

Dose-dependent Incorporation of Tea Catechins, (–)-Epigallocatechin-3-gallate and (–)-Epigallocatechin, into Human Plasma

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Tea catechins, (–)-epigallocatechin-3-gallate (EGCg) and (–)-epigallocatechin (EGC), have been reported to suppress oxidation of plasma low density lipoprotein (LDL) *in vitro*. If dietary catechins can be efficiently incorporated into human blood plasma, anti-atherosclerotic effects in preventing oxidative modification of LDL would be expected. In this study, a newly developed chemiluminescence detection-high pressure liquid chromatography (CL-HPLC) method for measuring plasma catechins was used and the incorporation of EGCg and EGC into human plasma was investigated. Healthy subjects orally ingested 3, 5, or 7 capsules of green tea extract (corresponding to 225, 375, and 525 mg EGCg and 7.5, 12.5, and 17.5 mg EGC, respectively). The plasma EGCg and EGC concentrations before the administration were all below the detection limit (<2 pmol/ml), but 90 min after, significantly and dose-dependently increased to 657, 4300, and 4410 pmol EGCg/ml, and 35, 144, and 255 pmol EGC/ml, in the subjects who received 3, 5, and 7 capsules, respectively. Both EGCg and EGC levels detected in plasma corresponded to 0.2–2.0% of the ingested amount. Catechin intake had no effect on the basal level of endogenous antioxidants (α -tocopherol, β -carotene, and lycopene) or of lipids in plasma. These results suggested that drinking green tea daily would contribute to maintain plasma catechin levels sufficient to exert antioxidant activity against oxidative modification of lipoproteins in blood circulation systems.

Key words: epigallocatechin gallate; epigallocatechin; absorption; plasma; human

Evidence is accumulating that oxidized low density lipoproteins (LDL) critically contribute to atherogenesis and that lipid peroxidation of plasma lipoprotein plays a key role.^{1–3)} Therefore, the inhibition of lipoprotein lipid peroxidation by antioxidants is of major interest to those developing methods of atherosclerosis prevention.

Tea catechins, such as (–)-epigallocatechin-3-gallate (EGCg; Fig. 1A) and (–)-epigallocatechin (EGC; Fig. 1B), have been reported to act as antioxidants and to inhibit Cu^{2+} -mediated LDL oxidation *in vitro* by scavenging oxygen radicals and chelating metal ions.^{4–7)} In particular, EGCg and EGC have been reported to have higher antioxidant activity.⁸⁾ Therefore, if they can be efficiently incorporated into human plasma, EGCg and EGC are expected to suppress lipoprotein lipid peroxidation and to act as anti-atherosclerotic agents.

In earlier studies we⁹⁾ and other investigators^{10,11)} have shown the occurrence of tea catechins in human plasma after their oral ingestion. However, it is not known whether EGCg and EGC are incorporated dose-dependently into human plasma.

In this study, various amounts of EGCg and EGC were orally ingested by healthy volunteers, and plasma EGCg and EGC levels were investigated by a newly established chemiluminescence detection-high pressure liquid chromatography (CL-HPLC)⁹⁾ to estimate the amount of catechins that can be absorbed into human plasma. Also, plasma endogenous antioxidant levels and plasma lipid profiles were examined to evaluate the effects of tea catechin intake.

Materials and Methods

Reagents. EGCg and EGC (both above 95% purity) extracted from green tea leaves, and Sunphenon DCF-1 capsule® (containing 75 mg EGCg and 2.5 mg EGC/capsule), were obtained from Taiyo Kagaku Co. (Yokkaichi, Japan). β -Carotene was purchased from Sigma Chemical Co. (St. Louis, MO). Lycopene was from Wako Pure Chemical Co. (Osaka,

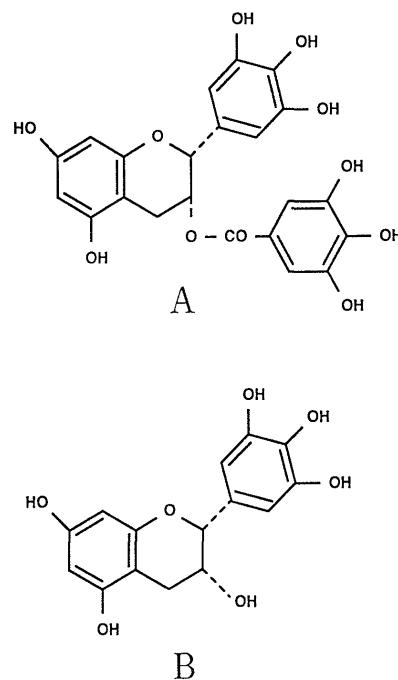


Fig. 1. Structure of Tea Catechins.

