Inhibitory effect of Chinese green tea on endothelial cell-induced LDL oxidation

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Abstract

Green tea has been shown to inhibit Cu\(^{2+}\)-induced LDL oxidation and suppress lipoxygenase activity. Since LDL oxidation is a characteristic feature of atherogenesis and lipoxygenase is involved in the disease process, the effect of Lung Chen Tea, a non-fermented Chinese green tea, on LDL oxidation induced by human umbilical cord vascular endothelial cell was investigated in the present study. Lung Chen Tea was extracted with methanol and the dried powder was redissolved in water before extraction with chloroform and then ethyl acetate. Lung Chen Tea, chloroform and ethyl acetate fractions dose-dependently reduced LDL oxidation and decreased its relative electrophoretic mobility \( (P < 0.001) \) when compared to the oxidized LDL. The lipid peroxidation products, thiobarbituric acid reactive substances, and cellular cholesterol were also significantly lowered by 5 and 10 \( \mu g/ml \) Lung Chen Tea \( (P < 0.001) \) in a dose-dependent manner. The remaining aqueous layer, which was devoid of catechins after chloroform and ethyl acetate extractions, did not prevent LDL oxidation. The results of this study demonstrated that Lung Chen Tea and catechin-rich fractions significantly prevented endothelial cell induced LDL oxidation. The consumption of Lung Chen Tea may therefore lower the risk of coronary heart diseases. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Chinese green tea; Lung Chen Tea; LDL oxidation; Cholesterol; Relative electrophoretic mobility; TBARS

1. Introduction

Lipid laden foam cells are characteristic of atherogenesis and it is widely accepted that oxidative modification of low-density lipoprotein is necessary for foam cell formation [1]. Native LDL does not induce foam cell formation in cell culture condition and it has been suggested that LDL must be modified before it can be taken up into cells. Monocyte-derived macrophage, indeed, took up excess acetylated or oxidized LDL and formed the lipid laden foam cell [2,3]. It has been reported that antioxidants, e.g. \( \beta \)-carotene and \( \alpha \)-tocopherol, prevented LDL oxidation and delayed the development of atherosclerotic plaques in animals [4,5]. Miura et al. [6] have demonstrated that epigallocatechin gallate (EGCG), the major catechin in green tea, inhibited Cu\(^{2+}\)-ion induced LDL oxidation, and that tea catechins also acted as radical scavengers against propagating lipid peroxyl radical species [7].

Previously, we have shown that Lung Chen Tea, a non-fermented Chinese green tea, was effective in lowering both serum and liver cholesterol levels in diet-induced hypercholesterolemic rats [8]. Since cholesterol is the major factor in atherogenesis, reduction in cholesterol level may prevent coronary heart diseases. Antioxidants have also been shown to delay atherogenesis and Serafini et al. [9] have demonstrated an in vivo anti-oxidative effect of green tea in man. It is thus interesting to investigate if Chinese green tea has any effect on LDL oxidation. We have investigated five kinds of Chinese tea, namely Lung Chen Tea, Jasmine Tea, Iron Buddha, Oolong Tea and Pu erh Tea, and found that Lung Chen Tea has the highest content of catechins [8]. Since catechins have been shown to produce an anti-oxidative effect, Lung Chen Tea was chosen. This is also the most widely consumed Chinese green tea. In the present study, the effect of Lung Chen Tea and its extracts on LDL oxidation induced by human umbilical cord vascular endothelial cell was examined.
2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS) and RPMI-1640 culture medium were obtained from Gibco BRL (Gaithersburg, MD). Thiobarbituric acid, sodium dodecyl sulphate (SDS), 1,1,3,3-tetramethoxypropane (TMP), trypsin, penicillin, streptomycin, bovine serum albumin, trypan blue, butylated hydroxytoluene, phenol reagent, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium bicarbonate, sodium hydroxide, EDTA.Na₄, acetic acid, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) were purchased from Sigma (St Louis, MO). Methanol, n-butanol, ethyl acetate and chloroform were from Merck (Darmstadt, Germany). Lung Chen Tea was imported from West Lake of Hangzhou, China. It was prepared from the youngling buds of the tenderest leaves sprouting in the spring season.

