbic acid being found there (data not shown).

Catechin. The effect of catechin was tested in duplicates at four different levels, ranging from 0 to 5 mM, at constant Fenton reagent concentrations of 1 and 0.1 mM. The amounts of dG preserved (**Figure 2A**) increased in the presence of 1 mM or more of catechin at H:1-F:1 ($p < 0.01$), whereas the measured concentrations of dG at H:0.1-F:0.1 did not differ significantly from the initial concentration of 400 μM ($p = 0.13$), independent of the catechin concentration.

The inhibition of the primary oxidation products 8,5′-cyclodG is shown in **Figure 2B**, its being about 50% at 1 mM catechin and 80% at 5 mM, independent of the Fenton reagent concentrations applied, but at 0.1 mM catechin, there was no significant effect ($p > 0.14$). Catechin exerted a different effect on 8-oxodG formation (**Figure 2C**), where 0.1 mM catechin increased the amount of adduct slightly ($p < 0.10$) and where a 5 mM concentration inhibited formation of the adduct markedly. Gh (**Figure 2D**) was strongly inhibited by 0.1 mM catechin at H:0.1-F:0.1 ($p < 0.01$), and when the concentration was increased further up to 5 mM, no Gh could be detected at all. A similar inhibitory effect was also found at H1:F1, but first it was detected at 1 mM and higher catechin concentrations. The inhibition of Gh^{ox} was even stronger, as shown in **Figure 2E**. Already at 0.1 mM catechin at H1:F:1, the inhibition was about 75%, and at higher concentrations (1 mM and higher), no Gh^{ox} could be detected at all. At H:0.1-F:0.1, no Gh^{ox} was detected, regardless of the catechin concentration. In most samples, the amount of unreacted catechin found after oxidation was about half the amount of catechin that was added, independent of its initial concentration (data not shown).

DISCUSSION

In evaluating antioxidative effects, the most relevant parameters to measure are the amount of the sensitive substrate of interest (lipids, proteins, nucleosides, or whatever) that has reacted and the amounts of the oxidation products produced. Since at low levels of oxidation it is difficult to measure the decrease in the concentration of the sensitive substrate accurately, it is more common there to quantify the oxidation products. However, in most cases, several oxidation products are formed, and it is difficult to quantify all of them. It is common, therefore, to measure only one oxidation product or a few of them, these serving as markers of the total oxidation level. 8-oxodG, the marker often used for monitoring oxidation of DNA and its building blocks, is clearly prone to oxidize further in vitro. The question is if this is also important for the in vivo situation. Although the probability seems very low that the same base could be oxidized twice in genomic DNA, its role could still be important here since oxidative damage has been reported to be able to migrate over long distances in DNA, so that readily oxidized lesions such as 8-oxodG can act as sinks for the trapping of electron holes (*5*). Since it could be misleading, therefore, to only use 8-oxodG, measuring other oxidative lesions and secondary oxidation products of 8-oxodG as well could provide a better estimate of the oxidation level (*7*, *23*−*25*). Since in the present study high oxidation levels were obtained, the decrease in dG concentration that occurred could be measured rather accurately, at least when the oxidation was extensive. Through the quantification of several oxidation products in parallel, it was possible to compare the effects observed, in terms of the dG level that remained, with the amounts of oxidation products obtained.

