Effects of Oolong Tea on Plasma Antioxidative Capacity in Mice Loaded with Restraint Stress Assessed Using the Oxygen Radical Absorbance Capacity (ORAC) Assay

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In the present study, we investigated the antioxidative effect of oolong tea *in vitro* and *in vivo* using the oxygen radical absorbance capacity (ORAC) assay. An oolong tea extract, catechin and related compounds suppressed the oxidation of fluorescence induced by AAPH in a dose-dependent manner, that is, they prolonged the antioxidant time *in vitro*. Oral administration of the oolong tea extract to mice treated with restraint stress increased ORAC activity in plasma as compared with a stress control group. The extract also increased plasma vitamin C levels, and there was a good relationship between ORAC activity and the vitamin C level in plasma. The elevation of plasma ORAC and vitamin C level may have been related to the stress-relieving effect of oolong tea. These effects are probably due to the antioxidative properties of the tea. Thus, these findings suggested that oolong tea has beneficial effects on health related to its antioxidative action.

Key words oolong tea; antioxidant; oxygen radical absorbance capacity (ORAC)

The role of free radicals in the pathogenesis,[1] and progression of disease[2] and in the aging process has been much discussed.[3] Nutritionists have sought to understand the body's oxidation process and to prevent damage caused by rogue oxygen molecules. Studies have indicated that certain antioxidants may have additional activities, such as reducing the energy of free radicals or suppressing the generation of free radicals by interrupting the oxidizing chain reaction.[4] Antioxidants trapping free radicals and lipid peroxides may delay the onset of lipid peroxidation, stall the further production of free radicals, and inhibit damage by certain enzymes that can degrade connective tissues.[5] However, the overall antioxidative capacity in the blood seems to be of greatest significance.[6] This relationship has led to considerable interest in assessing the antioxidative capacity of foods and other nutritional antioxidant supplements.[7] In recent years, the oxygen radical absorbance capacity (ORAC) assay has been widely accepted as a tool for antioxidant assessment, and has been proposed as a method for comparing and standardizing nutritional supplements.[8]

ORAC was measured using disodium fluorescein as fluorescence, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, which is relevant to biological systems because the peroxyl radical is the most abundant free radical. Trolox, a water-soluble analogue of vitamin E used as a reference standard, and the loss of fluorescence were monitored.[9] The antioxidative effects of oolong tea were expressed in ORAC, where one ORAC unit equals the fluorescence decay inhibited by 1 mM trolox. The assay calculates the ability of a product to protect against damage induced by oxygen free radicals.[10]

Tea is the most popular beverage in the world.[11] 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. It 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.[12] 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.[13] The quantities of ingredients depend on the extent of fermentation. Oolong tea contains a large quantity of polyphenols, different from green or black tea.[14]

Polyphenols, chemical compounds produced by plants to protect themselves from foreign invasion, inhibit lipid oxidation and scavenge superoxide radicals.[15] The polyphenols in oolong tea give the drink its unique flavor and also improve beauty and health.[16] In China, oolong tea has traditionally been considered to have anti-obesity[17] and hypolipidemic effects,[18] and habitual ingestion is thought to be effective in enhancing the metabolic rates of fat oxidation.[19] According to research, there are many other compounds in the tea that can protect against oxidative damage. Tea polyphenols also reduce DNA damage caused by oxidative agents *in vitro*,[20] prevent the formation of peroxide free radicals, and inhibit the oxidation of LDL cholesterol.[21]

However, it is not known which components of oolong tea are responsible for its ORAC, and little attention has been given to the influence of oolong tea on antioxidative capacity. Therefore, an evaluation of the antioxidative effect of oolong tea was performed using the ORAC assay *in vitro* and *in vivo*.

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. The leaves were treated with 15 parts of boiling water for 5 min. After filtration, and evaporation of the water, the residue was powdered under frozen-decompression conditions. The recovery rate was 21.3%.

