Inhibitory Effect of Oolong Tea on the Oxidative State of Low Density Lipoprotein (LDL)

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In the present study, we investigated the anti-oxidant activity of oolong tea in an oxidation model using human low-density lipoprotein (LDL). Oolong tea suppressed the oxidation of LDL induced by 2,2'-azobis 4-methoxy-2,4-dimethylvaleronitrile (V70) in a dose-dependent manner, that is, it prolonged the lag time to 114.3%, 138% and 199.9% as compared with the control group at 0.5 μg/ml, 1.0 μg/ml, and 2.5 μg/ml, respectively. We also determined the scavenging effect of oolong tea on active oxygen radicals using the electron spin resonance (ESR) technique with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trapping agent. The intensity of the ESR signals for the DMPO–OOH adduct formed by the hypoxanthine/xanthine oxidase reaction system with DMPO decreased in the presence of oolong tea. The IC50 of oolong tea was 19.9 μg/ml. These findings suggested that oolong tea has beneficial effects on health related to its anti-oxidative action.

Key words oolong tea; antioxidant; low density lipoprotein (LDL); lag time; electron spin resonance (ESR)

Tea is the most popular beverage in the world. Green tea and oolong tea are consumed mostly in Japan and China, while black tea is preferred in America and Europe. All types of tea are manufactured from the same plant species, *Camellia sinensis* L., which was first discovered in China where it has been used as a daily beverage known to have beneficial effects on health for thousands of years. The various kinds of tea are produced through different processing methods. Oolong tea is semi-fermented, green tea is not fermented, and black tea is well fermented. In China, oolong tea traditionally has been considered to have anti-obesity and hypolipidemic effects and it has been thought that habitual ingestion is effective in enhancing metabolic rates and fat oxidation in humans.

Superoxide radicals are known to damage cell components causing aging and several serious diseases, such as cancer and heart disease. Evidence has emerged that the oxidative modification of low density lipoprotein (LDL) alters the physicochemical and biological properties of LDL particles, which is thought to cause atherosclerosis. The rapid uptake of oxidatively modified LDL through a scavenger receptor leads to the formation of foam cells and oxidized LDL has a number of other atherogenic properties similar to cholesterol-laden macrophages or smooth muscle cells. One approach to reducing the atherogenicity associated with modified LDL might be the use of anti-oxidants to prevent the formation of oxidized LDL. So it is very important to find effective scavengers of superoxide radicals. Early studies indicate that some tea catechins could inhibit lipid oxidation and scavenge superoxide radicals.

Less attention has been given to the influence of oolong tea on the oxidation of LDL and superoxide radicals. Therefore, we determined *in vitro* the susceptibility of LDL to oxidation in the presence of an oolong tea extract and the scavenging effect using electron spin resonance (ESR) techniques.

MATERIALS AND METHODS

**Tea Samples and Preparation of Tea Extracts** Oolong tea was purchased from the Fujian Tea Import & Export Co., Ltd., Fujian province, China. Black tea and green tea were purchased at a local market. The black tea was Lipton Red Label, and the leaf green tea was packaged in a metallic box (Shizuoka, Japan). The leaves were treated with 20 parts of hot water for 15 min at 90°C. After filtration and evaporation of the extracted water, the residue was powdered under frozen-decompression conditions. The recovery rate was 19.6% (green tea), 20.1% (oolong tea) and 27.2% (black tea), respectively. The extracts were dissolved in distilled water immediately before use in the present experiment.

**Measurement of LDL Oxidation** In the LDL oxidation test, a blood sample was taken by venapuncture in the morning from a non-smoking, healthy young male following an overnight fasting from 21:00 on the previous day, at the Kishigakakinai clinic (Mishima-gun, Osaka). The study was approved by the ethical committee of the Institute for Health Care Science, Suntory Ltd., and written informed consent was obtained. LDL was isolated from plasma according the method described previously. Briefly, blood was added to a tube containing 5 mM ethylenediaminetetraacetic acid (EDTA). Then, the tube was centrifuged at 3000 rpm for 10 min to obtain supernatant. Next, 0.3575 g of solid KBr was added to 1.1 ml of plasma to adjust the density to 1.21 kg/l, and then layered under 2.8 ml of LDL isolation solution (0.15 M NaCl, 0.1% EDTA, pH 7.4 with NaOH; d=1.009) in 3.9 ml quick seal tubes. The tubes were ultracentrifugated for 40 min at 100000 rpm using a Beckman optima™ TL model ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, U.S.A.) with a TCA-100.4 rotor at 4°C. The LDL was isolated at a density of 1.006 to 1.063 g/ml, and the concentration of LDL protein was determined using BioRad protein assay reagent (BioRad, San Fransisco, CA, U.S.A.). The LDL was diluted to a final concentration of 70 μg protein/ml with 10 mM phosphate buffered saline (PBS) buffer containing 1 mg/ml of EDTA based on a curve of the standard protein solution, and
a given concentration of oolong tea extract was added to the LDL solution, then, incubated at 37 °C. Next, 10 µl of 40 mM 2-2′-azobis 4-methoxy-2,4-dimethylvaleronitrile (V70) was dissolved in acetonitrile, and was added to the LDL solution to give 400 µM of V70 as a final solution. The oxidation of LDL was examined by measuring the production of conjugated dienes as the increase in absorbance at 234 nm of the LDL solution for 150 min according to the method originally described by Esterbauer et al. The resistance of LDL to oxidation is expressed as lag time. The inhibitory action of the oolong tea extract was determined by measuring the oxidation sensitivity of LDL. Results were expressed as the mean±standard deviation (S.D.).

