The Protective Role of Different Green Tea Extracts after Oxidative Damage Is Related to Their Catechin Composition

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The antioxidant activities of three different green tea extracts were investigated and compared by two different methods. By the first method, which evaluated the direct protective effect of the green tea extracts on lipid peroxidation, the extracts were added, at different concentrations, to a lipid model system, made by refined peanut oil, freshly submitted to a further bleaching and subjected to forced oxidation at 98 °C, by an oxidative stability instrument. By the second method, the effectiveness of the same extracts was checked in cultures of neonatal rat cardiomyocytes exposed to a free radical-generating system by evaluating conjugated diene production and lactate dehydrogenase release. All of the extracts revealed a strong antioxidant activity by both the methods, and a particular effectiveness was demonstrated by the extracts having higher amounts of (−)-epigallocathechin-3-gallate and (−)-epigallocathechin, as analyzed by reverse-phase HPLC analysis.

Keywords: *Green tea extracts; catechins; oxidative damage; lipid oxidation; cardiomyocytes*

INTRODUCTION

Free radical intermediates contribute to a wide range of diseases (Halliwell, 1987; Kritchewsky, 1992), including atherosclerosis, emphysema, ulcerative colitis, diabetes, multiple sclerosis, rheumatoid arthritis, Parkinson's disease, and cancer. Tissue damage induced by radiation exposure, xenobiotics, and ischemia/reperfusion is also a direct result of free radical-mediated toxicity.

The prevention of oxidative damage has been the subject of considerable investigation. The ultimate goal is to develop chemical and dietary intervention methods to attenuate or prevent the resulting pathological state of free radical damage.

Antioxidants are substances that are capable of inhibiting oxidation and are classified into two categories: the preventive inhibitors (i.e., superoxide dismutase) and the free radical chain-breaking antioxidants (i.e., ascorbate, α-tocopherol, *β*-carotene, polyphenols, etc.).

The average diet contains a great number of compounds with antioxidant activity: natural and synthetic phenols, furanones/furfurals, and polyfunctional acids (Bonorden and Pariza, 1994). Polyphenols are plant metabolites occurring widely in plant foods (Harbone, 1989); they possess outstanding antioxidant and free radical-scavenging properties, suggesting a possible protective role in man (Scott et al., 1993).

Green tea is an excellent source of polyphenol antioxidants, particularly of a group of polyphenol compounds known as green tea catechins (GTCs) (Zhu et al., 1997). Green tea refers to a nonfermented product in which GTCs are more preserved than in partially fermented (oolong or paochong tea) or fully fermented teas (black or pu-erh tea). The content of the different GTC isomers could vary among different green teas, depending on the species, the climate, the cultural practices, and, in the case of green tea extracts (GTEs), the condition and the technology used for the extraction and the conservation. Therefore, the antioxidant ability of different GTEs could vary according to their GTCs' quali-quantitative composition.

In this light, the purpose of this study was to evaluate the ability of different GTEs in reducing free radical-mediated damage in comparison to well-known antioxidant agents, using two different experimental model systems. The first one was a method in which the antioxidant ability of the different GTEs was tested by subjecting to forced dynamic oxidation by an oxidative stability instrument (OSI) a lipid model system, in the absence or in the presence of GTEs at different concentrations (0.02−1% w/w) or butylated hydroxytoluene (BHT), a common synthetic antioxidant food additive. This method allows one to evaluate the antioxidant ability of the different GTEs added either to lipid model systems or to lipid-containing food. The second experimental model system was an in vitro method in which primary cultures of neonatal rat cardiomyocytes were exposed to a free radical-generating system (FRGS) catalyzed by xanthine oxidase. Cardiomyocytes were supplemented with different GTEs and α-tocopherol (αTC), utilized as an agent with known protective effect in the myocardial cells (Massey and Burton, 1990), and the protective effect of the different supplements against free radical damage was evaluated by measuring the conjugated diene production and the lactate dehydrogenase (LDH) release from cell cultures.

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The results obtained with the two experimental methods were then compared to verify whether the different GTEs had the same efficiency as antioxidants in an edible oil and in a living cell.

Finally, GTEs were analyzed by reverse phase HPLC, and their qualitative and quantitative composition was correlated to their antioxidant ability.