The concentrations of caffeine, gallic acid, flavanols, and other polyphenols in the oolong tea were analyzed by high-

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Table 1. Concentrations of Caffeine and Polyphenols in Oolong Tea

Components	Oolong tea (mg/100 ml)
Gallic acid	2.19
Caffeine	23.51
Gallocatechine	6.68
Epigallocatechine	16.14
Catechine	1.65
Epicatechine	5.08
Epigallocatechine gallate	25.73
Allocatechine gallate	1.85
Epicatechine gallate	5.73
Catechine gallate	0.60
Polymerized	33.65
Total polyphenols	99.32

Data are mean amounts of oolong tea components consumed daily.

performance liquid chromatography (HPLC) with UV detection at 280 nm.[14] The analysis was performed with a Cosmosil 5PE-MS column (4.6 mm internal diameter ×150 mm; Nakalai Tesque, Kyoto, Japan) at 40 °C. Compounds were eluted (eluent A: 0.05% trifluoroacetic acid in water; eluent B: 0.05% trifluoroacetic acid in acetonitrile) at a flow rate of 2 ml/min using a gradient program (eluent B content: 10% for 5 min, 21% for 8 min, 90% for 1 min, and 90% for 6 min). The quantification of caffeine, gallic acid, and flavanols was made using standard calibration curves for marketed compounds. Other polyphenols were quantified using a calibration curve that was derived from polyphenols isolated from tea by HPLC. The concentrations of caffeine and polyphenols in oolong tea are shown in Table 1.

Animals Male ICR mice, 7 weeks old, were purchased from Charles River Japan, Inc. (Tokyo, Japan). The animals were kept in a specific pathogen-free animal room at 23± 1 °C with a 12-h light–dark cycle (lights on from 0600 to 1800 h), and were fed standard laboratory chow (CE-2; CLEA Japan, Inc.) and tap water. The animals were kept under these conditions for 1 week before the experiment. In the restraint stress experiment, each mouse was confined to an oval metal restraint cage for 6 h before the plasma ORAC assay. The care and treatment of the animals conformed to the guidelines established by the Japanese Society of Nutrition and Food Science (Law No. 105 and Notification No. 6 of the Japanese government).

Chemicals and Preparation Ascorbic acid, disodium fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue as a control standard), and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, as a peroxyl radical generator) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Catechin, (−)-epicatechin (EC), and (−)-epigallocatechin gallate (EGCG) were purchased from Sigma Chemical Co., and used as a positive control in the present experiment. Ascorbic acid and fluorescein were directly dissolved in an acetone/water mixture (50 : 50, v/v), and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Trolox, AAPH, and the oolong tea extract were dissolved in the potassium phosphate buffer immediately before the ORAC assay. This provide fluorescein and AAPH in final concentrations of 63 nM and 12.8 mM, respectively. Catechin, EC, and EGCG were dissolved at 1 mg/ml in ethanol before being appropriately diluted with the same buffer solution. For the *in vivo* experiment, the oolong tea extract was dissolved in distilled water before use.

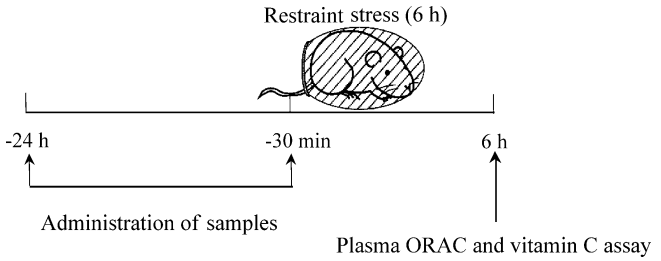

Fig. 1. Experimental Schedule for ICR Mice Exposed to Restraint Stress

ICR mice were fixed in the restraint cage for 6 h before the plasma ORAC and vitamin C assay.