(–)-Epigallocatechin-3-gallate (EGCg; A) and (–)-epigallocatechin (EGC; B).

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Abbreviations: EGCg, (–)-epigallocatechin-3-gallate; EGC, (–)-epigallocatechin; LDL, low density lipoprotein; CL-HPLC, chemiluminescence-high pressure liquid chromatography; EDTA, ethylenediamine tetraacetic acid disodium salt; HRP, horseradish peroxidase; ECD, electrochemical detector.

Japan). *d*- α -Tocopherol was obtained from Eisai Co. (Tokyo, Japan). Other reagents and chemicals were commercially available extra-pure grade products.

Human subjects. Two female and one male adult volunteers (21–23 years old, non-smokers) participated in this study. After fasting for 12 h, each volunteer orally ingested 3, 5, or 7 capsules of Sunphenon DCF-1 (corresponding to 225, 375, and 525 mg EGCg and 7.5, 12.5, and 17.5 mg EGC, respectively). Blood from the subjects was collected into heparinized tubes before and at 90 min after the ingestion. The plasma was obtained by centrifuging the heparinized blood at $1000 \times g$ for 15 min at 4°C.

Generally, one tea cup of green tea contains about 100 mg of catechins. Therefore, the total amount of EGCg and EGC provided by 3, 5, and 7 capsules of Sunphenon DCF-1 is roughly comparable to that of tea catechins in 2, 4, and 6 cups of green tea, respectively.

CL-HPLC. The CL-HPLC system used in the EGCg and EGC assay was the same as that reported in the method paper.⁹⁾ Briefly, the CL-HPLC system consisted of reversed phase HPLC and a chemiluminescence detector, in which separated EGCg and EGC generated chemiluminescence at a post column, successively reacting with the following two chemiluminescence cocktails; 8.2 M acetaldehyde in 50 mM phosphate buffer (pH 7.4, contained 108 mg horseradish peroxidase/liter) and 8.8 M hydrogen peroxide aqueous solution.

A mixture of standard EGCg and EGC solution was made by dissolving EGCg and EGC in a Vc-EDTA solution which consisted of 2% ascorbic acid and 0.1% ethylenediamine tetraacetic acid disodium salt (EDTA) in 0.4 M NaH_2PO_4 buffer at pH 3.9. The concentrations of EGCg and EGC in sample solutions were measured from a calibration curve made with standard solution.

EGCg and EGC assay. To measure EGCg and EGC levels, plasma (250 μl) diluted with the same volume of Vc-EDTA solution was used. To each plasma sample, 500 μl of acetonitrile was added. The mixture was then vortexed for 5 min, and 3 ml of ethyl acetate was added. The mixture was vortexed again vigorously for 4 min and centrifuged ($1000 \times g$) at 4°C for 15 min. The supernatant ethyl acetate layer was collected. This ethyl acetate extraction was repeated three times. The combined ethyl acetate layer was evaporated to dryness with a rotary evaporator. The dried extract was redissolved in 900 μl of methanol-water (8:1, v/v) and passed through a HPLC chromatodisc (GL chromatodisc 13A, pore size 0.45 μm ; GLC Science Co., Tokyo, Japan) with 4 ml of methanol as eluant to exclude impurities. The methanol filtrate was evaporated to dryness and was dissolved in an appropriate amount of 10% acetonitrile aqueous solution. A portion of this acetonitrile aqueous solution was injected into CL-HPLC to measure the EGCg and EGC concentrations.

A mixture of 460 pmol of EGCg and 560 pmol of EGC was added to the plasma (250 μl) of a catechin-untreated control subject and the extracts were analyzed by CL-HPLC. EGCg was detected at 380 pmol and EGC at 450 pmol, indicating that the recovery from human plasma with the CL-HPLC assay was 84% for EGCg and 82% for EGC.