MATERIALS AND METHODS

Materials. GTEs were a kind gift of Di Minno D.&C. S.r.l. (Milano, Italy) and Indena (Milano, Italy). GTE 1 was defined by the producer as an aqueous spray-dried extract containing caffeine (identified by TLC); GTE 2 was defined by the producer as having a polyphenol content >60% (w/w), an (−) − epigallocatechin-3-gallate (EGCG) content >40% (w/w), and a caffeine content <0.1% (w/w) (by HPLC); GTE 3 was defined by the producer as having a polyphenol content of 75 ± 5% (w/w), an EGCG content of 30 ± 5% (w/w), a content of other catechins of 40 ± 10% (w/w), and a caffeine content <8% (w/w) (by HPLC). The refined peanut oil was purchased from a local market.

Gallic acid monohydrate (GA), minimum 98% purity (HPLC), (−)-epigallocatechin (EGC), minimum 98% purity (HPLC), (+)-catechin (C), minimum 98% purity, EGCG, minimum 95% purity, (−)-epicatechin (EC), minimum 98% purity (HPLC), (−)-gallocatechin gallate (GCG), minimum 98% purity (HPLC), (−)-epicatechin gallate (ECG), minimum 98% purity (HPLC), anhydrous caffeine (CAF) USP grade, horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade methanol, formic acid, and BHT were from Carlo Erba (Milano, Italy), and acetonitrile was from Prolabo (Paris, France). All of the other chemicals and solvents were of the highest analytical grade.

Methods. *HPLC Analysis of Green Tea Extracts.* The HPLC analyses were performed on a Jasco model LC 1600 (Tokyo, Japan), equipped with a binary pump delivery system model PU1580, a Rheodyne injection valve model; 7125 (Cotati, CA), with a loop of 20 μL capacity, and a Jasco diode-array UV−vis detector model MD1510. Integration and data elaboration were performed by Borwin Chromatography software, version 1.50. A Luna 5 μm C18 column (Phenomenex, Torrance, CA) was used. A linear gradient elution was carried out using the following solvent systems: mobile phase A, double-distilled water/methanol/formic acid (74.7:25:0.3, v/v); mobile phase B, acetonitrile/formic acid (99.7:0.3, v/v). The linear gradient of eluents A and B was formed as follows: 100% A for 8 min, 0−100% B from 8 to 33 min (at a rate of 4% B per min); 100% B was then maintained for a further 5 min. The flow rate was 1.0 mL/min. The green tea extracts were dissolved in distilled water/formic acid (99.7:0.3, v/v; 1 mg/mL) and sonicated for 10 min at 30 °C at 50−60 Hz by an Elma Transsonic T310 sonicator (Singen, Germany). Twenty microliters of each GTE solution was injected after filtration through a 0.45 μm filter disk (Millipore, Bedford, MA), and catechins were revealed at 270 nm. Identification of catechins, gallic acid, and caffeine was performed by comparison of the retention times of the unknown peaks to reference standards. A standard mixture made by GA (0.014 mg/mL), EGC (0.038 mg/mL), C (0.070 mg/mL), EGCG (0.014 mg/mL), EC (0.070 mg/mL), GCG (0.014 mg/mL), ECG (0.014 mg/mL), and CAF (0.038 mg/mL) in double-distilled water/formic acid (99.7:0.3, v/v) was prepared, as previously described for the GTE solutions, and analyzed. Calibration curves were made for GA ($R^2 = 0.999$), EGC ($R^2 = 0.996$), C ($R^2 = 0.999$), EGCG ($R^2 = 0.999$), EC ($R^2 = 0.999$), GCG ($R^2 = 0.998$), ECG ($R^2 = 0.997$), and caffeine ($R^2 = 0.999$) using standard solutions.

Forced Oxidation of a Lipid Model System by OSI. The lipid model system was prepared from a refined peanut oil, purchased from a local market. After the addition of 10% (w/w) bleaching earth (previously activated at 110 °C for 18 h), the oil was kept for 15 min at 80 °C under stirring and weak vacuum in a Rotavapor and filtered.