Assessment of Antioxidative Capacity *in Vitro* and Plasma Antioxidant Status The automated ORAC assay was carried out on a Labsystems Fluoroskan Ascent plate reader (Helsinki, Finland) with fluorescent filters (excitation wavelength: 485 nm, and emission filter: 527 nm), as previously described.[22] Fluorescein was used as a target of free radical attack, with AAPH as a peroxyl radical generator. Trolox was used as a control standard. Final results were calculated based on the difference in the area under the fluorescein decay curve between the blank and each sample. An oolong tea solution was administered to animals orally at 0.1 ml/10 g body weight 24 h and 30 min before the restraint stress. Ten animals in each group were sacrificed for analysis of antioxidative capacity after the restraint stress. Blood samples were taken from the heart, under anesthesia with diethyl ether, 6 h after the stress (Fig. 1), into a tube containing 2% sodium heparin. Each tube was centrifuged at 5000 rpm for 5 min, and the plasma was immediately placed in an ice bath until appropriately diluted with pH 7.4 phosphate buffer before the ORAC assay. To measure the ORAC in the nonprotein fraction, the plasma was treated with 60% perchloric acid (PCA) to adjust the concentration to 3%, and the sample was centrifuged at 15000 rpm for 15 min at 4 °C. Then, the supernatant was removed as the plasma nonprotein fraction, and appropriately diluted with pH 7.4 phosphate buffer for the analysis.

Assessment of Vitamin C level in Plasma Plasma vitamin C analysis was carried out using an automatic serum analyzer (model 7070, Hitachi Co., Ltd., Japan) and by the ascorbate oxidase method.[23]

Statistics Data are expressed as the mean±S.D. Statistical analyses of data were performed with Student's *t*-test. Differences were considered to be significant when the p value was less than 0.05.

RESULTS

Antioxidative Capacity of Oolong Tea Extract *in Vitro* Figure 2 shows the working curves of fluorescein oxidation used as an index of resistance time for the oxidative reaction. The linear relationship between the net area and different concentrations of the antioxidant was evaluated. Figures. 2A and B represent the trolox and vitamin C, a single antioxidant, and fluorescence decay curves, respectively. A linear regression curve of two concentrations (125, 65 ng/ml) versus the net area under the curve was obtained. Trolox in-

