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Size Effect of Se-Enriched Green Tea Particles on in Vitro Antioxidant and Antitumor Activities

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The antioxidant and antitumor activities (in vitro) of superfine regular and Se-enriched green tea particles with different sizes (3.52 μm and 220 nm) were investigated in this paper. The vitamin C and tea polyphenol contents of green tea in different sizes were significantly different, and amino acid and chlorophyll just changed a little. The antioxidant activity of green tea particles was evaluated by DPPH radical scavenging and linoleic acid peroxidation inhibition methods, and the antitumor activity was evaluated by antiproliferation assay on HepG2, A549, and MGC803 cells. The results indicated that enrichment of selenium endowed green tea with higher antioxidant activity and antitumor activity on HepG2 and A549 cells but not on MGC803 cells. The DPPH radical scavenging rates of regular and Se-enriched green tea of 220 nm (67.87% and 69.49%, respectively) were significantly greater than that of 3.52 μm, but the inhibition of linoleic acid peroxidation for green tea of 220 nm was lower. The inhibitory rates of green tea of 220 nm on HepG2, A549, and MGC803 cells achieved 77.35%, 80.76%, and 87.54% for regular green tea, and 82.51%, 88.09%, and 74.48% for Se-enriched green tea at the dose of 100 μg mL^{-1}, values that were all significantly higher compared to that of 3.52 μm.

KEYWORDS: Superfine Se-enriched green tea; particle size; antioxidant; antitumor

INTRODUCTION

Green tea is the second most common beverage next to water in terms of worldwide popularity. Consumption of tea has been closely related to many health benefits including cancer prevention abilities in a wide range of organs (*1*). Epidemiologic studies revealed that green tea or its extracts possess beneficial activities such as antioxidant, hepatoprotective, antimutagenic, anticancer, and application in treatment of obesity (*2–6*). Selenium has been reported to be protective to oxidative damage of the liver, kidney, and heart (*7*). Some literature showed enrichment of selenium could significantly enhance the quality and bioactivities of green tea (*8–11*). At present, Se-enriched green tea is being increasingly produced in China and has been well-known as a bioactive drink due to its high content of active components (*10*).

Recently, superfine comminution technology has been applied widely to Chinese medicine and food processing. The surface characteristics of superfine particles such as size, surface charge density, surface hydrophobicity, and so on were regarded as the key factors for their activities (*12*) and the particle size is crucial (*13*). The absorptions and bioavailabilities of nutriment could be enhanced after treatment with superfine comminution (*14, 15*). For instance, water-soluble extracts and the three kinds of lipophilic-active ingredient amounts of micronized powder of *Salvia miltiorrhiza Bge* were 1.23, 1.54, 1.48, and 1.90 times those of crude powder (*16*), and others found a size-dependent cytotoxicity effect of *realgar* particles on the endothelial cell line ECV-304 and particle sizes smaller than 150 nm were able to induce apoptosis (*17*). Thereby, superfine comminution technology has raised much interest on particle effect that could influence or alter the original characteristics of materials and shown great dominance in the fields of agriculture and food. However, such reports on the biological activity of superfine Se-enriched green tea particles are very limited.

The chemical composition of green tea is complex with 15−20% of proteins and more than 30% of phenolic compounds, compared to herbal medicine. With the tradictional drinking way of hot-water infusion only 4.5% of phenolic compounds and trace protein or pigments were contained and the loss of many water-insoluble nutrition compositions took place during the process of infusion, such as dietary fiber, protein, fat-soluble vitamins, and pectin. Considering the loss of active ingredients in tea infusion and the revolution in the field of agriculture and food brought about by superfine comminution technology, the present study has been designed to investigate the characteristics and antioxidant and antiproliferation activities (in vitro) of superfine regular and Se-enriched green tea of different particle sizes.