The OSI time of the lipid model system was checked on 5 ± 0.1 g of bleached oil ($n = 16$) by an eight-channel OSI instrument (Omnion Inc., Decatur, IL) at 98 °C with a stream of air bubbling through the oil of 120 mL/min (Jebe et al., 1993). The different GTEs, solubilized in 150 μL of ethanol, were added (0.02−1% w/w) to the freshly bleached peanut oil, and the OSI times were measured as described above. BHT, solubilized in 150 μL of ethanol, was used as standard antioxidant and was added to the oil at concentrations of 0.02 and 0.04% (w/w). Ethanol up to 3% (v/w) did not show any significant effect on the OSI time.

Analyses were performed in triplicate, and data are reported as means ± SE.

Cell Cultures and in Vitro Peroxidation. Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2−4-day-old Wistar rats, as previously reported (Bordoni et al., 1991). Cells were grown in Petri dishes until confluence in nutrient mixture Ham F10 supplemented with 10% v/v FCS and 10% v/v HS. At confluence, cardiomyocytes were supplemented with 20 μM αTC or 10 or 50 μg/mL of the different acqueous solutions of GTEs. Twenty-four hours later, cultures were exposed to the FRGS for 1 h. In the FRGS, containing 2.3 mM purine (7*H*-imidazo[4,5-*d*]pyrimidine), 2.4 μM Fe^{3+} loaded transferrin and 0.01 unit/mL xanthine oxidase, superoxide and hydroxyl radicals were generated, as reported by Burton et al. (1984). Appropriate control groups were processed in the same way in the absence of αTC and GTEs and/or FRGS.

Cells were washed three times with Ham F10 medium supplemented with 10% HS and 10% FCS and two times with PBS. All of the cells were scraped off in ice-cold methanol, and the appearance of conjugated diene-containing lipids was evaluated as an index of lipid peroxidation using the method of Burton et al. (1990). Briefly, cells, scraped from the culture plates, were extracted in chloroform/methanol/water (2:1:1, v/v). The chloroform layers from two extractions were combined and then dried under nitrogen. Samples were resuspended in a known volume of acetonitrile, and absorbance was determined at 235 nm.

Lactate dehydrogenase (LDH) release was monitored before cell scraping by collecting aliquots of medium from each dish; these aliquots were then analyzed spectrophotometrically for LDH activity by measuring NADH levels at 340 nm (Korzeniewski and Callewaert, 1983).

Data are means ± SD of five different cultures. Statistical differences were evaluated using Student's *t* test.

RESULTS

HPLC Analysis of GTEs. HPLC analysis was performed to determine the catechin composition of the three GTEs. The total catechin contents were 93.0 ± 3.73, 697.5 ± 16.56, and 757.6 ± 12.34 mg/g of GTE for GTEs 1, 2, and 3, respectively. Figure 1 represents the composition in single catechins of the three GTEs. GTE 1, revealing the lowest total catechin content, is clearly an unconcentrated extract. EGCG and ECG were the most representative catechins in GTEs 2 and 3, EGCG being higher in GTE 2 than in GTE 3 and ECG being higher in GTE 3 than in GTE 2. Furthermore, GTE 3 was the only extract with a high level of GCG, whereas GTE 2 revealed a higher level of EGC than GTEs 1 and 3. Caffeine was not detected in GTE 2.

Peroxidation in a Food Model. The antioxidant activity of the three different GTEs was tested by measuring the OSI time of a freshly bleached peanut oil supplemented with increasing concentrations of GTEs (0.02−1% w/w). The model system, freshly prepared to avoid oxidation, presented an OSI time value of 14.4 ± 0.75 h (coefficient of variation = 3.9%).