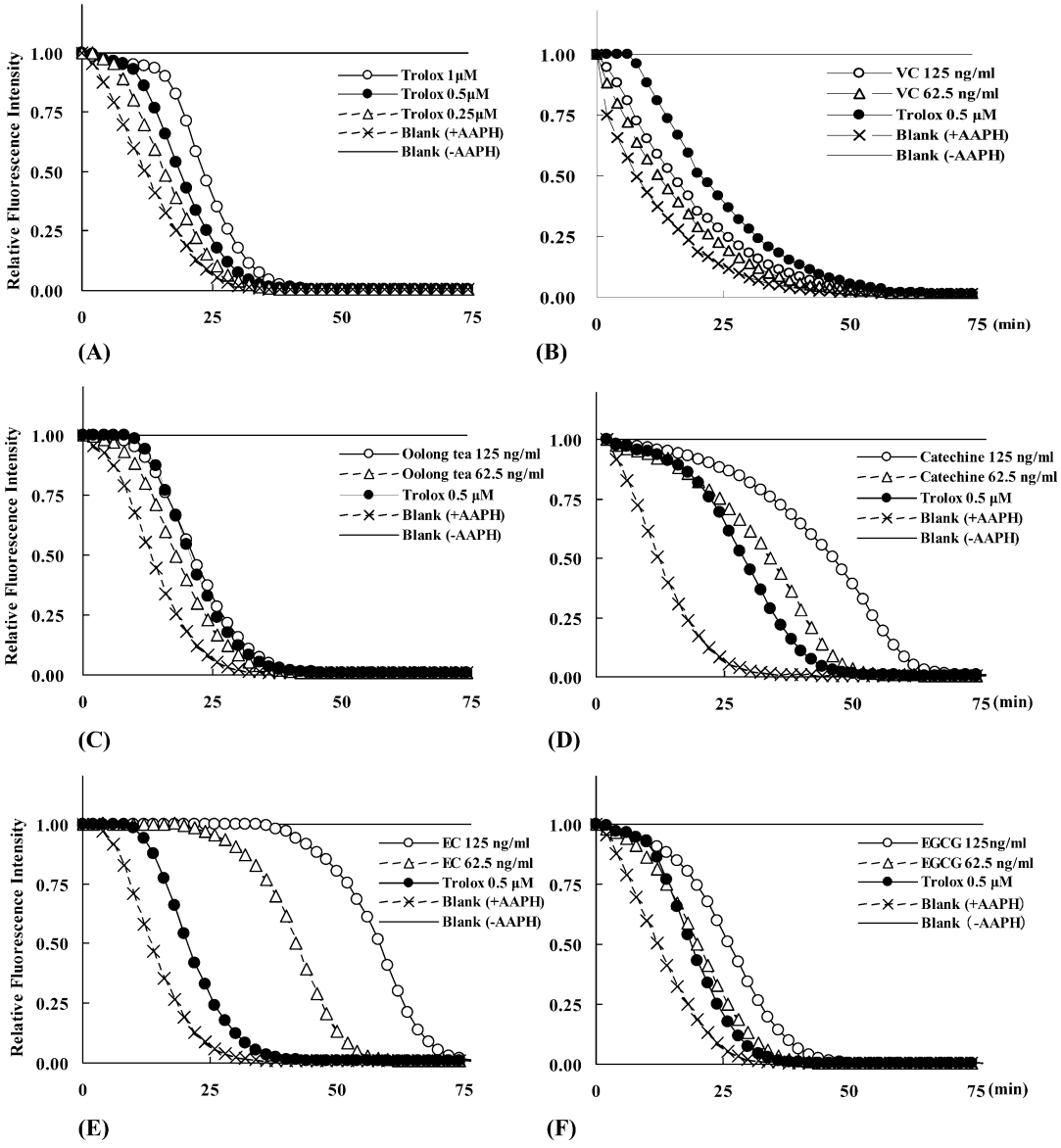

Fig. 2. Dose-Dependent Inhibition, by Oolong Tea Extract and Catechines, of Fluorescence Decay Induced by AAPH

Curves of fluorescence decay induced by AAPH as a peroxyl radical generator in the presence of oolong tea extract of or catechin compounds at different concentrations are shown. Trolox, a water-soluble vitamin E analogue, was used as a control standard. The antioxidative activity of a sample is expressed as the net area under the curve. Data are expressed as means of three experiments.

creased the level of scavenging activity for the oxidation of Fluorescein in a dose-dependent manner, and the antioxidative capacity of vitamin C was weaker than that of trolox. In Fig. 2C, quenching curves of disodium fluorescein illustrate the ability of oolong tea to absorb the peroxyl radical as compared to that of the standard trolox. The two concentrations of oolong tea extract gave a good fit to the linear regression equation. As seen in Fig. 2D, when the ORACs of catechin, EC (Fig. 2E), and EGCG (Fig. 2F), which are polyphenolic compounds contained in the tea extract, were compared using the area under the curve, a good correlation was obtained between catechin content and ORAC activity. From the curves, it was clear that the catechins inhibited the process, and levels remained high until 75 min compared to the basal and Trolox curve. The antioxidative capacity of the catechins was greater than that of the oolong tea extract, and the catechin content correlated with their antioxidant capacity. The capacity of the catechins was in the order of EC, catechin, EGCG and oolong tea extract. The order is similar to that for their radical scavenging effects.[24)]

Effect of Oolong Tea Extract on Plasma Antioxidative Capacity Whole plasma and protein free plasma were analyzed using the same procedure, but with different sample preparation procedures. ORAC in the plasma treated with PCA reflects the antioxidative capacity of the nonprotein antioxidants in blood, because PCA preserves water-soluble antioxidants, including vitamin C.

The ORAC for whole plasma *in vitro* increased in a dose-dependent manner, as shown in Fig. 3A. However, when the plasma protein was removed, the ORAC value was about 1/10 that for whole plasma (Fig. 3B). As shown in Fig. 4, 250 mg/kg of oolong tea extract and 250 mg/kg of vitamin C, doses determined in preliminary experiments, were administered orally 24 h and 30 min before the stress, respectively.