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Table 1. Composition of Green Tea Samples with Different Particle Sizes

samples	Se[a] (mg kg^{-1})	vitamin C (g kg^{-1})	tea polyphenols (g kg^{-1})	amino acid (g kg^{-1})	chlorophyll (g kg^{-1})
superfine regular green tea (3.52 μm)	0.34 ± 0.02b	1.94 ± 0.08c	159.31 ± 0.98d	17.61 ± 1.14b	3.04 ± 0.06 b
superfine Se-enriched green tea (3.52 μm)	17.92 ± 0.19a	2.28 ± 0.03a	228.89 ± 0.39a	24.28 ± 0.98a	3.11 ± 0.01ab
superfine regular green tea (220 nm)	0.34 ± 0.02b	1.01 ± 0.06d	201.45 ± 2.15c	24.50 ± 0.91a	3.08 ± 0.02ab
superfine Se-enriched green tea (220 nm)	17.92 ± 0.19a	2.16 ± 0.05b	217.08 ± 1.25b	25.45 ± 0.78a	3.24 ± 0.17 a

[a] Values were means of three determinations ± standard deviation ($n = 3$). Values within the same column followed by different letters are significantly different at $P < 0.05$.

MATERIALS AND METHODS

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic acid (ca. 99%) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka, USA. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco, USA, and fetal bovine serum (FBS) was from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). 5-Fluorouracil (5-Fu) was provided by Nantong Pharmacy Factory (Nantong, China). Other reagents were of analytic grade and were purchased from Nanjing Chemical Industry (Nanjing, China).

Preparation of Se-Enriched Green Tea. Se-enriched green tea and regular tea were prepared in April 2006 at Fukun Tea Plantation of Yixing, Jiangsu Province. The process of enriching selenium was according to the protocols reported previously (*8, 9*). Briefly, selenium-enriched fertilizer prepared by Hu et al. was first dissolved in deionized water and afterward sprayed on old leaves of the tea trees. Eventually, tea leaves with one newly growing bud and two young leaves were collected, immediately cleaned, and processed into commercial roasted green tea.

Preparation of Superfine Regular and Se-Enriched Green Tea of Different Particle Sizes. The regular and Se-enriched green tea were milled with a Mikro-ACM air-classifier mill (Hosokawa Micron Corp., Japan) and the median particle size (D_{50}, the particle size when the cumulative value is 50% by volume in the particle size cumulative distribution profile) was 3.52 μm measured by Mastersizer Microplus (Malvern Instruments Ltd., UK) (*18, 19*). A 3.52 μm sample of regular green tea (mRGT) and 3.52 μm of Se-enriched green tea (mSeGT) were further ground respectively, using a QM-DK low-temperature planetary ball mill (air-cooled) (Nanjing University Instrument Plant, China). The median particle size determined by Zetasizer 3000HS (Malvern Instruments Ltd., UK) was 220 nm (*20, 21*). The superfine regular green tea particle of 220 nm (nRGT), the Se-enriched green tea particle of 220 nm (nSeGT), mRGT, and mSeGT were all stored at −20 °C for further experiments.

Ingredients Assay of Different Green Tea Samples. The ingredients of different green tea samples, including Se, vitamin C, tea polyphenol (TPP), amino acid, and chlorophyll, were assayed according to previous literature (*9, 22, 23*).

Assay of Antioxidant Activity by the DPPH Radical-Scavenging Activity Method. The radical scavenging capacity of mRGT, mSeGT, nRGT, nSeGT, and α-tocopherol was determined by using the stable radical DPPH (*24*). Four milligrams of different samples were suspended in 100 mL of ethanol, and then dispersed by ultrasonic instrument under the condition of 25 °C, 60 W for 20 min. A fixed quantity (2 mL) of each solution was added to 2 mL of 2×10^{-4} mol L^{-1} ethanolic DPPH solution. The mixture was shaken vigorously with a Vortex Mixer MS2 (Che Scientific Company Ltd., Hong Kong) and the absorbance was monitored spectrophotometrically at 517 nm immediately and recorded at 5 min intervals until the absorbance reached a steady state. The mixture without the addition of sample served as the control. All the tests were performed in triplicate and the inhibitory rate was calculated according to the formula of Yen and Duh (*25*).