Table 1 shows the OSI times of the lipid model system supplemented with the three different GTEs or with

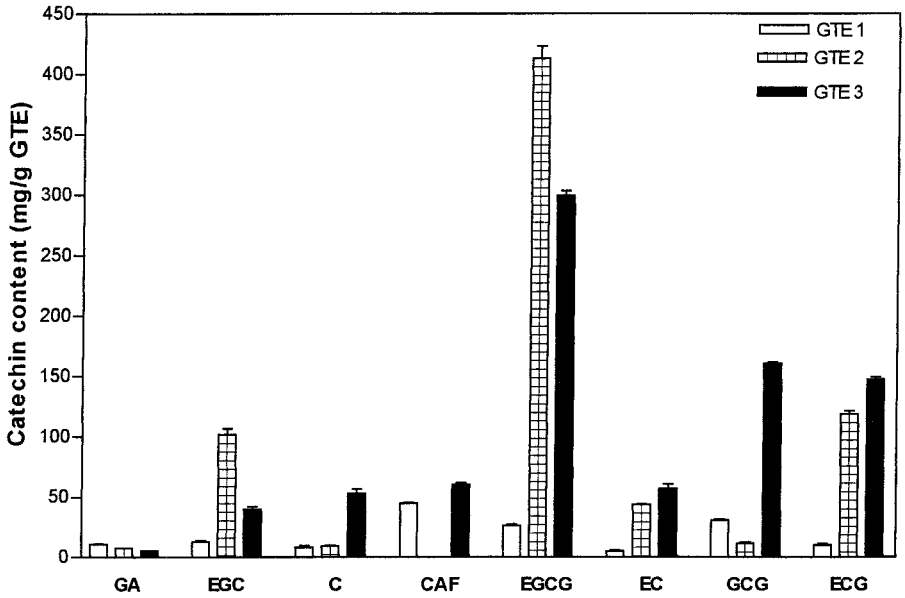

Figure 1. Catechin composition of the different GTEs. Catechin composition analysis was performed by reverse phase HPLC as reported under Methods. Values are reported as milligrams per gram of dry GTE and are means ± SE of four analyses.

Table 1. Antioxidant Activity, Measured as OSI Time, of the Three Different GTEs Supplemented to a Lipid Model System[a]

% of antioxidant (w/w)	OSI time (h)			
	BHT	GTE 1	GTE 2	GTE 3
0 ($n = 16$)	14.4 ± 0.75	14.4 ± 0.75	14.4 ± 0.75	14.4 ± 0.75
0.02 ($n = 3$)	23.22 ± 0.34	16.63 ± 1.10a	33.45 ± 2.55a	64.10 ± 4.35ab
0.04 ($n = 3$)	27.37 ± 0.95	19.45 ± 3.15a	58.55 ± 5.65a	86.30 ± 3.55ab
0.5 ($n = 3$)		36.30 ± 2.93	120.75 ± 4.50	116.25 ± 13.70
1 ($n = 3$)		44.00 ± 3.05	140.80 ± 9.25	157.40 ± 4.80

[a] The antioxidant activity of the different GTEs was tested by measuring the OSI time as reported under Methods. BHT was used to compare GTE effectiveness to a well-known antioxidant molecule. Data are means ± SE for the number of analyses reported in parentheses. Statistical analysis, performed with the Student's *t* test, revealed significant differences in comparison to BHT supplementation at the same concentration (a, at least $p < 0.05$). Comparison between GTEs 2 and 3 by the Student's *t* test revealed significant differences only at 0.02 and 0.04% w/w supplementation (b, at least $p < 0.05$).

BHT. BHT was used as a standard of antioxidant activity; it was added at 0.02 and 0.04% w/w because higher percentages are generally not allowed for human food and food ingredients (Giese, 1996; Hamama and Nawar, 1991).

Apart from GTE 1, the other extracts, just at the lowest concentration (0.02% w/w), exhibited an antioxidant activity higher than BHT. The OSI times of the oil supplemented with 0.04% w/w GTEs 2 and 3 were ~2 and ~3 times higher than that of BHT and ~4 and ~6 times higher than in the unprotected lipid model system, respectively. At these concentrations, GTE 1 demonstrated only a weak protective effect. At GTE concentrations ≥0.5% w/w the protective effects of GTEs 2 and 3 were comparable (no statistical differences in the OSI time values) and the OSI times were 8−10 times higher than that of the pure model system. On the other hand, the antioxidant effect of GTE 1 appeared to be weaker than those of the other two GTEs at all of the concentrations used. A significant linear correlation was found between the concentration of total catechins added to the model system and the corresponding OSI times (GTE 1, $r = 0.974$, $p < 0.01$; GTE 2, $r = 0.930$, $p < 0.02$; GTE 3, $r = 0.886$, $p < 0.05$). Similar correlations were also found between individual catechin concentrations and the corresponding OSI times (data not shown).