2.2. Cell culture

Human umbilical cord vascular endothelial cell (HUVEC) was purchased from American Type Culture Collection (Rockville, MD). Endothelial cells were maintained on RPMI-1640 medium supplemented with 10% (v/v) FBS, 50 U/ml penicillin and 50 µg/ml streptomycin. One millilitre of the cell suspension at a density of 10⁵ cells per ml was added to each of the wells in a six-well culture cluster (Costar, Cambridge, MA) and incubated at 37°C in 5% CO₂ until confluent.

2.3. Cell viability

Cell viability was assessed with the trypan blue exclusion test [10,11]. Two hundred cells were randomly examined microscopically and the number of cells stained blue was determined.

2.4. LDL isolation

Blood was obtained from healthy volunteers after overnight fasting. LDL (density 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation at 4°C in a L8-70 Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA) using a 70.2 rotor at 40,000 rev./min for 20 h [12]. The LDL was dialysed for 24 h at 4°C against three changes of phosphate-buffered saline (NaCl 140 mM, KCl 3 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 2 mM) before use. EDTA (0.3 mM) was added to all buffers to prevent auto-oxidation of the lipoproteins.

2.5. LDL oxidation by endothelial cells

At confluence, the original medium was removed and cells were washed three times with Ca²⁺, Mg²⁺-free Hank’s Balanced Salt Solution. RPMI-1640 medium containing LDL (100 µg LDL protein per ml) was added to the culture cluster and incubated for 24 h. The oxidation was stopped by the addition of 1 mmol/l EDTA and 0.02 mmol/l butylated hydroxytoluene. The culture medium was collected and centrifuged at 1000 rev./min for 10 min to remove the endothelial cells.

2.6. Lipid peroxidation

The extent of lipid peroxidation was determined by measuring the quantity of thiobarbituric acid reactive substances (TBARS) generated in the culture medium [13]. The reaction mixture was composed of 750 µl of 0.8% thiobarbituric acid, 750 µl of 20% acetic acid, 100 µl of 8.1% sodium dodecyl sulphate, and 400 µl of the standard 1,1,3,3-tetramethoxypropane or samples in 2.0 ml. It was incubated at 95°C for 1 h and then cooled in tap water before extraction with 3 ml of n-butanol. The absorbance of n-butanol extracts at 532 nm (DU 650 UV spectrophotometer, Beckman Instruments) was recorded against a reagent blank. The amount of malondialdehyde (MDA) was determined from the calibration curve and the extent of lipid peroxidation was expressed as nmol equivalent of MDA.

2.7. Electrophoretic mobility of LDL

The electrophoretic mobilities of native and modified LDL were determined by agarose gel electrophoresis [14]. The electrophoresis was performed with a Chiron Diagnostics Lipoprotein System (Lipoprotein Kit/8) connected to a LKB power supply. The agarose gel (1.0% agarose and 1.3% barbital buffer) was electrophoresed (90 V, 40 mA) in universal buffer containing 1.67% sodium barbital and 0.25% barbital for 35 min. The electrophoretic mobility was expressed as the ratio of the distance moved by the modified LDL to that of the native LDL.

2.8. Preparation of Lung Chen Tea

Tea extract was prepared by brewing Lung Chen Tea leaves (2% w/v) in freshly boiled water for 0.5 h, and the tea was then filtered. The filtrate was freeze-dried to obtain the Lung Chen Tea extract.

2.9. Preparation of tea solvent extracts

Tea catechins were extracted (Fig. 1) with by a modified method of Ho et al. [15]. Tea leaves were finely ground and extracted with 3 vol. of methanol in...
a round-bottomed flask under reflux at 50°C for 3 h. The extraction procedure was repeated twice and the methanolic fractions were evaporated to dryness at 50°C under reduced pressure in a rotary evaporator (Büchi Rotavapor-EL, Switzerland). The residues obtained were re-dissolved in distilled water and subjected to sequential extractions with chloroform and ethyl acetate. The chloroform, ethyl acetate and the remaining aqueous layers were separated and evaporated to dryness under reduced pressure. These residues were re-dissolved in water and freeze-dried to produce the chloroform, ethyl acetate and remaining aqueous extracts. The freeze-dried extracts were desiccated and stored at −20°C until use.