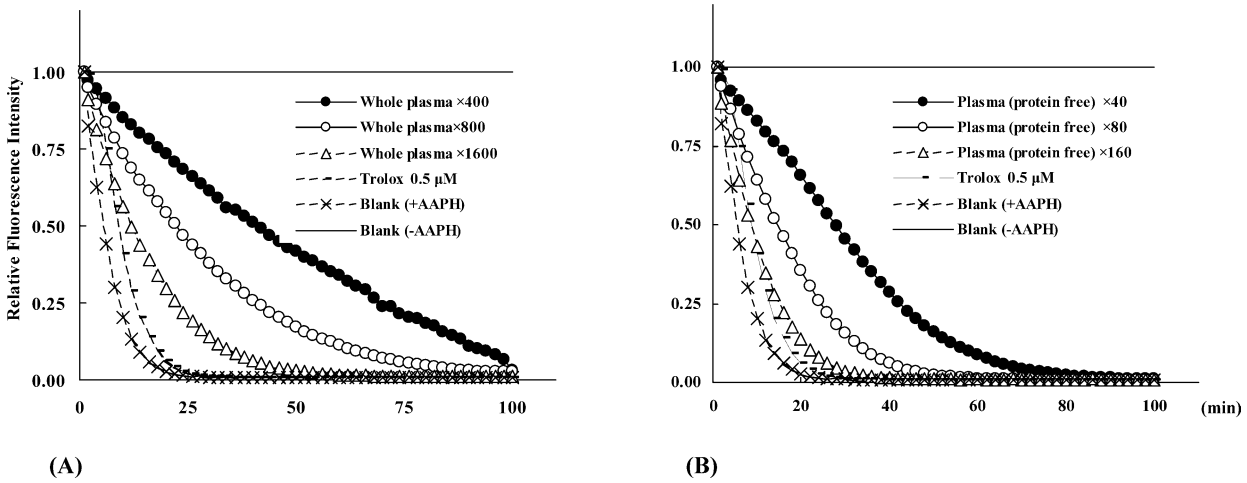

Fig. 3. Dose-Dependent Inhibition by Plasma of Fluorescence Decay Induced by AAPH

Curves of fluorescence decay induced by AAPH as a peroxyl radical generator in the presence of oolong tea extract, or catechin compounds at different concentrations, are shown. Trolox, a water-soluble vitamin E analogue, was used as a control standard. The antioxidative activity of a sample is expressed as the net area under the curve. Data are expressed as means of three experiments.

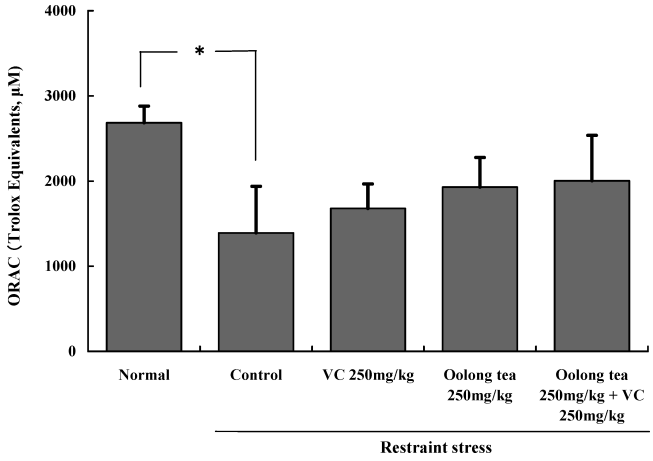

Fig. 4. Effect of Oolong Tea Extract on Plasma Antioxidative Capacity

The ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 μM. The area under the curve for the sample is compared to the area under the curve for Trolox, and the antioxidative value is expressed in micromoles of Trolox equivalent per liter. The results represent the mean±S.D. of values obtained from 7 animals in each group. The significance of differences from the normal control mice at $*p<0.05$ was determined with Student's *t*-test.