In Vitro Peroxidation. Conjugated diene levels are reported in Figure 2 as the fold increase in conjugated diene production in nonsupplemented and antioxidant-supplemented cell cultures exposed to the FRGS, in comparison to their respective counterparts not exposed to the FRGS. No differences in diene content were found between different groups when cardiomyocytes were not exposed to the oxidative stress ($A_{235nm} = 0.067 \pm 0.003$). Exposure to the FRGS caused a 4-fold increase in conjugated diene production in cardiomyocytes not supplemented with antioxidants. When cardiomyocytes were supplemented with αTC, a significant attenuation of conjugated diene formation was detected; conjugated diene production was only slightly increased with respect to control cells not exposed to FRGS and highly significantly lower than in cells exposed to FRGS without the protection of antioxidants. Supplementation with the different GTEs revealed the ability of these natural antioxidants in protecting cardiomyocytes against peroxidative damage, although to different extents. The protective effect of GTEs was dependent on the concentration used, and it was higher using GTE 3 than GTEs 2 and 1.

The release of LDH from unsupplemented and antioxidant-supplemented cardiomyocytes exposed to the FRGS is reported in Figure 3 as the fold increase in comparison to their counterparts not exposed to the oxidative stress. No differences in LDH release were detected in cultures not exposed to the FRGS (37.5 ± 2.5 units/mL). A significant LDH release was demonstrated in cardiomyocytes exposed to the oxidative stress in the absence of any antioxidants. In cardiomyocytes

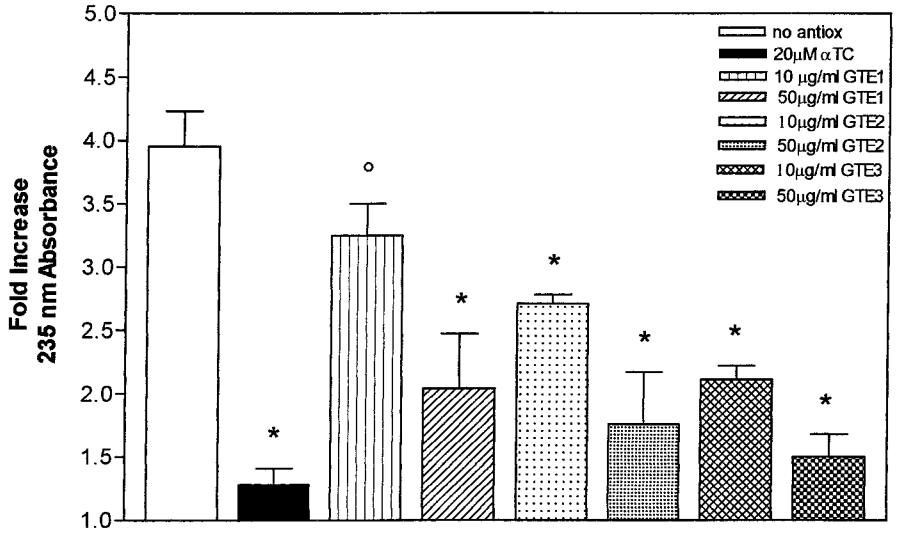

Figure 2. Fold increase in conjugated diene content of neonatal rat cardiomyocytes exposed to the free radical-generating system for 1 h. Conjugated diene production was measured as 235 nm absorbance as reported under Methods. The value of 235 nm absorbance of cells not exposed to FRGS was 0.067 ± 0.003, independent of the presence of antioxidants. Fold increase was calculated as the ratio between the absorbance in cells exposed and not exposed. Data are means ± SD of five different cell cultures. Statistical analysis was performed with Student's *t* test comparing cardiomyocytes treated and nontreated with the different antioxidants and exposed to FRGS (○, $p < 0.01$; ∗, $p < 0.001$). In comparison to the corresponding non-FRGS-exposed cells, statistical analysis revealed a significant increase in conjugated diene production: $p < 0.05$ in αTC and 50 μg/mL GTE 3 treated cells; $p < 0.01$ in 10 μg/mL GTE 3 and 50 μg/mL GTE 1 and 2 treated cells; $p < 0.001$ in nontreated and in 10 μg/mL GTE 1 and 2 treated cells.