2.10. Quantitation of tea catechins

The quantities of tea catechins, i.e. (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG), in the solvent extracts were analyzed by reverse phase high performance liquid chromatography using a Beckman Ultrasphere ODS column (4.6 × 250 mm, 5 μm) [16]. Twenty microlitres of the diluted extract solution were injected into the column via a 20-μl sample loop and water/methanol/acetic acid (74.9:25:0.1) was used as the mobile phase (1 ml/min). The absorbance at 280 nm was monitored with a variable wavelength detector (LDC Milton Roy SpectroMonitor 3100, USA).

2.11. Cholesterol determination

Monolayer endothelial cells were trypsinized and washed with Hank’s Balanced Salt Solution. The isolated cells were extracted with chloroform and dried under nitrogen. The cellular cholesterol content was determined with a cholesterol enzyme kit (Cholesterol liquicolor, Human Gesellschaft für Biochemica und Diagnostica mbH, Germany) as described by Roeschlauf et al. [17]. The content of cellular cholesterol was expressed as mg cholesterol/mg cell protein.

2.12. Protein assay

Protein concentrations in the final LDL and endothelial cell culture preparations were determined with Lowry’s method [18]. Bovine serum albumin was used as the protein standard and the absorbance was recorded at 660 nm.

2.13. Statistical analysis

The results were expressed as means ± S.E.M. (the standard errors of means) and the data were analyzed for significance of differences using the unpaired Student’s t-test [19].

3. Results

3.1. Viability of endothelial cells

The viability of endothelial cells in all culture wells was greater than 95%.

3.2. Quantities of catechins in the solvent extracts

The quantities of catechins in the solvent extracts are shown in Fig. 2. Ethyl acetate extract has the highest amount of catechins while the least amount was found in the remaining aqueous extract. Its contents of EGCG and ECG were also lower than the chloroform extract (P < 0.001) but its EGC content was higher (P < 0.05). The amounts of EGC in the ethyl acetate extract and remaining aqueous extract were similar, however the former contained more EGCG, EC and ECG.

3.3. Amount of TBARS in culture medium

The extent of LDL oxidation after incubation with endothelial cells was determined from the amount of...
Fig. 2. Quantities of the four major tea catechins, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG), in the solvent extracts of Lung Chen Tea. Values are expressed as the means ± S.E.M.; *P < 0.05, **P < 0.001 when compared with the chloroform extract.

TBARS generated in the cell culture medium, and the results are shown in Fig. 3a,b. Endothelial cells significantly increased the amount of TBARS when compared with the cell-free control (P < 0.001, Fig. 3a). Lung Chen Tea extract, at 5 and 10 μg/ml, dose-dependently inhibited endothelial cell induced LDL oxidation (Fig. 3a) and significantly lowered the TBARS values (P < 0.001). The anti-oxidative substances in Lung Chen Tea were concentrated in the chloroform and ethyl acetate fractions which also dose-dependently lower the TBARS content (Fig. 3b). Furthermore, the anti-oxidative effect of 10 μg/ml ethyl acetate extract was significantly stronger than the same concentration of chloroform extract (P < 0.05), while the left over aqueous extract was not effective.

3.4. Cellular cholesterol content in endothelial cells

Lung Chen Tea dose-dependently reduced cholesterol content in endothelial cells (Fig. 4a). The cellular content of cholesterol in 5 and 10 μg/ml Lung Chen Tea groups were lower than the oxidized LDL control and 1 μg/ml Lung Chen Tea group (P < 0.001, P < 0.01 respectively). Wells containing 10 μg/ml chloroform extract, 5 and 10 μg/ml ethyl acetate extracts have significantly less cholesterol accumulation when compared with the oxidized LDL control (Fig. 4b; P < 0.01, P < 0.01 and P < 0.001, respectively). There was a dose-dependent relationship for the ethyl acetate extract while no effect was observed in the remaining aqueous extract.