Regarding the ability of antioxidants to inhibit the oxidation of dG, these obviously are unable to suppress oxidation if the concentrations of the Fenton reagents are a magnitude higher than that of the antioxidants. The lowest concentration of H_2O_2 applied (0.1 mM) in the experiments is actually in the range of the physiological concentration reported to be found in human urine and ocular tissue (*26*). Catechin, however, appeared able to reduce or slow the continuing oxidation of 8-oxodG since the amount of Gh^{ox} was found to already be significantly ($p < 0.01$) reduced when 0.1 mM catechin was present. This could be related to catechin's chelating effect, especially if iron needs to be directly bound to 8-oxo-guanine for secondary oxidation to take place, as Colwell et al. has suggested (*27*). When the antioxidants and oxidative reagents were present in equimolar concentrations, ascorbic acid showed a pro-oxidative effect at H:0.1-F:0.1, indicated by remaining dG ($p = 0.04$), 8,5′cyclodG ($p = 0.02$), and 8-oxo-dG ($p = 0.03$) This effect was, however, not reflected in the secondary oxidation products. At H:1-F:1 and 1 mM ascorbic acid, contradicting results were observed. There was no change in the total oxidation of dG, while there was a significant increase in 8,5′cyclodG formation ($p = 0.02$). However, 8-oxodG (nonsignificant $p = 0.13$) and its secondary oxidation into Gh (significant $p < 0.01$) were inhibited, indicating a possible shift toward more sugar-based oxidation. Catechin, on the other hand, proved to be effective at H:1-F:1, clearly reducing the amounts of oxidized dG, 8,5′-cyclodG, and secondary oxidation products of 8-oxodG when present at this concentration ratio ($p < 0.01$ in all cases). At H:0.1-F:0.1, the effects were less marked. A positive effect was indicated by a decrease in 8′5-cyclo-dG ($p = 0.14$) and in a significant reduction in the levels of the secondary oxidation products ($p < 0.01$). In contrast, the amount of 8-oxo-dG was significantly increased ($p = 0.02$), thus indicating a pro-oxidative effect of catechin. Ascorbic acid and catechin, when present at 10 or at 5 times higher antioxidant than Fenton reagent concentrations, respectively, repressed most of the oxidation, as could be expected. It can thus be seen, when looking at the results from all four oxidation products and remaining dG, that catechin proved to be the better antioxidant, in protecting dG from iron-mediated Fenton oxidation. In the study, ascorbic acid was present mainly as acorbic acid (not ascorbate), due to the acidity of the iron mediated Fenton reaction. The pH varied between