Determination of Antioxidant Activity by Inhibition of Linoleic Acid Peroxidation. The inhibition of linoleic acid peroxidation was performed to evaluate the antioxidant activity of different tea samples according to Kikuzaki and Nakatani with a slight modification (*26*). Briefly, 1 mL of a 50 μg mL^{-1} of sample solution (pretreated as above), 2 mL of 2.51% (w/v) linoleic acid in ethanol, 4 mL of 0.05 mol L^{-1} of phosphate buffer (pH 7.0), and 2 mL of distilled water were mixed in a 10 mL tube with a screw cap. The peroxidation was initiated by addition of 0.417 mL of 0.1 M 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and then incubated in a 37 °C water bath in the dark. The above mixture (0.1 mL) was added to 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. After 5 min, 0.1 mL of 0.02 mol L^{-1} ferrous chloride in 3.5% (v/v) hydrochloric acid was added to the above mixture and then mixed. The absorbance of the mixture was recorded at 50, 100, and 200 min at 500 nm with a spectrophotometer. α-Tocopherol used at the same concentration and linoleic acid mixture without the addition of samples served as the positive control. All the tests were performed in triplicate and the results were averaged.

Cell Lines and Cell Culture. Three different cell lines, including human hepatoma cells (HepG2), human lung cancer cells (A549), and human gastric cancer cells (MGC803), were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were respectively incubated in 90% DMEM medium supplemented with 10% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assayed by the MTT Method. In vitro cytotoxicity of mRGT, mSeGT, nRGT, and nSeGT was assayed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (*27*). In brief, cells in a logarithmic growth-phase were seeded into 96-well culture plates (5×10^3 cells per well) at room temperature for 24 h, and then exposed to the various concentrations of different green tea powders suspended in dimethyl sulfoxide (DMSO) and incubated again for 48 h. The culture medium was removed and MTT reduction was initiated by adding a 20 μL MTT solution (5 mg mL^{-1}) per well. After 4 h of incubation, the supernatant was discarded and 100 μL of DMSO was added to each well to terminate the reaction. The mixture was shaken and the optical density was measured at 570 nm, using a Universal Microplate Reader (EL800, BIO-TEK Instruments Inc., USA). Three parallel wells of each concentration of different samples were repeated for the average result, and the vehicle (DMSO) treatments served as the control (*17, 28*).

Statistical Analysis. The data were presented as means ± standard deviations ($n = 3$). Statistical analysis was performed by using Student's *t*-test and one way analysis of variance. Multiple comparisons of means were done by the LSD (least significance difference) test. A probability value of <0.05 was considered significant. All computations were made by employing the statistical software SAS (version 8.0).

RESULTS AND DISCUSSION

Ingredient Analysis of Different Green Tea Samples. The contents of ingredients of superfine regular and Se-enriched green tea with different particle sizes are shown in **Table 1**. The contents of selenium, vitamin C, and tea polyphenols were all significantly enhanced by foliar spray with selenium fertilizer ($P < 0.05$), which was in agreement with the reports by Xu et al. (*11*). Selenium could bind with protein in the form of selenocysteine and selenomethionine, and with TPP as well to form a stable complex (*29*). However, no significant difference was observed in the contents of chlorophyll and amino acid by

Table 2. Antioxidant Activities of Superfine Regular and Se-enriched Green Tea Particles by DPPH assay

samples	inhibition (%)[a]		
	16 min	30 min	100 min
α-tocopherol	79.57 ± 0.20a	82.36 ± 0.24a	84.88 ± 0.16a
superfine regular green tea (3.52 μm)	28.46 ± 0.29d	31.77 ± 0.20d	38.15 ± 0.05e
superfine Se-enriched green tea (3.52 μm)	35.54 ± 0.24b	38.59 ± 0.29c	45.79 ± 0.63d
superfine regular green tea (220 nm)	55.74 ± 0.72b	59.86 ± 0.62b	67.87 ± 0.88c
superfine Se-enriched green tea (220 nm)	56.45 ± 0.89b	60.63 ± 0.89b	69.49 ± 0.54b

[a] Values were means of three determinations ± standard deviation ($n = 3$). Values within the same column followed by different letters are significantly different at $P < 0.05$.

selenium enrichment, except for the amino acid content of 3.52 μm of green tea particles.

