Effects of Japanese Black Tea on Atherosclerotic Disorders

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The atherogenic index was found to be significantly better in rats fed a high-cholesterol diet supplemented with black tea extract than in the ones not given the extract. It was also evident that black tea inhibited the proliferation of smooth muscle cells involved in the development and progression of atherosclerosis, and suppressed the production of oxidized low-density lipoprotein, a cause of lipid accumulation. It thus seems likely that black tea has an antiatherosclerotic action.

Key words: black tea; kurocha; atherosclerosis; rat

Birudan, in Asahi-machi, Niikawa-gun, Toyama Prefecture, is a mountain village with a population of about 600 people in about 120 households. People in Birudan have a traditional tea ceremony in which frothy Japanese black tea (Camellia sinensis L., Kurocha, is prepared. The tea used for this ceremony falls under the category of late-fermented tea, being processed in a different way from green tea or regular black tea. The tea leaves are steamed immediately after picking and fermented in a process that lasts about 20 days. Green tea is prepared by steeping tea leaves in hot water for a few minutes, while black tea is an extract prepared by boiling for several hours. For this reason, the contents of (−)-epigallocatechin 3-O-gallate and the antioxidant vitamins in black tea are much lower than those in green tea. In recent years, surveys on the residents of Birudan have been done from the viewpoint of public health nutrition as well as in its folkloric aspects. However, there is no report on the biological activity of the tea prepared by prolonged boiling. In this study, black tea was examined for its effects on serum cholesterol levels, oxidized low-density lipoprotein (LDL), and vascular smooth muscle cells in relation to atherosclerosis.

Materials and Methods

Medium and reagents. RPMI 1640 medium and fetal bovine serum were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Cell Culture Laboratories (Cleveland, Ohio), respectively. [Methyl-3H]thymidine (248 GBq/mmol) was obtained from New England Nuclear Corp. (Boston, MA). LDL was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Black tea. One hundred grams of commercial black tea (cultivated in Toyama Prefecture) was boiled gently in 1,000 ml of water for 5 h. The extract was evaporated to dryness under reduced pressure. The yield was about 15.8%.

Animal experiment. Male LWH: Wistar rats weighing 150–160 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). The rats were kept in wire-bottomed cages under a conventional lighting regimen with a dark night. The room temperature (about 23°C) and humidity (about 60%) were controlled automatically. The animals were pair-fed a laboratory chow diet (powdered CE-2 of CLEA Japan Inc., Tokyo, Japan) containing 1% cholesterol and 0.5% cholic acid. Black tea was dissolved in water, and given to rats orally every day as drinking water. The dose was adjusted to 100 or 200 mg/kg body weight by regulating its concentration in response to water consumption. Control rats were given access to water correspondingly. Six rats were used for each experimental group. After 20 days, the rats were killed by decapitation between 1300 and 1400 to avoid any effect of circadian variation. The blood samples were allowed to clot at 4°C, and then centrifuged. The sera obtained in this manner were used for measurements of their chemical parameters.

Measurement of serum cholesterol. Total cholesterol and free cholesterol were measured using commercial assay kits (Cholesterol E-Test Wako and Free Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan). LDL- and high-density lipoprotein (HDL)-cholesterol were measured by the method of Noma et al. Cell culture. Smooth muscle cells were isolated from bovine aorta by scraping the surface of intimae. The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum in a 60-mm dish at 37°C under a humid atmosphere of 5% CO₂ in air until confluent. The cells were then transferred to 35-mm dishes at about 10⁶ cells/well, cultured in the presence of 0.1–25 μg/ml black tea extract for 24 h, and labelled with 0.037 MBq/ml [3H]thymidine during the last 6 h of culture. After culture, the cell layer was washed twice with Ca, Mg-free phosphate-buffered saline (CMF-PBS) and the cells were scraped off with a rubber policeman in the presence of CMF-PBS. The cell homogenate was prepared by sonication and a sample was used for the measurement
of DNA by the method of Kissane and Robins.\(^4\) The incorporation of \(^{3}H\)thymidine into the 5\% trichloroacetic acid (TCA)-insoluble fraction of the cell homogenate was measured by liquid scintillation counting using a portion of the cell homogenate. All assays were done with 5 measurements.