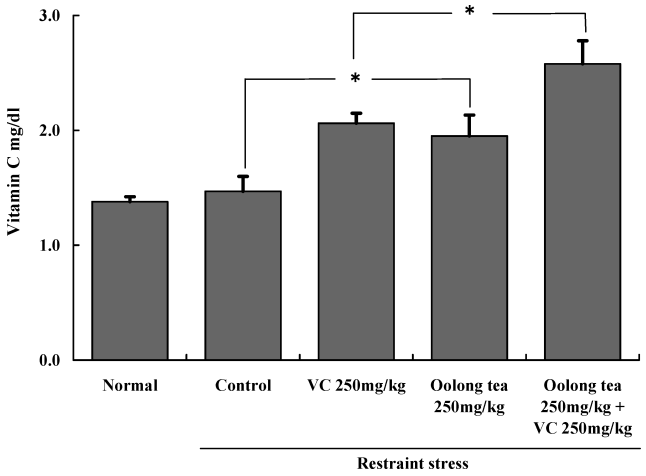

Fig. 5. Effect of Oolong Tea Extract on Plasma Vitamin C Levels

Plasma vitamin C was analyzed using the ascorbate oxidase method. The results represent the mean±S.D. of values obtained from 7 animals in each group. The significance of differences from the normal control mice at $*p<0.05$ was determined with Student's *t*-test.

The same volume of water was given as a control. The plasma ORAC value was calculated as the ratio of the area under the fluorescence decay curve for 0.5 μM Trolox as a standard. In normal mice, the plasma ORAC level was 2684.2±519.1 mM Trolox eq., while the ORAC value observed 6 h after stress was remarkably decreased (1390.3±547.7 mM trolox eq., $p<0.05$), as shown in Fig. 4. The results suggest that the stress plays an important role in increasing oxidative stress. Average values for ORAC of vitamin C, oolong tea, and vitamin C plus oolong tea were 1678.1±288.0, 1929.2±346.7, and 1954.0±569.1 mg/g, respectively. The respective total antioxidative capacity of plasma increased 20.7%, 38.8% and 40.5% compared with the stress control, although the change was not significant.

Effect of Oolong Tea Extract on Plasma Levels of Vitamin C Vitamin C concentrations of blood samples were analyzed 6 h after the restraint stress (1.45±0.13 mg/dl). The control value (restraint mice) was not significantly decreased compared with normal mice (1.38±0.04 mg/dl). As shown in Fig. 5, when mice were given 250 mg/kg of the oolong tea extract, their plasma vitamin C levels increased significantly compared with the stressed mice (1.95±0.18 mg/dl, $p<0.05$). Levels in the vitamin C alone group were 2.06±0.08 mg/dl, which was 141.1% the value for stress control mice. Our results also show that the vitamin C concentration in mice treated with oolong tea extract plus vitamin C was 2.58±0.20 mg/dl, a remarkable increase as compared with the group given vitamin C alone ($p<0.05$).

DISCUSSION

In this study, we estimated the scavenging effect of oolong tea extract *in vitro*, and its effect on the antioxidative capacity plasma, with the ORAC assay. As shown in Fig. 2C, the tea extract scavenged the free radicals generated by AAPH in a

dose-dependent manner. In addition, we also examined ORAC values of plasma in the restraint mice and in mice treated with the oolong tea extract by oral-administration. The results showed that the extract changed the oxidative status of plasma in mice subjected to stress. Oolong tea may decrease the oxidative level of plasma by reducing stress, because the total antioxidative capacity of plasma is tightly regulated in part through a homeostatic mechanism. Additionally, the result following the consumption of oolong tea is also partly due to the antioxidants in the tea.