The content of chlorophyll was stable even when the particle size varied both in regular green tea and in Se-enriched green tea, but significant changes was observed for other ingredients. For regular green tea, vitamin C decreased greatly while TPP and amino acid showed a significant elevation with the decrease in particle size. For Se-enriched green tea, however, we found a reduction in both the contents of vitamin C and TPP as the particle size decreased while the content of amino acid was stable. Superfine particles exhibited different properties compared with those of corresponding regular material in terms of the huge surface effect (*30*). The particle size effect and breakage of cellular membrane and structure by comminuting could accelerate the dissolution of ingredients. Likewise, these effects may also contribute greatly to the accelerated oxidation for some labile components such as vitamin C and TPP. Therefore, the changes of the ingredient contents could be attributed to the action of various factors given the different sensitivity of each ingredient to these factors.

Antioxidant Activity of Superfine Regular and Se-Enriched Green Tea of Different Particle Sizes. In our studies, the DPPH radical scavenging assay and FTC method were performed to compare the antioxidant capacity of various tea samples. The antioxidant activity of green tea samples was first assessed by the DPPH radical-scavenging method and inhibition percentages were measured at 16 (*25*), 30 (*31, 32*), and 100 min until the absorptions reached a stable plateau (**Table 2**). A higher inhibition rate is an indication of higher antioxidant activity. The results indicated that nRGT and nSeGT exhibited a significantly higher capacity of scavenging the DPPH radical compared with mRGT and mSeGT during the experiment time. At 16 and 30 min, no significant difference on inhibition rate was observed between nSeGT and nRGT while the former showed significantly higher antioxidant activity (69.49%) at 100 min. The absorptions reached stability at 100 min and the radical scavenging abilities were observed in the following order: α-tocopherol > nSeGT > nRGT > mSeGT > mRGT.

The antioxidant activity of different samples was also assessed by inhibition of linoleic acid peroxidation and a low absorbance indicated a low concentration of formed peroxides and a high level of antioxidant activity. As shown in **Figure 1**, the antioxidant activity of various samples was revealed in the following order at the reaction end point (200 min): α-tocopherol = mSeGT > mRGT = nSeGT > control > nRGT. Specially, absorption of nRGT at 200 min was higher than the negative control, which indicated that nRGT exhibited prooxidation.

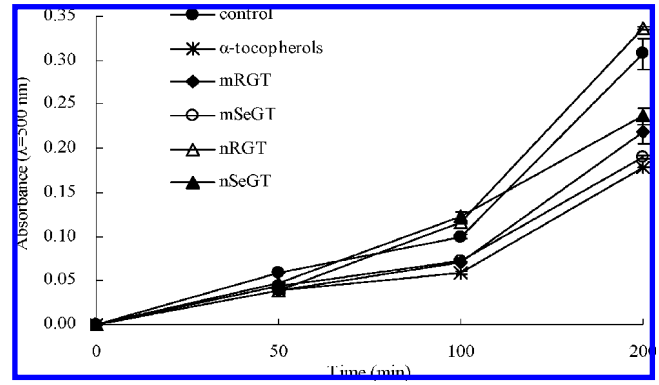

Figure 1. Antioxidant activity of superfine regular and Se-enriched green tea particles assessed by inhibition of linoleic acid peroxidation. mRGT, superfine regular green tea of 3.52 μm; mSeGT, superfine Se-enriched green tea of 3.52 μm; nRGT, superfine regular green tea of 220 nm; and nSeGT, superfine Se-enriched green tea of 220 nm.