**Measurement of oxidized LDL.** By the method reported by Kuzuya et al.,\(^3\) LDL was diluted with 0.15 M NaCl (pH 7.4) to a concentration of 150 \(\mu\)g protein/ml, and incubated with 20 \(\mu\)M CuSO\(_4\) at 37\(^o\)C for the stated periods. The extent of lipid peroxidation was estimated fluorometrically as thiobarbituric acid (TBA)-reactive substances.\(^5\) For this purpose, oxidized LDL (20 \(\mu\)g protein) was mixed with 1.5 ml of 0.67\% TBA and 1.5 ml of 20\% TCA. After this was heated at 100\(^o\)C for 45 min, the fluorescent reaction products were assayed on a spectrofluorometric detector (Shimadzu RF-550, Kyoto, Japan) with excitation at 515 nm and emission at 553 nm. Malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane was used as a standard. The values were obtained as MDA equivalents (nmol) for five measurements.

**Spin-trapping assay.** Electron spin resonance (ESR) spectroscopy combined with spin-trapping using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used to identify radical species. Twenty microliters of DMPO was added to 100 \(\mu\)l of 100 \(\mu\)M homoaarginine solution and 200 \(\mu\)l of 10 \(\mu\)g/ml black tea extract, followed by stirring for 10 s. The ESR spectra of this mixture were measured with a JEOL FE-3X type spectrometer (JEOL, Tokyo, Japan: X band, 100 kHz modulation) at 30\(^o\)C at 5 min after the addition of DMPO. Microwave power, modulation amplitude, and sweep time were set at 8 mW, 0.1 mT, and 0.5 min, respectively. Two peaks of external manganese dioxide appearing at \(g=1.981\) and \(g=2.034\) were used for measurement of both the g-value and the amount of each DMPO-adduct. The radical species was assigned by comparing the observed spectra with the calculated ones. The previously reported values of the hyperfine splitting constants of the DMPO-adduct of a carbon-centered radical (DMPO-C) (\(a(N) = 1.58\) mT and \(a(\beta H) = 2.42\) mT), the DMPO adduct of the hydroxyl radical (DMPO-OH) (\(a(N) = 1.49\) mT and \(a(\beta H) = 1.49\) mT), and the DMPO adduct of the hydrogen radical (DMPO-H) (\(a(N) = 1.66\) mT and \(a(\beta H) = 2.25\) mT) were used for the calculation. g-Factors for all the spin adducts were 2.006.\(^7\,8\)

**Statistics.** Data are presented as mean \(\pm\) S.E. Differences among groups were analyzed by Dunnett’s method,\(^9\) and considered significant at \(p < 0.05\).

**Results**

**Body weight and food intake**

There were no significant differences in body weight changes between the control and black tea-treated groups, nor were there any significant differences in food intake.

**Serum cholesterol**

Table I compares the levels of serum cholesterol in the black tea-treated rats with those in the control ones. Administration of black tea at a dose of 100 mg/kg body weight/day for 20 days tended to decrease the level of total cholesterol, although not significantly. A further increase in the dose to 200 mg produced a further decrease in the total cholesterol level. A statistically significant difference in the free cholesterol level between the black tea-treated rats and non-treated rats was obtained at a daily dose of 200 mg/kg body weight, its level being 0.79 mmol/L. Similarly, administration of black tea to rats resulted in a decrease of LDL-cholesterol from 3.51 to 3.11 mmol/L at the 100-mg level and from 3.51 to 2.81 mmol/L at the 200-mg level. In contrast, the HDL-cholesterol level, which was 0.78 mmol/L, increased significantly at both the 100 and 200 mg dosage levels as compared with that in the control rats. These changes were inversely related to those in total cholesterol, free cholesterol, and LDL-cholesterol, as shown in Table I. The atherogenic index was 4.79 in the control rats not given black tea, and the corresponding value in rats given the preparation was decreased significantly to 3.51 at the 100-mg level (a 27% decrease, \(p < 0.001\)) and to 2.55 at the 200-mg level (a 47% decrease, \(p < 0.001\)).

**Cultured smooth muscle cells**

Figure 1 shows the proliferative activity of cultured smooth muscle cells in terms of \(^{3}H\)thymidine uptake. The \(^{3}H\)thymidine uptake into the TCA-insoluble fraction of smooth muscle cells was 1759 dpm/\(\mu\)g DNA in the absence of black tea. In the presence of 10 \(\mu\)g/ml black tea, the \(^{3}H\)thymidine uptake was significantly decreased by 16% to 1482 dpm/\(\mu\)g DNA. When the concentration of the black tea was increased to 25 \(\mu\)g/ml, the proliferation of smooth muscle cells was decreased further. However, the \(^{3}H\)thymidine uptake showed no significant change in the presence of 0.1 and 1 \(\mu\)g/ml black tea.