Measurement of Superoxide Anion Radical Activity
Superoxide anion radical scavenging activity was measured, using the 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adduct generated in the hypoxanthine (HPX) and xanthine oxidase (XOD) reaction system by the spin-trapping method. The sampling procedure was as follows. All reagents were dissolved in 100 mM sodium phosphate buffer solution (pH 7.4). Specifically, 50 µl of a sample solution was mixed with 50 µl of 2 mM HPX, and 35 µl of 5.5 mM diethylenetriamine-N,N,N′,N″,N‴-pentaacetic acid (DTPA), 15 µl of 8.97 M DMPO and 50 µl of 0.4 U/ml XOD solution were added to the reaction solution in a test tube. The measurement was started immediately after quickly stirring the mixture. A 200 µl aliquot of the mixture was taken into a flat cell. DMPO–superoxide anion (O$_2^-$) adduct spectra, which were generated within a quartz cell at 60 s after the addition of XOD, were measured. Spectral analysis was conducted using an ESR apparatus (Nippon Denshi JES-FR30 Free Radical Monitor) at a power of 4 mW, sweeping width of 335.5 mT, modulation of magnetic field of 0.1 mT, gain of 160 times, sweeping time of 0.5 min and reaction time of 0.1 s at room temperature. After the recording, the signal intensity of the lowest field peak of the spectrum was normalized as the relative height against the standard signal intensity of the manganese oxide marker. The absolute concentration of DMPO–O$_2^-$ was finally determined by a double-integration of the ESR spectrum. The scavenging activity of the sample is expressed in terms of the LC$_{50}$ value, the concentration required to give a 50% decrease in the signal intensity of the DMPO adduct of the superoxide anion radical.

Statistics Data are expressed as the mean±S.D. Statistical analyses of lag-time data were performed with Student’s t-test. Differences were considered to be significant when the p value was less than 0.005.

RESULTS

Inhibition of LDL Oxidation by Oolong Tea Extract
Figure 1A shows the temporal change in the formation of conjugated dienes in LDL on incubation with V70, which exhibits absorbance at 234 nm. These working curves of LDL oxidation were used as an index of resistance time for the oxidative reaction. Three concentrations (2.5 µg/ml, 1.0 µg/ml and 0.5 µg/ml) of oolong tea extract were used to clarify the dose-dependent effect on the generation of conjugated dienes during lipid peroxidation. From the curves, it is clear that the extract inhibited the process in a dose-dependent manner.

Fig. 1. Inhibition of LDL Oxidation by Oolong Tea Extract

Anti-oxidative effects of oolong tea extract are shown as the lag time for formation of conjugated dienes in LDL incubated in vitro. LDL at 70 µg protein/ml was incubated with the extract, and oxidation was initiated by the addition of V70 at a final concentration of 400 µM. The formation of conjugated dienes was determined from changes of absorbance light at 234 nm for 150 min continuous monitoring (A). The lag time was obtained from working curves of LDL oxidation, and the results represent the mean±S.D. of three experiments. LDL was significantly protected against oxidation compared with the control (B) *Significantly different from the control at p<0.005, ++p<0.001 as determined by Student’s t-test.

Lag time, as a measure of the susceptibility of LDL to oxidation determined using a spectrophotometer at 234 nm, was at 2.5 µg/ml of oolong tea extract, prolonged to 199.9% (p<0.001), and at 1.0 µg/ml and 0.5 µg/ml, prolonged to 138.6% (p<0.005) and 114.3% (p<0.005), respectively. The extract remarkably suppressed the oxidation of LDL, and the results were obtained from three experiments (Fig. 1B).