Data from two assays indicated the green tea enriched with selenium exhibited higher antioxidant capacity compared with the regular one at the same particle size. The result was consistent with previous work indicating that selenium could enhance the antioxidant activity of green tea (or its extracts) (*11, 22*), which could be due to the fact that selenium plays an important role in antioxidases and enrichment of selenium could enhance their activities. The DPPH radical scavenging ability of green tea particles of 220 nm was higher than that of 3.52 μm, but the inhibition of linoleic acid peroxidation for green tea of 220 nm was lower and nRGT without higher content of selenium even exhibited prooxidation. The antioxidant activity of flavonoids might be reduced by autoxidation. Most plant polyphenol compounds, especially the flavonoids, possess both antioxidant and prooxidant properties, depending on its backbone structure, environmental factors such as the presence of transition metal ions (Cu^{2+}, Fe^{3+}, et al.) and H_2O_2, and the concentration of polyphenol, which might be attributed to metal chelating activity, radical scavenging activity, or activity to generate some reactive oxygen species (*33–35*). Joubert et al. (*36*) found the prooxidant activities of a crude polyphenol in linoleic acid peroxidation could be terminated with ascorbate added to regenerate Fe^{3+} to Fe^{2+} and the extracts displayed hydroxyl radical scavenging activity. The autoxidation of polyphenol compounds and the metal chelating activity of green tea particles of 220 nm might be accelerated due to its huge surface effect and new structure brought about by treatment of superfine comminution. An accurate mechanism of prooxidation of nRGT needs to be studied in further work.

Cell Proliferation Assay of Superfine Regular and Se-Enriched Green Tea of Different Particle Sizes. All green tea samples and 5-fluorouracil (5-Fu) presented inhibition activities against proliferation on the HepG2 cells, A549 cells, and MGC803 cells, and the antitumor activities were dose-dependent in the range of 1 to 100 μg mL^{-1} (**Figure 2**).

The inhibition of green tea samples on HepG2 cells growth was shown in **Figure 2A**. nSeGT gave the highest inhibition rate ((82.51 ± 4.41)%) compared with mRGT and 5-Fu, which was similar to that of nRGT and mSeGT at the dose of 100 μg mL^{-1}. Enrichment of selenium could significantly enhance the inhibitory potency of green tea particles of 3.52 μm in HepG2 cells growth. The activities of superfine regular green tea were enhanced significantly by reducing the particle size but those of the Se-enriched green tea were not. The inhibitory rate at 100 μg mL^{-1} decreased in the following sequence: nSeGT = mSeGT = nRGT > 5-Fu > mRGT.