**Table 1. Effects of Black Tea on Serum Cholesterol Levels**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg B.W./day)</th>
<th>T. Chol. (mmol/L)</th>
<th>Free Chol. (mmol/L)</th>
<th>LDL-Chol. (mmol/L)</th>
<th>HDL-Chol. (mmol/L)</th>
<th>A.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>4.53 (\pm) 0.15</td>
<td>0.91 (\pm) 0.06</td>
<td>3.51 (\pm) 0.13</td>
<td>0.78 (\pm) 0.03</td>
<td>4.79 (\pm) 0.24</td>
</tr>
<tr>
<td>Black tea</td>
<td>100</td>
<td>4.33 (\pm) 0.16</td>
<td>0.83 (\pm) 0.05</td>
<td>3.11 (\pm) 0.10a</td>
<td>0.96 (\pm) 0.06a</td>
<td>3.51 (\pm) 0.19a</td>
</tr>
<tr>
<td>Black tea</td>
<td>200</td>
<td>4.00 (\pm) 0.12a</td>
<td>0.79 (\pm) 0.05a</td>
<td>2.81 (\pm) 0.10a</td>
<td>1.13 (\pm) 0.09a</td>
<td>2.55 (\pm) 0.18a</td>
</tr>
</tbody>
</table>

T. Chol., total cholesterol; Free Chol., free cholesterol; LDL-Chol., low-density lipoprotein-cholesterol; HDL-Chol., high-density lipoprotein-cholesterol; A.I., atherogenic index. A.I. was calculated on the basis of T. Chol. and HDL-Chol., using the following equation: A.I. = (T. Chol.-HDL-Chol.)/HDL-Chol. Statistical significance: \(p < 0.05\), \(p < 0.01\), \(p < 0.001\) vs. control value.
**Fig. 1.** Effects of Black Tea on the Proliferation of Cultured Smooth Muscle Cells.
Statistical significance: *p* < 0.001 vs. control value.

**LDL peroxidation**
LDL was time-dependently oxidized by CuSO4, leading to peroxidation. This peroxidation was suppressed when black tea was present in the incubation mixture. Table II shows the course of formation of TBA-reactive substance in the reaction medium supplemented with 4 different concentrations of black tea extract, of which 1 and 10 μg/ml (final concentration) had no effect. However, as the concentration increased, the slopes of the curves decreased, demonstrating dose-dependent inhibitory activity.

**Spin trapping**
Figure 2 shows the influence of the presence of 10 μg/ml black tea extract on the ESR spectrum of a mixture of 100 mM homoarginine aqueous solution (100 μl) and DMPO (20 μl). The ESR spectrum obtained in the absence of black tea extract is shown in Fig. 2(A). Some of the peaks assigned to DMPO-C, DMPO-OH, and DMPO-H are shown at the bottom of the figure. Although all the peaks of DMPO adducts are not evident, DMPO-C, DMPO-OH, and DMPO-H appear in this spectrum. With regard to the spectral intensities of Mn2+ used as a standard, the signals from Fig. 2(B) demonstrate that these peaks were reduced in the black tea-treated group.

**Discussion**
Previous reports concerning the biological activity of tea have documented its beneficial effects such as anticancer, antioxidant, anticaries, intestinal flora-improving, antibacterial, antiviral, antihyperlipemic, antiobesitic, antiaging, antiabetic, antihypertensive, and renal failure-improving effects.10-17 However, these studies refer to green tea in most cases, and few data are available for the biological activity of black tea prepared by a special process. The benefits of black tea as a healthy drink are now more generally appreciated, along with the recent health food boom. In this connection, we investigated the effects of black tea against hypercholesterolemia, using rats fed a high cholesterol diet, and found a significant decrease in the serum total cholesterol in rats given black tea extract. A similar effect was observed for the serum levels of free and LDL-cholesterol, suggesting that black tea would exert an antihypercholesterolemic effect i.e. an antiatherosclerotic effect.