Scavenging Activities of Oolong Tea, Green Tea and Black Tea Extracts against Superoxide Radicals
The anti-oxidant activity of the oolong tea extract was evaluated by making comparisons with a typical ESR spectrum of the DMPO–OOH spin adduct in the HPX–XOD reaction system. The ESR spectra changed when different concentrations of the extract were added to the system. At concentrations from 0.04 mg/ml to 1 mg/ml, there was a dose-dependent increase in the level of scavenging activity (Fig. 2). IC$_{50}$ values, the concentration needed to scavenge 50% of superoxide radicals, for the three types of tea extract are shown in Table 1. The IC$_{50}$ of oolong tea extract is about 19.9 µg/ml. It was found that the scavenging ability of the oolong tea extract was greater than that of the green and black tea extracts, which were 30.08 µg/ml and 28.1 µg/ml, respectively. Oolong tea is therefore the most effective anti-oxidant among them.
artery wall. These deposits partly derive from oxidative stress. Atherosclerosis is an arterial disease characterized by lipid deposits in the artery wall. The uptake of oxidized LDL into macrophages is uncontrolled. Modified LDL, not recognized by apolipoprotein B/E receptors, binds to macrophages, which act as scavengers of LDL. The uptake of oxidized LDL into macrophages is uncontrolled. Then, the macrophages become foam cells, in which too much esterified cholesterol is accumulated. Oxidative modifications of LDL can be of various types, which too much esterified cholesterol is accumulated. Therefore, it is important to suppress the oxidation of LDL for reducing the risk of atherosclerosis, and some compounds possessing anti-oxidative activity could have beneficial effects in the prevention of the disease. Based on our results, oolong tea water extract which suppressed LDL oxidation as compared with the control group may help to prevent the development of atherosclerosis.

DISCUSSION

In the present study, we determined the lag time of LDL oxidation by continuously monitoring the formation of conjugated dienes. The results clearly indicate that oolong tea water extract affects the susceptibility of LDL to peroxidation, prolonging the lag time of LDL oxidation in a dose-dependent manner. Recent studies have indicated a correlation between LDL oxidation and atherosclerosis. Atherosclerosis is an arterial disease characterized by lipid deposits in the artery wall. These deposits partly derive from oxidative modified LDL. Oxidative modifications of LDL can be of importance in the development of atheromatous lesions. Modified LDL, not recognized by apolipoprotein B/E receptors, binds to macrophages, which act as scavengers of LDL. The uptake of oxidized LDL into macrophages is uncontrolled. Then, the macrophages become foam cells, in which too much esterified cholesterol is accumulated. Therefore, it is important to suppress the oxidation of LDL for reducing the risk of atherosclerosis, and some compounds possessing anti-oxidative activity could have beneficial effects in the prevention of the disease. Based on our results, oolong tea water extract which suppressed LDL oxidation as compared with the control group may help to prevent the development of atherosclerosis.

Usually, the generation and scavenging of active oxygen free radicals are balanced in the human body. If there is an imbalance in the mechanism regulating anti-oxidant enzymes such as superoxide dismutase or catalase, excessive amounts of active oxygen radicals can be generated in the body. Previous studies demonstrated that stress can cause free radical reactions to produce deleterious modifications in membranes, proteins, enzymes and DNA, which may be correlated with life-style related diseases such as cancer, diabetes and atherosclerosis. So it is important to find effective scavengers of active oxygen radicals. On the one hand, it has been reported that the spin trapping method is useful for the detection of unstable radicals, which can be observed with an ESR spectrometer. In this study, we investigated the scavenging effect of oolong tea water extract on free radicals with ESR techniques. As a result, we found that the extract could effectively scavenge the free radicals generated by the hypoxanthine/xanthine oxidase reaction system. The anti-oxidant activity of oolong tea is also supported in LDL oxidation experiments. Although the active substances in the extract were not identified, studies suggest that the anti-oxidant action of oolong tea is due to active components such as polyphenols which are abundant in the tea. It has been reported that the ingestion of polyphenolic compounds, such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-gallocatechin gallate (GCG) and (-)-epicatechin (EC), which are contained in tea enhanced the anti-oxidative activity in both humans and animals, and it was discussed that the biological effects of tea are due to the anti-oxidative activities of tea catechins. Green tea and catechins have been reported to have many pharmacological properties such as hypolipidemic effects, anti-oxidative effects and scavenging effects on free radicals, thereby preventing LDL oxidation. But to our knowledge, these properties in oolong tea have never been taken notice of. The oolong tea extract was found to have strong superoxide scavenging activity as compared to green tea and black tea extracts in the present study. This may be due to the different recovery rates of water extracts in the three types of tea, or may be affected by the contents of tea extract from different localities and varieties of teas. Yan et al. calculated the amount of catechins in various tea extracts and found the greatest amount in green tea (26.7%) followed by oolong tea (23.2%) and black tea (4.3%). Ohe et al. reported that catechins are not the major components of oolong tea responsible for anti-genotoxic effects against nitroarenes. This suggests that other unique polyphenols may be contained in oolong tea. Recently, Zhu et al. found that phenolic compounds in oolong tea also showed strong anti-oxidative activities. Our results clearly demonstrated that oolong tea is a powerful anti-oxidant, which is the major factor in preventing LDL oxidation, and scavenger of active oxygen free radicals. The results encourage us to investigate the pharmacology of oolong tea for life-style-related diseases and for general health.

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