3.5. Electrophoretic mobility of LDL

Fig. 5 shows the electrophoretic mobility of LDL after 24-h incubation with HUVEC. The relative electrophoretic mobility was expressed as the ratio of the...
the increase in relative electrophoretic mobility of LDL induced by endothelial cell in a dose-dependent manner ($P < 0.001$, Table 1). Chloroform and ethyl acetate extracts that were enriched with water soluble catechins were effective while the remaining catechin

distance moved by the oxidized LDL to that of native LDL. Incubation with endothelial cells increased the electrophoretic mobility of LDL (lane 2) when compared with native LDL (lane 1). Five and ten microgram per ml of Lung Chen Tea significantly prevented

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**Fig. 4.** Effect of Lung Chen Tea and the three extracts on endothelial cell cholesterol content after incubation with LDL for 24 h. Values are expressed as means ± S.E.M. of eight determinations. (a) *$P < 0.001$ when compared with the oxLDL; † $P < 0.01$ when compared with the 1 μg/ml Lung Chen Tea extract. (b) *$P < 0.01$, ‡ $P < 0.001$ when compared with the oxLDL; † $P < 0.02$ when compared with the 5 μg/ml EA. Ex.; oxLDL = oxidized LDL (cell + LDL).

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**Table 1**

Effect of Lung Chen Tea and its solvent extracts on the relative electrophoretic mobility of LDL after overnight incubation with endothelial cells

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Relative electrophoretic mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>1.00</td>
</tr>
<tr>
<td>Control (oxidized LDL)</td>
<td>3.467 ± 0.08</td>
</tr>
<tr>
<td>Cell free control</td>
<td>1.250 ± 0.01*</td>
</tr>
<tr>
<td>Lung Chen Tea extract (1 μg/ml)</td>
<td>3.465 ± 0.10</td>
</tr>
<tr>
<td>Lung Chen Tea extract (5 μg/ml)</td>
<td>2.648 ± 0.07*†</td>
</tr>
<tr>
<td>Lung Chen Tea extract (10 μg/ml)</td>
<td>1.324 ± 0.05*‡, †</td>
</tr>
<tr>
<td>Chloroform extract (5 μg/ml)</td>
<td>1.324 ± 0.05*§</td>
</tr>
<tr>
<td>Chloroform extract (10 μg/ml)</td>
<td>1.327 ± 0.06*§</td>
</tr>
<tr>
<td>Ethyl acetate extract (5 μg/ml)</td>
<td>1.320 ± 0.03*§</td>
</tr>
<tr>
<td>Ethyl acetate extract (10 μg/ml)</td>
<td>1.420 ± 0.05*§</td>
</tr>
<tr>
<td>Remaining aqueous extract (5 μg/ml)</td>
<td>3.374 ± 0.03</td>
</tr>
<tr>
<td>Remaining aqueous extract (10 μg/ml)</td>
<td>3.772 ± 0.22</td>
</tr>
</tbody>
</table>