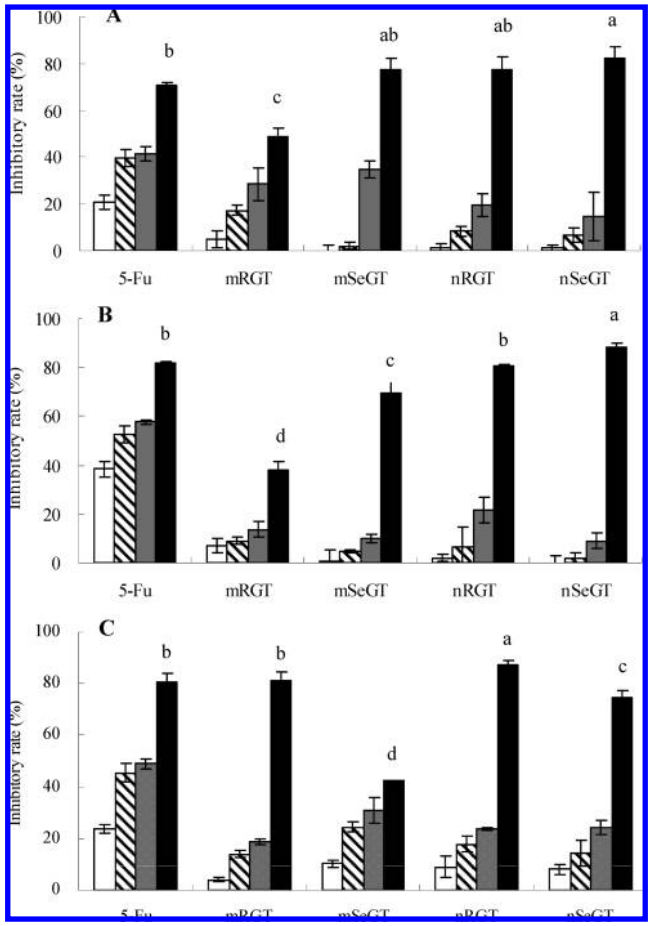

Figure 2. Suppressive effects (in vitro) of superfine regular and Se-enriched green tea particles on tumor cells: (**A**) HepG2 cells; (**B**) A549 cells; and (**C**) MGC803 cells. Histograms marked with different letters are significantly different ($P < 0.05$) at the concentration of 100 μg mL^{-1}. mRGT, superfine regular green tea of 3.52 μm; mSeGT, superfine Se-enriched green tea of 3.52 μm; nRGT, superfine regular green tea of 220 nm; nSeGT, superfine Se-enriched green tea of 220 nm. Open bar, 1 μg mL^{-1}; slashed bar, 10 μg mL^{-1}; solid gray bar, 20 μg mL^{-1}; solid dark bar, 100 μg mL^{-1}.

The antiproliferation of green tea samples on A549 cells was similar to that on HepG2 cells (**Figure 2B**). At the dose of 100 μg mL^{-1}, the highest inhibitory rate was (88.09 $\pm$ 2.19)% by nSeGT, which was significantly higher than that of 5-Fu and other treatments. Moreover, the antitumor activities could be enhanced both by enrichment of selenium and reduction of particle size. The inhibitory rates at 100 μg mL^{-1} were as follows: nSeGT > 5-Fu = nRGT > mSeGT > mRGT.

The inhibitory effects of green tea samples (100 μg mL^{-1}) on MGC803 cells were in different sequence compared with the above cell lines (**Figure 2C**): nRGT > mRGT = 5-Fu > nSeGT > mSeGT. The inhibitory effect at 100 μg mL^{-1} could be improved by reducing the particle size, but impaired by increasing the selenium content, which was different from that of HepG2 and A549 cells.

Tea polyphenol was the main contributor to cancer prevention followed by other components. The induction of cell cycle arrest and apoptosis could possibly be the important mechanism for cancer prevention by tea. Tea polyphenol and other components were able to significantly inhibit telomerase activity in HepG2 cells to inhibit proliferation and thus suggest an efficient means of lung cancer prevention. The mechanism of cancer prevention by tea has been reported but is still not fully understood (*37, 38*). That the addition of selenium intake could lower the risk of some cancers might be due to the enhancement of antioxidases by selenium. Se-enriched food could significantly inhibit the growth of hepatocellular carcinoma in both in vitro assays and animal models (*39–41*).

In our study, the antiproliferation potencies of superfine regular and Se-enriched green tea could be significantly improved by reducing the particle size. This result is consistent with the previous study by Deng et al. that realgar with a smaller particle size of 150 and 100 nm could remarkably inhibit cell viability through apoptosis compared with a particle size of 200 and 500 nm (*17*). The resulting antitumor activity might be explained in that superfine green tea particles of 220 nm provided a huge interface to contact with cancer cells and more active components were diffused into cells. Superfine Se-enriched green tea particles could be more effective in antiproliferation of HepG2 and A549 cells, but be less for MGC803 cells, and this might be attributed to the distinguishing sensitivity of different cells to medicine (*42*) or that the inhibitory mechanism on MGC803 was probably via another pathway rather than enhancing the activities of selenoenzymes.

In summary, this study demonstrated that reduction of particle size could lead to significantly increased antioxidant and antitumor activity of green tea particles in vitro. The quality and biological activities of green tea could be improved by enrichment of selenium as shown for HepG2 and A549 but not for antiproliferation of MGC803 cells. These findings warrant further investigation on the proper size and selenium content most effective in vivo. The application of superfine comminution technology in Se-enriched green tea processing might be a promising prospect for increased utilization of green tea.

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