Carotenoids and tocopherol assay. β -Carotene and lycopene were extracted from plasma by the methods of Stahl *et al.*,¹²⁾ and analyzed by UV-HPLC.¹³⁾ HPLC separation was done with a C18 column (Lichrospher RP-18(e), 4×250 mm, Merck), and the mobile phase was acetonitrile-methanol-dichloromethane-water (70:70:20:0.16, v/v) at 1.0 ml/min flow rate. The analysis was done by setting the UV detector at 450 nm. Plasma α -tocopherol was measured by fluorescence-HPLC as reported by Abe *et al.*¹⁴⁾

Plasma lipid profile. Plasma total cholesterol, free cholesterol, HDL-cholesterol, triacylglycerol, and phospholipid levels were measured using the cholesterol-E-test, free cholesterol-E-test, HDL-cholesterol-E-test, triglyceride-E-test, and phospholipid-C-test (Wako Pure Chemical Co., Osaka, Japan), respectively. Plasma cholesterol ester was calculated by subtracting free cholesterol from total cholesterol.

Statistical analysis. The data were expressed as the mean and standard deviation (SD). Statistical comparisons were made with Student's *t*-test. Statistical significance was accepted at a *p* value of <0.05 .

Results

CL-HPLC chromatogram of EGCg and EGC

Figure 2 shows a typical CL-HPLC chromatogram of a

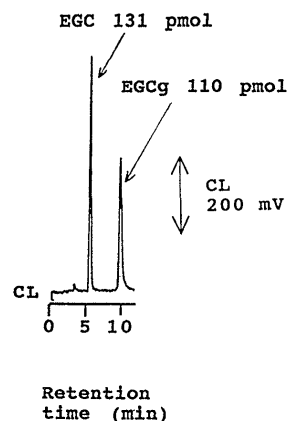


Fig. 2. Chemiluminescence Chromatogram of a Mixture of Standard EGCg (110 pmol) and EGC (131 pmol) with CL-HPLC.

The conditions for CL-HPLC are as given in our method paper.⁹⁾

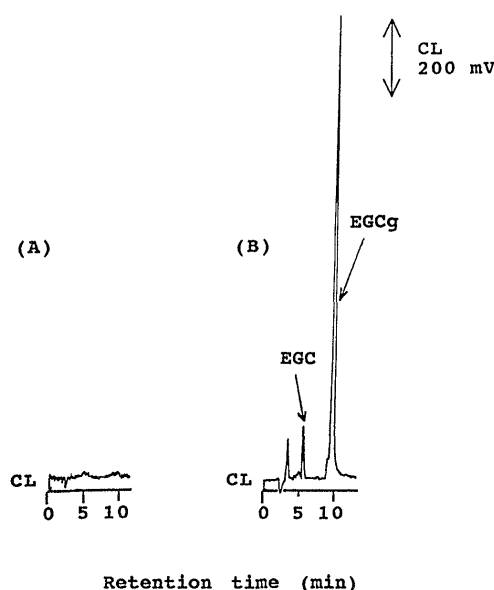


Fig. 3. CL-HPLC Chromatograms of Human Plasma EGCg and EGC.

The plasma extract (B) from a healthy subject 90 min after a single oral administration of seven capsules of Sunphenon DCF-1 (equivalent to 525 mg EGCg and 17.5 mg EGC/subject) and (A) from the same subject before catechin ingestion, were analyzed by CL-HPLC.

mixture of standard EGCg (injected amount 110 pmol) and EGC (131 pmol). Two intense chemiluminescence peaks ascribed to EGCg (10.7 min of retention time) and EGC (6.1 min) were found, and no other peaks were detected.