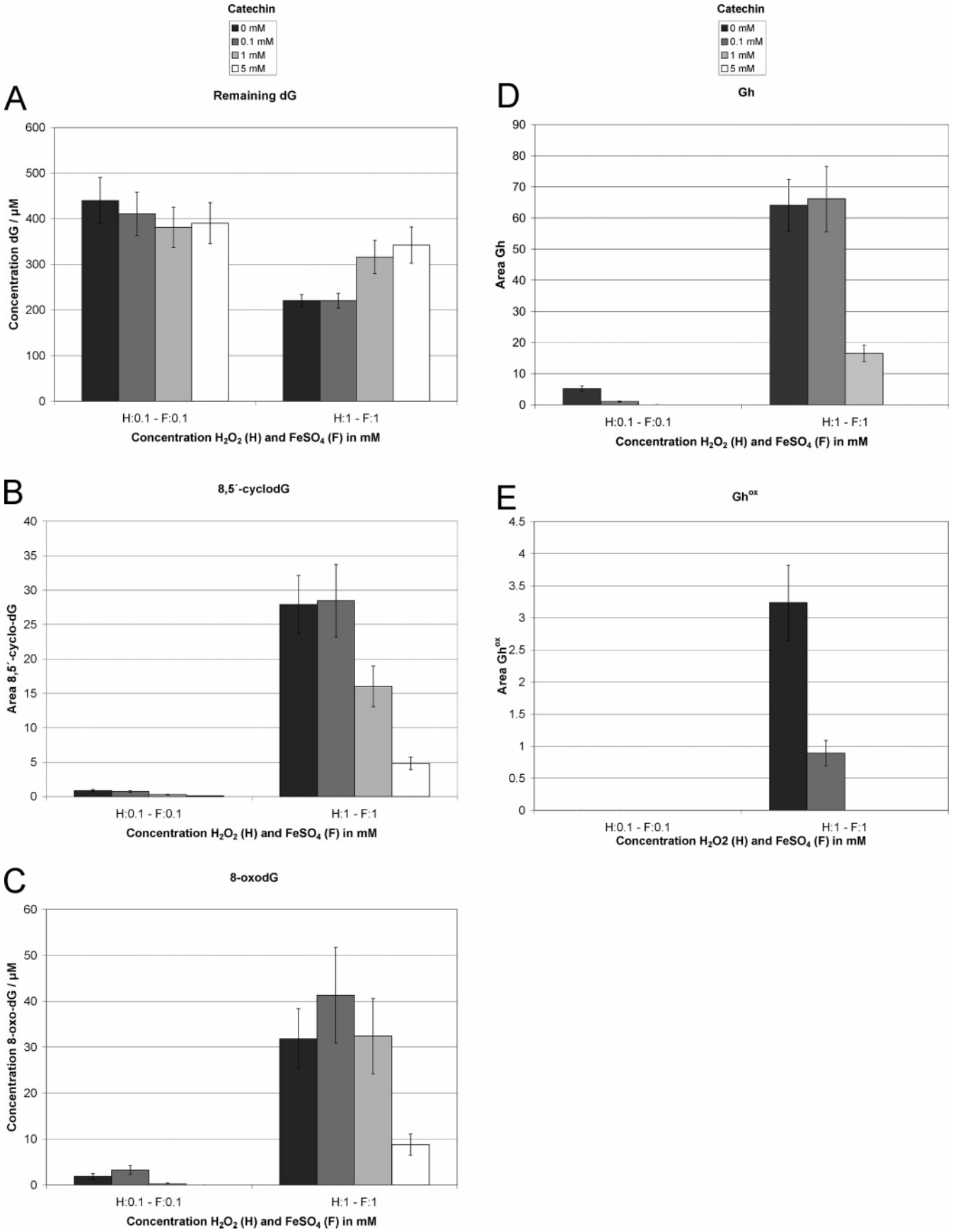


Figure 2. Effect of (+)catechin on the amount of (**A**) Remaining dG, (**B**) 8,5′-cyclodG, (**C**) 8-oxodG, (**D**) Gh, and (**E**) Gh^{ox}. The different concentrations (mM) of H_2O_2 (**H**) and $FeSO_4$ (**F**) are shown, and the concentration of (+)catechin applied is indicated by the color in the legend. The error bars represent the calculated 95% confidential interval.

2.6 and 4 at H:4-F:4 and H:01-F01, respectively. Ascorbic acid (10 mM) itself lowered the pH to 3 at H:01-F:01, but at higher Fenton reagent concentrations, its influence on the pH was rather small. We chose to avoid the use of buffer salts since those would have interfered in various ways, for example, by interacting with metal ions and reacting with reactive radicals.

Although ascorbic acid is known to be highly effective in scavenging radicals, its antioxidative mechanism may not be particularly effective in the present case. Since hydroxyl radicals, because of their high degree of reactivity, react with most organic substances at will, the place where they are formed is of considerable importance. If metal ions are associated with the phosphate backbone of DNA or with the imidazole group of guanine, hydroxyl radicals are generated in close proximity to the oxidation-sensitive substrate. Thus, to scavenge them before they cause damage, the radical scavenger in question needs to be present in high concentrations. In our study, 10 mM ascorbic acid was needed to achieve effective protection. The intracellular concentration of ascorbic acid is reported to be somewhere between 1 and 5 mM (*28*), which was not enough under our conditions. In fact, there exists no compelling evidence that the addition of ascorbic acid decreases the levels of oxidative DNA damage in vivo, except perhaps when there is a deficiency in ascorbic acid (*13*). Most of the studies showed a null result. However, it is interesting that the few studies indicating a positive effect used 8-oxodG as an oxidative marker (*29*). Antioxidants that are metal chelators can remove metal ions from oxidation-sensitive substrates and thus increase the chances for scavenging of the hydroxyl radicals before they cause damage. Catechin is an iron chelator strong enough to remove iron from the Fe^{2+}-EDTA complex. It also chelates Cu^{2+} readily, but not Fe^{3+} (*16*, *17*). Also, if the metal chelator works as an effective radical scavenger, such as it does in the case of catechin, many radicals can be trapped immediately after being formed, due to the distance between the scavenger and the radical formation site being so short. The presence of catechin in combination with ascorbic acid could be beneficial, due in part to ascorbic acid being able to regenerate oxidized catechin (*30*). The oxidized form of catechin might also be able to act as an antioxidant in itself. This was observed in epicatechin, an enantiomer of catechin, when it was used to inhibit lipid peroxidation (*15*).

The results of the present study clearly indicate that the chelating ability rather than the scavenging property alone plays a major role in the observed antioxidative efficiency of catechin. To more adequately assess its effectiveness as an antioxidant in vivo, its bioavailability and the antioxidant potential of its metabolites needs also to be considered. Several studies show that catechin can undergo significant metabolism and is actually mostly present as metabolites in vivo (*31*). In the small intestine, mostly glucuronide conjugates and O-methylated forms are formed, and in the colon, the microflora degrade catechin into phenolic acids, which may be absorbed (*32*). The fact that catechin shows a positive effect, already at relatively low concentrations of both antioxidant and oxidants, indicates that it could have a beneficial effect in vivo by decreasing the DNA damage caused by radicals formed in metal-ion catalyzed reactions.