* Values are expressed as means ± S.E.M. of eight determinations.
† $P < 0.001$ when compared with the Control group (oxidized LDL).
‡ $P < 0.01$ when compared with the 1 μg/ml Lung Chen Tea extract.
§ $P < 0.001$ when compared with the 5 μg/ml Lung Chen Tea extract.
rats was found to be more resistant against Cu²⁺-induced delayed atherogenesis [5,24]. Green tea has been reported to be anti-oxidative and to inhibit lipoxygenase in vitro [15]. Plasma from epicatechin-treated rats was found to be more resistant against Cu²⁺-induced oxidation compared to control. This finding was based on cholesteryl hydroperoxide accumulation and the consumption of α-tocopherol during oxidation [25]. Since ingestion of green tea has also been shown to significantly increase the total plasma antioxidant capacity in man [9], its effect on endothelial cell induced LDL oxidation was studied in the present experiment. Endothelial cells oxidized polyunsaturated fatty acid of LDL, resulting in an elevation of lipid peroxides. In the absence of endothelial cells, the oxidation was quite limited and was mainly due to the oxygen in the air. Incubation of LDL with endothelial cells for 24 h significantly increased the TBARS content, electrophoretic mobility of LDL and cholesterol accumulation in endothelial cells. The amount of lipid peroxidation products (TBARS) in culture medium was significantly increased after overnight incubation of LDL with endothelial cell (P < 0.001). Lung Chen Tea dose-dependently lowered the amount of lipid peroxidation products in the culture medium and the oxidation was totally inhibited at the concentration of 10 μg/ml. These results clearly demonstrated that Lung Chen Tea is a powerful antioxidant in inhibiting endothelial cell induced LDL oxidation (Fig. 3a). Accumulating evidence supports the role of lipoxygenase in endothelial cell- and macrophage-induced LDL oxidation [26,27]. Tea catechins which inhibit lipoxygenase and act as scavengers for radicals and chain-breaking antioxidants [7,15] may suppress endothelial cell induced LDL oxidation through these mechanisms. Moreover, it was found that the water soluble catechins, that were selectively concentrated in the ethyl acetate and chloroform extracts, prevented LDL oxidation while the remaining aqueous layer, which was deprived of tea catechins, has no preventive effect. This strongly suggested that tea catechins may be the major antioxidants present in Lung Chen Tea.

The electrophoretic mobility of LDL was increased by incubation with endothelial cells (Table 1). The binding of aldehydic lipid peroxidation products to the ε-amino groups of lysine residues in apo B during oxidation and the conversion of histidine and proline residues to negatively charged aspartic acid and glutamic acid by reactive oxygen species increased the negativity of LDL [28]. Since derivatization of apo B was a prerequisite for scavenger receptor recognition [1], inhibition of LDL oxidation and derivatization of apo B can prevent foam cell formation. In the present study, it was shown that Lung Chen Tea prevented the increase in negative charge of LDL after incubation with endothelial cell and so it may be effective in preventing the uptake of LDL by macrophage scavenger receptor and foam cell formation. The results of the relative electrophoretic mobility were also consistent with TBARS contents. Both the ethyl acetate and chloroform extracts prevented the increase in TBARS content in culture medium and decreased electrophoretic mobility of LDL. These effects could be due to the inhibition of lipid peroxidation by the presence of tea catechins in the extracts.

It has been shown that modification of LDL is necessary for the scavenger receptor uptake, and triggers the accumulation of cellular cholesterol in macrophages [1]. The scavenger receptor is different from native LDL receptor in that it cannot be down-regulated. It takes up modified LDL continuously, leading to foam cell formation. Incubation of LDL with endothelial cells dramatically increased the content of cellular cholesterol in the endothelial cells. In the presence of Lung Chen Tea extract, endothelial cell accumulated less cholesterol (Fig. 4a). The reduction in cellular cholesterol content was also concentration-dependent. Furthermore, ethyl acetate and chloroform extracts that contained water soluble catechins were effective in preventing cholesterol accumulation in endothelial cells (Fig. 4b). The remaining aqueous extract that has its catechins removed has no preventive effect. There was a direct correlation between the amount of cholesterol accumulation and the extent of LDL oxidation and electrophoretic mobility. This is because an increase in LDL oxidation will lead to more apo B derivatization and more LDL-cholesterol would be taken up by the macrophage through interaction with the scavenger receptors.

The present study demonstrated that apart from lowering serum and liver cholesterol, Lung Chen Tea prevented LDL oxidation by endothelial cells. It is possible that Lung Chen Tea may delay atherogenesis and lower the risk of coronary heart disease by inhibiting LDL oxidation and foam cell formation. Lung
Chen Tea therefore may be an ideal beverage in preventing the development of coronary heart disease.

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References