Figure 3 shows the CL-HPLC chromatograms of EGCg and EGC in human plasma extract. The extracts were prepared 90 min after a single oral administration of seven capsules of Sunphenon DCF-1 (equivalent to 525 mg EGCg and 17.5 mg EGC/subject) (Fig. 3B) and from the same subject before the administration (control plasma, Fig. 3A), and analyzed by CL-HPLC. Although no chemiluminescence peak was detected in the control plasma (Fig. 3A), the plasma from the subject who received catechin capsules had two intense chemiluminescence peaks ascribed respectively to EGCg and EGC (Fig. 3B). The EGCg (10.7 min of retention time) and EGC (6.1 min) peaks were identical to the retention times of standard EGCg and EGC. When plasma was treated with tannase, which hydrolyzes the galloyl ester from EGCg, and extracted and analyzed by CL-HPLC, the EGCg peak in the plasma extract disap-

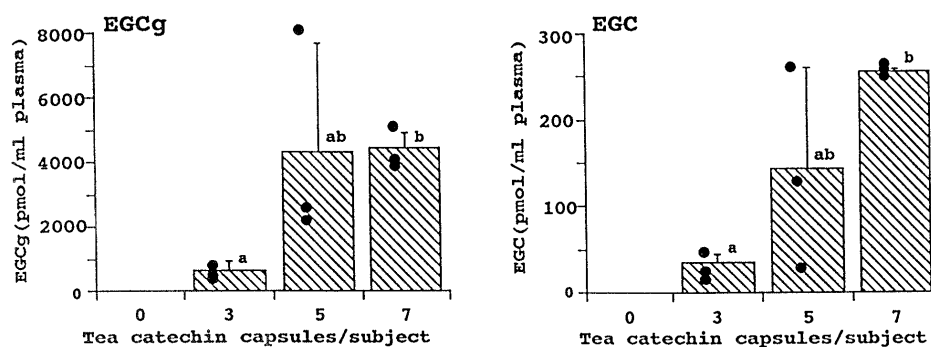


Fig. 4. Plasma EGCg and EGC Concentrations before and 90 min after Administration of Sunphenon DCF-1 as Determined by CL-HPLC.

Each subject received 3, 5, or 7 capsules of Sunphenon DCF-1 (corresponding to 225, 375, and 525 mg EGCg and 7.5, 12.5, and 17.5 mg EGC/subject) as a single oral supplementation after 12 h of fasting. Values are the mean \pm SD ($n=3$ subjects).

^{a, b} Values with different superscript letters are significantly different at $p < 0.05$.

peared (data not shown). No interference peaks were observed on the chemiluminescence chromatograms of the human plasma extracts.

EGCg and EGC in human plasma

Figure 4 shows plasma EGCg and EGC concentrations at 90 min after a single oral administration of 3, 5, or 7 capsules of Sunphenon DCF-1. Plasma EGCg and EGC levels before the administration were below the detection limit (<2 pmol/ml plasma). Ninety minutes after a single oral intake, EGCg was significantly increased to 657, 4300, and 4410 pmol/ml (300, 1970, and 2020 ng/ml) in the subjects who received 3, 5, and 7 capsules, respectively. EGC also showed a significant increase to 35, 144, and 255 pmol/ml (10, 44, and 78 ng/ml) in the subjects who received 3, 5, and 7 capsules, respectively. The results suggested a dose-dependent incorporation of EGCg and EGC in the free forms into human plasma. The total amount of EGCg in the blood mass was calculated to be 450–7500 μ g/subject, accounting for 0.2–2.0% of ingested EGCg when the whole blood mass was estimated to be 4 liters/subject. Similarly 0.2–1.3% of the ingested EGC was calculated to be incorporated into human plasma.

Plasma endogenous antioxidants and lipid profile

Table I shows the plasma endogenous β -carotene, lycopene, and α -tocopherol concentrations of human subjects before and 90 min after the ingestion of 3, 5, or 7 capsules of tea catechin. Catechin supplementation had no effect on the basal levels of plasma β -carotene, lycopene, or α -tocopherol.

Table II shows the effects of tea catechin ingestion on human plasma lipids. No significant influences were observed on the levels of total cholesterol, free cholesterol, cholesterol ester, HDL-cholesterol, triacylglycerol, or phospholipids.