Although the strong reducing potential of ascorbic acid is essential for its antioxidant characteristics, this also makes it pro-oxidative under certain conditions, through the reduction and release of metal ions (*33*). The overall effect of ascorbic acid is highly dependent on the concentration of both ascorbic acid as well as Fe^{2+}. If the ascorbic acid/iron ratio is low, ascorbic acid is known to act as a pro-oxidant, but when this ratio is higher, the antioxidant effect dominates (*34*). This double nature can clearly be seen in the results presented here. A strong antioxidant effect was observed in mixtures containing 2 or 4 mM Fenton reagents and 10 mM ascorbic acid since this increases the concentration of the dG that remains and decreases the amounts of the oxidation products. In the reaction mixtures containing 1 mM ascorbic acid, the same antioxidative effect was observed for the oxidation products, but the change in the amount of dG that remained was less. In addition, ascorbic acid was found to have a pro-oxidative effect at low Fe^{2+}-concentrations (0.1 mM). The concentration of remaining dG in the samples containing H:0.1-F:0.1 decreased due to the presence of ascorbic acid, and a similar trend was observed in the H:4-F:0.1 samples, except for the case when 0.1 mM ascorbic acid was employed. At equimolar Fenton reagent concentrations of 1 mM or higher, the crossover between antioxidative and pro-oxidative behavior of ascorbic acid was somewhere in the region of 1−10 mM, according to the 8,5′-cyclodG results, although this pro-oxidative effect was not reflected in any of the remaining dG results. The pro-oxidative effect that was observed was probably based on the fact that the scavenging property of ascorbic acid had an ancillary role as compared to its ability to reduce Fe^{3+} to Fe^{2+}, supporting the Fenton reaction. Nevertheless, in vivo studies have not shown ascorbic acid to have any pro-oxidative tendency, even at high iron loadings (*14*, *35*, *36*). Catechin has also been reported to sometimes have pro-oxidative effects through the reduction of metal ions (*37*, *38*). It has been shown to be able to reduce Cu^{2+} but not Fe^{3+} (*16*, *17*). Accordingly, catechin should be unable to enhance the iron-mediated Fenton reaction, as the data presented also showed.

During the oxidation reaction, the decrease in the dG that remained was accompanied by an increase in some of the oxidation products, although the pattern was rather complicated. 8,5′-cyclodG correctly indicated the pro-oxidative effects of a high concentration of ascorbic acid in H:4-F:0.1 and of 1 mM ascorbic acid in H:0.1-F:0.1. The corresponding effects on the concentration of 8-oxodG were either much smaller or nonexistent. Also, the Gh and Gh^{ox} concentrations indicated an antioxidative effect above 1 mM ascorbic acid in H:4-F:0.1, which contradicts the measured levels of 8,5′-cyclodG and remaining dG. In the reaction mixture H:0.1-F:4, the amount of 8-oxodG decreased strongly when the concentration of ascorbic acid increased. The corresponding trend was not observed in the other oxidation products. This effect was probably caused by ascorbic acid, either through its enhancing the further oxidation of 8-oxodG to a greater extent than the oxidation of dG or perhaps through its altering the oxidation pattern towards more sugar based adducts, this leading to an overall reduction in the observed 8-oxodG concentration. Other contradicting results were observed for 8-oxodG in H:4-F:4 and 1 mM ascorbic acid and also in H:0.1-F:01 and H:1-F:1 containing 0.1 mM catechin. An apparent pro-oxidative effect was seen there. The effect was caused by the antioxidative effect of ascorbic acid and catechin, by decreasing the secondary oxidation of 8-oxodG. A similar apparent pro-oxidative effect was observed by Suzuki et al., who also detected an increase in 8-oxodG formation when a related antioxidant, epigallocatechin, was used in an oxidation assay in which dG and hypochlorous acid were employed (*23*).

The lack of correlation being found between the amounts of the different oxidation products that were produced is probably due to the complexity of the kinetics involved in the formation of primary oxidation products and their possible further oxida-

tion. Since results based on different oxidation products can lead to contradictory conclusions, one should be careful in interpreting pro- and anti-oxidative effects by the monitoring of a single oxidation product. More reliable conclusions can be obtained by monitoring several different oxidation products simultaneously. If one is limited to the measurement of only a single oxidation product, one should be chosen that is not particularly prone to further oxidation. 8,5′-cyclodG appears to be a good alternative, or one that at least is much better than the 8-oxodG that is frequently employed. Although the biological importance of 8,5′-cyclodG is still largely unknown, it has been suggested that cyclic purines accumulate in DNA repair-deficient cells and inhibit gene expression and DNA-polymerases with possible pathological consequences (*8*, *9*).

ABBREVIATIONS USED

dG, 2′-deoxyguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-5′-cyclodG, 8,5′-cyclo-2′-deoxyguanosine; Gh, guanidinohydantoin; Gh^{ox}, dehydro-guanidinohydantoin; ROS, reactive oxygen species; H, hydrogen peroxide concentration in mM; F, iron(II) sulfate concentration in mM.

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