In the pathogenesis of atherosclerosis, a localized change in the tunica intima, intimal thickening, induces

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**Table II.** Effects of Black Tea during Oxidation of LDL.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>—</td>
<td>0.170±0.014</td>
</tr>
<tr>
<td>1</td>
<td>0.168±0.012</td>
</tr>
<tr>
<td>10</td>
<td>0.171±0.012</td>
</tr>
<tr>
<td>25</td>
<td>0.169±0.013</td>
</tr>
<tr>
<td>50</td>
<td>0.172±0.015</td>
</tr>
</tbody>
</table>

Values given are in MDA nmol/ml. Statistical significance: *p* < 0.05, *p* < 0.001 vs. pre-incubation value; *p* < 0.01, *p* < 0.001 vs. non-black tea additive value.
luminal stenosis and sclerosis of the artery. A fundamental factor involved in atherogenesis is the proliferation of smooth muscle cells. Although there are hardly any smooth muscle cells in the normal arterial intima, they migrate from the tunica media to the intima in response to certain stimuli from the intimal side, and are stimulated by various factors to proliferate and produce connective tissue. This causes intimal thickening, and hence luminal stenosis. Therefore, it is generally considered that the migration of smooth muscle cells from the media to the intima and their proliferation in the intima are major events in the formation of an atherosclerotic lesion. On the other hand, according to Chamley-Campbell et al.,19,20 migrating smooth muscle cells and cells cultured in serum-supplemented medium proliferate rapidly, secreting a growth factor that acts on the cells themselves to induce a metabolic pathway of degenerative LDL involved in foam cell formation, thereby contributing to both cell proliferation and cholesterol accumulation in the atherosclerotic lesion. Using [3H]thymidine uptake as an index, we measured the effects of black tea extract on the proliferation of smooth muscle cells, which would develop into a pathologic condition. The proliferation of smooth muscle cells was suppressed in the presence of black tea extract at a concentration of 10 μg/ml or higher; the suppression of cell proliferation was statistically significant when the concentration of black tea extract was 25 μg/ml. These results suggest that black tea prevents foam cell formation by suppressing the accumulation of lipids.

On the other hand, smooth muscle cells are known to essentially have a metabolic pathway in which LDL is incorporated via LDL-receptors. However, Steinberg et al.21 have found that smooth muscle cells derived from the atherosclerotic intima have scavenger receptors for modified LDL which are free from down-regulation, suggesting that LDL modification is a cause of lipid accumulation in smooth muscle cells. At present, no biological factors besides lipooxygenase in endothelial cells are definitely known to elicit LDL modification. However, Parthasarathy et al.22 have reported that Cu²⁺ directly elicits modification of LDL in vitro (and Fe³⁺ indirectly, working on glycated LDL). In this experiment, we used this reaction system for identifying the effects of black tea, and we observed concentration-dependent inhibition of LDL oxidation. This observation suggests that black tea extract has its antiatherosclerotic effect by inhibiting not only the proliferation of smooth muscle cells in atherogenesis but also the production of oxidized LDL, which accumulates in the atherosclerotic lesion. However, these results also suggest that the peroxidation is not a one-step reaction but a series of steps, as reported by Esterbauer et al.23 Therefore, the inhibiting action of black tea extract on MDA generation from oxidized LDL may include more than a single mechanism. Considering that a chelation reaction might be involved, we did a preliminary test by adding an equimolar amount of CuSO₄ to the extract, in the absence of LDL, to observe the resulting alteration of the ultraviolet spectrum. To obtain peaks with a higher resolving power, since separate resolution was not possible in the general spectra following addition of copper, the spectra were studied by quadratic differential analysis. The black tea extract produced obvious alterations in the absorption peaks from 200 to 230 nm, indicating that they induced some reaction with Cu²⁺.

Superoxide (O₂⁻) or other free radicals derived from O₂ can be generally regarded as factors causing LDL modification. Parthasarathy et al.22 have shown that there is no direct oxidation of LDL in the O₂⁻ production system, with minimal true inhibition of LDL modification by superoxide dismutase (SOD). On the other hand, Bedwell et al.24 have demonstrated that ·OH and ·OOH induce oxidation of LDL, indicating the important role of these radicals in LDL oxidation. Based on the fact that ·OH is generated in homoaergine solution,25 we investigated the action of black tea extract and confirmed its ·OH-eliminating action. We have also demonstrated using the ESR technique that black tea extract is an antioxidant that inhibits the formation of radicals. Although it is generally considered that cells play a minor role in the modification of LDL, merely providing an oxidative environment, we believe it is necessary to further investigate the effects of black tea extract on cultured smooth muscle cells.

Vitamin E reportedly serves as the initial barrier against oxidation of LDL, and when it is depleted, the antioxidant action of carotenoids takes effect. It is also reported that the presence of water-soluble antioxidants (uric acid, ascorbic acid) delay the destruction of primary antioxidant substances in LDL.26 We intend to study the difference between these antioxidants and black tea in a future investigation.

References

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