Discussion

Recent studies have found tea catechin in human plasma and urine^{10,11} and in rat plasma¹⁵ and portal blood¹⁶ after ingestion. For the tea catechin level in human plasma, 100–585 pmol/ml (46–268 ng/ml)¹⁰ and 65–175 pmol/ml (30–80 ng/ml)¹¹ of EGCg was estimated after 60 min of single oral intake of green tea extract containing respectively 88 mg and 105 mg EGCg. The plasma EGC concentration was also reported to be from 268 to 673 pmol/ml (82–

Table I. Human Plasma Endogenous Antioxidant Concentrations before and 90 min after Tea Catechin Ingestion^a

Dose	β -Carotene	Lycopene	α -Tocopherol
Capsule ^b	(nmol/ml)	(nmol/ml)	(nmol/ml)
0 (before ingestion)	0.94 ± 0.26	0.78 ± 0.10	28.6 ± 5.6
3	0.80 ± 0.11	0.75 ± 0.08	28.4 ± 2.5
5	0.86 ± 0.41	1.08 ± 0.39	34.5 ± 4.7
7	0.88 ± 0.24	1.03 ± 0.40	31.5 ± 5.0

^a Values are the mean \pm SD ($n=3$ subjects).

^b Each subject received 3, 5, or 7 capsules of Sunphenon DCF-1 (corresponding respectively to 225, 375, and 525 mg EGCg/subject and 7.5, 12.5, and 17.5 mg EGC/subject) as a single oral supplementation after 12 h of fasting.

206 ng/ml) 60 min after a single intake of tea extract containing 82 mg EGC.¹⁰ In such studies, catechins were measured by HPLC combined with an electrochemical detector (ECD) or a UV detector. However according to our preliminary studies, catechin peaks recorded on ECD and UV chromatogram concomitantly appeared with unknown peaks of low reproducibility, especially in biological samples. Consequently, we have newly developed a more selective and sensitive method, CL-HPLC, for measuring EGCg present in blood plasma⁹ and tissue organelles such as in liver and intestinal mucosa¹⁷. In a previous study,⁹ we found that plasma EGCg concentrations significantly increase 60 min after ingestion in humans, suggesting that the EGCg absorbed could act as effective antioxidant in the plasma. To prove this, it is necessary to establish whether tea catechin can be dose-dependently incorporated into human plasma in the free form; the free form structure would likely best show the function of the antioxidant. Therefore, in this study, different amounts of EGCg and EGC were given to humans, and their concentrations in plasma were investigated by using CL-HPLC.

The plasma EGCg and EGC concentrations were first shown to be significantly increased with the amount of catechin ingested (Fig. 4), indicating dose-dependent incorporation of tea catechins into human plasma. No difference was observed for calculated percentages of incorporated amount against dosage between EGCg (0.2–2.0%) and EGC (0.2–1.3%). This may imply that there is no difference in the absorption kinetics of EGCg and EGC, even though EGCg has a galloyl group. It is likely

Table II. Human Plasma Lipid Profile before and 90 min after Tea Catechin Ingestion^a

Dose	TC ^c	FC ^c	CE ^c	HDL-C ^c	TG ^c	PL ^c
Capsule ^b	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)
0 (before ingestion)	142 ± 17	47.9 ± 3.6	94.1 ± 20.9	49.8 ± 23.5	50.0 ± 5.8	192 ± 24
3	127 ± 6	39.3 ± 6.7	87.5 ± 7.5	50.2 ± 27.5	55.2 ± 0.5	167 ± 15
5	129 ± 41	42.5 ± 11.1	87.1 ± 33.4	78.9 ± 10.9	54.9 ± 8.9	170 ± 13
7	160 ± 24	51.5 ± 10.1	109.1 ± 15.5	64.6 ± 24.5	55.3 ± 8.0	188 ± 29

^a Values are the mean ± SD (*n* = 3 subjects).

^b Each subject received 3, 5, or 7 capsules of Sunphenon DCF-1 (corresponding respectively to 225, 375, and 525 mg EGCg/subject and 7.5, 12.5, and 17.5 mg EGC/subject) as a single oral supplementation after 12 h of fasting.

^c Abbreviations: TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; HDL-C, HDL-cholesterol; TG, triacylglycerol; PL, phospholipids.

that other tea catechins would be efficiently absorbed at a similar rate into human plasma. On the other hand, for the absorption rate of natural antioxidants into the human body, approximately 80–90% in ingested ascorbic acid, over 50% in β -carotene as the micelle form and 20% in α -tocopherol was known to be incorporated through the digestive tracts.