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.[25] Usually, the generation and scavenging of active oxygen free radicals are balanced in the human body. If there is a failure in the mechanism regulating antioxidant enzymes, such as superoxide dismutase or catalase, however, excessive amounts of active oxygen radicals can be generated.[26] So it is important to find effective scavengers of free radicals for general health, and it is also often assumed that antioxidant nutrients such as fruits, vegetables and tea contribute to the prevention of life-style-related diseases.

Oolong tea has been reported to have several pharmacological properties, including hypoglycemic effects,[27] antioxidative effects[28] and scavenging effects on free radicals,[29] thereby preventing the oxidation of LDL.[21] Although there are few reports on the absorption and metabolism of oolong tea, the increase in plasma ORAC following treatment with oolong tea extract indicated that the extract enhanced the production of endogenous antioxidants,[30] including an increase in SOD activity,[31] or that antioxidants contained in the extract suppressed the generation of oxidants in plasma. Some studies suggest that the major antioxidative action of oolong tea is due to active components such as polyphenols which are abundant in the tea.[32] In the present study, the total polyphenol content of oolong tea was analyzed, revealing that drinking 100 ml of oolong tea provides 99.3 mg of polyphenolic compounds. Therefore, oolong tea can be considered a better source of antioxidants than most beverages. According to research, the ingestion of polyphenolic compounds, such as EGCG, (−)-epigallocatechin (EGC), (−)-gallocatechin gallate GCG and EC, which are contained in tea, enhances antioxidative activity in both humans[33] and animals.[34] Our results showed that catechin, EC and EGCG are more powerful radical scavengers than the oolong tea extract, and in terms of antioxidative activity ranked in the order EC, catechin and EGCG. Moreover, there is much less EC than EGCG in the tea catechins. From these findings, it is concluded that the catechins are effective compounds, and play a major role in the antioxidative activity of oolong tea.

In general, the total antioxidative capacity of plasma appeared to be due to the total activity of a variety of compounds, including vitamin C, polyphenols, and possibly other endogenous components. In recent years, numerous studies have shown that endogenous antioxidants include enzymes, coenzymes and sulfur-containing compounds such as glutathione.[35] Exogenous antioxidants include vitamins C and E, and carotenes.[36] Vitamin C is the most famous antioxidant found in fruits and vegetables, and has promising applications in anti-aging. Epidemiological studies have shown that high vitamin C diets substantially decrease the risk of most diseases by neutralizing free radicals.[37] Recent studies have demonstrated that both lipid-soluble vitamin E and water-soluble vitamin C work together, and that a balance between them is needed to reduce free radical generation in the water and lipid layers in tissue.[38]

Plasma vitamin C as a marker for oxidative stress was not significantly decreased after 6 h of restraint stress. It has been reported that humans lack the ability to synthesize vitamin C because of a loss of function in the gene (Gulo) coding for a key synthetic enzyme, L-gulono-γ-lactone oxidase,[39] whereas mice having the functional gene can make vitamin C on their own. As a consequence, mouse tissues generally have high levels of vitamin C, which are only slightly influenced by exogenous vitamin C. On stress treatment, we found that the plasma vitamin C level increased slightly compared to the normal control. It was suggested that stress may induce the release of vitamin C from the body pool including neutrophils, leukocytes and tissues to plasma. Oxidative stress is known to increase the metabolic turnover of vitamin C due to oxidation by free radicals and reactive oxygen species generated by stress. In contrast, humans depend entirely on vitamin C derived from diet. Ingestion of the oolong tea extract in restrained mice significantly increased plasma vitamin C levels compared with the stress control. Although the efficacy of oolong tea was not made clear in this study, the results suggest that oolong tea suppresses the oxidative degradation of plasma vitamin C, and there was a sparing effect by the other antioxidants from the tea extract.

We conclude that the anti-stress activity of oolong tea is due to an increase in plasma antioxidative capacity achieved by protecting plasma vitamin C, as well as by a relaxing effect. Our study provides primary evidence *in vivo* of an increase in antioxidative capacity, as well as synergism, between vitamin C and oolong tea extract. The results encourage us to investigate the pharmacology of oolong tea for life-style-related diseases and for general health.

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