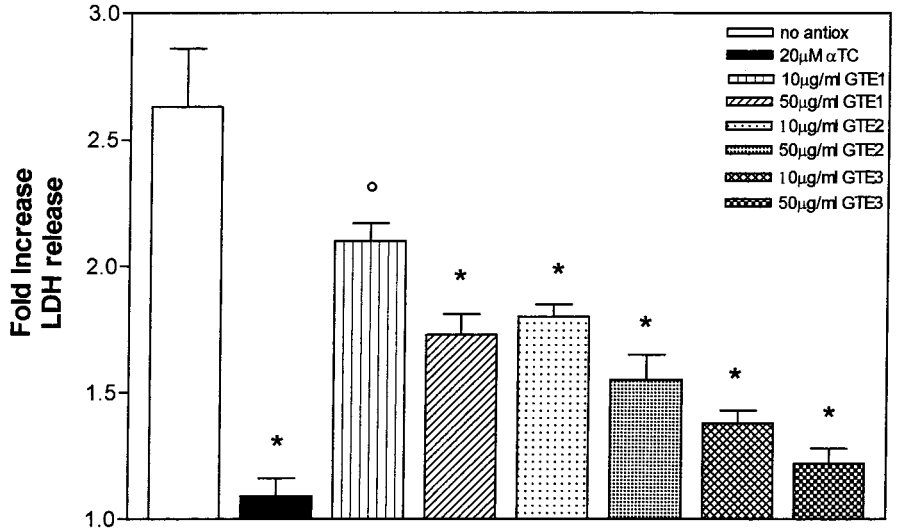

Figure 3. Fold increase in LDH release in the culture medium of neonatal rat cardiomyocytes exposed to the free radical-generating system for 1 h. LDH release was measured on aliquots of culture media as reported under Methods. The LDH release of cells not exposed to FRGS was 37.5 ± 2.5 units/mL, independent of the presence of antioxidants. Fold increase was calculated as the ratio between the LDH units in cells exposed and not exposed to FRGS. Data are means ± SD of five different cell cultures. Statistical analysis was performed with Student's *t* test comparing cardiomyocytes treated and nontreated with the different antioxidants and exposed to FRGS (○, $p < 0.01$; ∗, $p < 0.001$). In comparison to the corresponding non-FRGS-exposed cells, statistical analysis revealed a significant increase in LDH release apart from cells treated with αTC: $p < 0.05$ in 50 μg/mL GTE 3; $p < 0.01$ in 10 μg/mL GTE 3 and 50 μg/mL GTE 2 treated cells; $p < 0.001$ in nontreated 10 μg/mL GTE 1 and 2 and 50 μg/mL GTE 1.

supplemented with αTC, LDH release was similar to that of cells not exposed to the oxidative stress. Protection was also achieved by the addition of GTEs; in agreement with data on conjugated diene production, GTE 3 demonstrated the highest protective effect: when it was supplemented at 50 μg/mL, LDH release was similar to that obtained in the presence of αTC.

DISCUSSION

Normal human diet supplies a significant amount of compounds with antioxidant activity. Although the assimilation, metabolic fate, and toxicity of most of these compounds are not well-defined yet, their constant consumption warrants the consideration of their physiological role as antioxidants, especially when they are as effective as well-known antioxidants such as αTC.

In the in vitro model system we tested the protective effect of the different GTEs against the peroxidative damage induced by the exposure for 1 h to an FRGS. A similar generating system was previously reported to yield superoxide generation at levels comparable to that of in vivo production by stimulated neutrophils (Tate

et al., 1982). Moreover, hydroxyl radicals were generated in this system by the addition of iron-loaded transferrin according to a modified Harber−Weiss or Fenton reaction (Burton et al., 1984). The three GTEs were used at two different concentrations (10 and 50 μg/mL), and their protective effects were compared to those achieved by the addition of 20 μM αTC to the culture medium. The 20 μM dose used in this study has been reported to increase cellular αTC content ∼2−3-fold (Massey and Burton, 1990**)** and to reduce membrane lipid alteration, to enhance the recovery of contractile function, and to reduce the accumulation of calcium in isolated rat hearts exposed to global ischemia and reperfusion (Massey and Burton, 1989).