Several reports refer to the antioxidant effect of tea catechins in inhibiting the oxidative modification of LDL *in vitro*⁴⁾ and in preventing lipid peroxidation in rat plasma.¹⁸⁾ However, all these reports lacked quantitative data on the catechins. As shown here, EGCg in human plasma increased and attained a significant level (4300 pmol/ml, 1970 ng/ml, and 4410 pmol/ml, 2020 ng/ml) when a healthy subject received five and seven capsules of Sunphenon DCF-1 (equivalent respectively to 375 and 525 mg EGCg/subject) (Fig. 4). Such a high plasma EGCg level would be sufficient to exert an antioxidant effect against lipoprotein lipid peroxidation in plasma *in vivo*. A 500 pmol EGCg/ml (229 ng/ml) has been reported to inhibit Cu²⁺-mediated LDL peroxidation *in vitro*.⁴⁾ Okuda *et al.*¹⁹⁾ have showed the inhibitory effect of EGCg on lipid peroxidation induced by ADP and NADPH in rat liver microsome. The 50% inhibition by EGCg of microsomal lipid peroxidation was reported to be 900 ng EGCg/ml (2.0 μ M) of the reaction mixture. Therefore, we guess that catechins in the plasma at the concentrations over a 2.0–5.0 nmol/ml level, as shown in the plasma EGCg of humans who ingested 5 and 7 capsules of tea catechin, would have acted as effective antioxidant against lipid peroxidation *in vivo*. If tea catechin has dose-dependent antioxidant activity in human plasma *in vivo*, then it may also act as an anti-atherosclerotic agent. Studies to this effect are in progress in our laboratory.

Several epidemiological studies^{20,21)} have showed that individuals who consume four or more cups of black or green tea daily have a lower risk of atherosclerosis. The amount of tea catechins in four cups of green tea is roughly comparable to the EGCg and EGC provided by five capsules of the Sunphenon DCF-1 used in this study. Ingestion of five capsules of Sunphenon DCF-1 resulted in plasma EGCg and EGC concentrations of 4300 and 144 pmol/ml, respectively (Fig. 4). Thus, drinking four or more cups of green tea daily would maintain plasma levels of catechin high enough to be of therapeutic benefit against atherosclerosis. In Japan, some people drink more than 10 cups of green tea per day.²²⁾ In such people, plasma catechins would be maintained constantly at levels sufficient to show antioxidant activity.

Normal human plasma LDL is known to contain various hydrophobic antioxidants such as tocopherols and carotenoids.²³⁾ These endogenous antioxidants are suggested to protect the oxidative modification of lipoproteins *in vivo*. Therefore, if catechin intake results in a decrease in endogenous antioxidants, the results could be harmful to human health. For example, it has been reported that long term supplementation of β -carotene in rats decreases endogenous α -tocopherol levels in plasma.²⁴⁾ In this study, tea catechin supplementation caused no change in the levels of β -carotene, lycopene, or α -tocopherol in human plasma (Table I). This suggests that tea catechin ingestion is not harmful in this regard.

In atherosclerosis, plasma lipid profiles, especially of cholesterol, are important. An increase in the plasma cholesterol level is a causative factor in the development of atherosclerosis.²⁵⁾ Muramatsu *et al.*²⁶⁾ have reported that tea catechin reduced plasma cholesterol levels in cholesterol-fed rats and increased fecal elimination of cholesterol, and suggested that catechin exerts a hypocholesterolemic effect and has a protective effect against the atherosclerotic process. Presently, EGCg and EGC supplementation did not affect the levels of cholesterol and other plasma lipids 90 min after oral ingestion (Table II), but plasma cholesterol may decrease over a prolonged period, *i.e.*, 6–12 h, and with successive intake of tea catechin.

Other biological functions of tea catechin, in addition to antioxidant activity, may include inhibition of hydrolytic and oxidative enzymes (phospholipase A₂, cyclooxygenase, and lipoxygenase) and anti-inflammatory activity. Gerritsen *et al.*²⁷⁾ have reported that plant flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression, suggesting a potent anti-inflammatory mechanism.

In this study, various amounts of EGCg and EGC were administered to humans, and dose-dependent incorporation into plasma at levels sufficient to exert antioxidant effects was confirmed. The plasma endogenous antioxidants and plasma lipids were not affected by ingesting tea catechin. The taking of tea catechin as an antioxidative nutrient to prevent atherosclerosis can be recommended.

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