The exposure to FRGS of cardiomyocytes not supplemented with antioxidants caused a significant increase in conjugated diene production, used as an index of lipid peroxidation. The addition of 20 μM αTC protected almost completely cardiomyocytes from lipid peroxidation, in agreement with the findings of Massey and Burton (1990). All of the GTEs tested exerted a protection against peroxidative damage induced by FRGS, but to different extents, GTE 3 being more protective than GTEs 2 and 1. The protection by GTEs appeared to be dose-dependent, and 50 μg/mL GTE 3 had effects similar to those of 20 μM αTC. The release of LDH, utilized as an index of cell damage, showed a very high increase in this intracellular enzyme release in cells treated with FRGS in the absence of any antioxidants with respect to control cells. All antioxidants reduced LDH release, and even in this case, αTC and GTE 3 showed the highest protective effect.

Data obtained by the model system are in agreement with the in vitro data. In fact, GTE 3 revealed the highest antioxidant activity at low concentrations (0.02−0.04% w/w). In the model system, the protective effectiveness levels of GTEs 2 and 3 were maximal and comparable at the highest concentrations used (0.5−1% w/w).

It is known that different GTEs contain different amounts of GTCs, which are phenolic antioxidants. Their antioxidant activity is reported to be the direct result of the phenol acting as either a chain-breaking antioxidant (free radical scavenger), an inhibitor of singlet oxygen formation, a quencher of singlet oxygen, or an inactivator of prooxidant metals (Pokorny, 1987). As a general statement, the antioxidant activity of phenols increases as the number of hydroxyl groups increases; increasing the degree of alkyl substituents also enhances phenolic antioxidant activity (Pokorny, 1987).

As evidenced by HPLC analysis, GTEs tested in this study showed not only a different total catechin content but also significant differences in their qualitative-quantitative composition.

The weak antioxidant activity of GTE 1 appears to be related to its low total catechin content; otherwise, because GTEs 2 and 3 have similar total catechin contents, their different antioxidant effectiveness levels may be related to differences in single catechin content. Many studies have related the antioxidant activity of the different catechins to their chemical structure (Das and Pereira, 1990; Shahidi and Wanasundara, 1992). Salah et al. (1995) studied the antioxidant effectiveness of a series of polyphenols in both an aqueous and a lipid-based system; in the aqueous system, the antioxidant effectiveness was ECG > EGCG > GA > EC > C, whereas in the lipid system, it was ECG = EGCG = EC = C > EGC > GA. According to these data, the antioxidant activity of GTEs 2 and 3 could be related to their high contents of ECG and EGCG, which are the major polyphenol components of both GTEs. The higher antioxidant effectiveness of GTE 3 relative to GTE 2 could be tentatively related to its higher contents in C, EC, and GCG. Alternatively, because the antioxidant activity of the GTEs is probably due to the synergistic effect of all the components, the higher activity of GTE 3 could not be related to the highest content of a single catechin but to a most advantageous overall distribution of all components. Similar conclusions were reported by Halder and Bhaduri (1998) in human red blood cells in which black tea extracts seemed to be a better protective agent against various types of oxidative stress than single free catechins. Further studies based on the supplementation of the single catechins are needed to clarify this point.

The results obtained in the two different experimental systems used to investigate the protective effect of GTEs lead to similar conclusions, allowing one to determine an order of effectiveness for the different GTEs that is valid not only when GTEs are added to food but also in the living cell.

The evidence that dietary micronutrients protect against oxidative tissue damage is extensive. However, many important questions should be addressed before nutritional supplementation or food fortification is recommended as a chronic disease prevention strategy for the general public. Among primary concerns are the real protective effect of food antioxidants compared to those of well-defined antioxidants such as BHT or αTC and antioxidant organ specificity.

On the basis of our results, GTE 3 presents a protective effect higher than that of BHT in the model system and similar to that of αTC in vitro and could therefore be used in the protection of both unsaturated lipid rich food and living cells.

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