Anti-Atherogenic Effects of Fermented Fresh Coffee Bean, Soybean and Rice Bran Extracts

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Fermented fresh coffee bean, soybean and rice bran extracts (FCSRE, Guardox™) is a mixture of aqueous extract of fresh coffee beans, rice bran, and soybeans fermented with Bacillus subtilis Natto. Superoxide anion and hydroxy radical were effectively trapped by FCSRE, as shown by electron spin resonance experiments. Thiobarbituric acid reacting substance (TBARS) formation in human low-density lipoprotein (LDL) induced by copper ion was inhibited by adding FCSRE (0.1 mg/dl) in vitro. Male New Zealand white rabbits were assigned to two groups, control rabbits (n=8) were fed a diet containing 1% cholesterol for 12 weeks, and experimental rabbits (n=8) were fed a 1% cholesterol diet with added 5% FCSRE (2 g/kg body weight/day). Although plasma cholesterol levels rapidly increased after initiation of the high cholesterol diet, no statistical difference was observed between the control and experimental groups. After 12 weeks, sera were sampled, rabbits were sacrificed, and aortas were removed. TBARS formation in LDL induced by copper ion was significantly lower in the experimental group compared with the control group. Lag time of LDL oxidation was also significantly longer in the experimental group. FCSRE administration significantly inhibited the TBARS formation in the aorta compared with the control animals. There was a significant difference in the aortic area covered with atherosclerotic lesions between the experimental and the control groups (53.7±16.3 % vs. 36.2±8.18 %, p<0.05). These results suggest that FCSRE may be a promising material for the prevention of atherosclerosis.

Keywords: atherosclerosis, fermentation, Bacillus subtilis Natto, electron spin resonance, LDL oxidation

It has been shown that oxidative stress, caused by materials such as free radicals, active oxygens and lipid peroxidation products participates in various diseases such as atherosclerosis (Naito et al., 1995; Steinberg, 1997), diabetes mellitus, and cancer, and even in aging per se (Beckman & Ames, 1998; Finkel & Holbrook, 2000). Many studies, including ours (Kang et al., 1998; Kang et al., 1999; Kang et al., 2000; Naito et al., 2002), have been performed to examine the anti-atherogenic effects of antioxidative agents, using various models. Although many different types of natural antioxidants have been isolated and identified, the major natural antioxidants are plant phenols (Yamakoshi et al., 2000; Kaplan et al., 2001; Ling et al., 2001; Miura et al., 2001). Natural antioxidative substances contained in food, such as flavonoids, which we eat daily, not only inhibit the oxidation of food itself but may also be useful for eliminating active oxygen in the human body (Duthie & Crozier, 2000; Rice-Evans et al., 1997). Therefore, those natural antioxidant substances are expected to contribute to the prevention of diseases and the maintenance of our health. Several epidemiologic investigations showed that flavonoid intake is inversely associated with the mortality of coronary heart disease (Hertog et al., 1993; Knekt et al., 1996; Rimm et al., 1996).

Antioxidative compounds have also been shown to be produced by fermentation. The traditional fermented soybean products incubated with Aspergillus oryzae, Bacillus subtilis Natto and Rhizopus oligosporus are popular as “miso”, “natto” and “tempeh,” respectively, and have been proved to be more stable against lipid peroxidation than steam soybeans. The amount of the free isoflavones daidzein and genistein increased in miso and tempeh, and these isoflavones are believed to be principal antioxidants in these foods (Esaki et al., 1994). In the case of natto, there was little increase in the amount of free isoflavones, but the antioxidative activity of a water-soluble antioxidative fraction increased dramatically during incubation with Bacillus subtilis Natto.

The purpose of this study was to investigate the elimination of active oxygens and free radicals and the anti-atherogenic effects of newly manufactured fermented fresh coffee bean, soybean and rice bran extracts, which together form a new material (FCSRE) developed from a product of fermented rice germ and soybean extracts (Toyo Hakko, Ohbu). FCSRE was given orally to rabbits fed a high cholesterol diet to study the effects on oxidative stress and atherosclerosis. We also examined the effects of FCSRE on the oxidation of LDL and trapping of superoxide anion and hydroxy radical in vitro.
Materials and Methods

Chemicals Hypoxanthine (HX), 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO), diethylenetriaminepentaacetic acid (DETAPAC), and xanthine oxidase (XO) were purchased from Sigma (Tokyo). Ferrous sulfate and hydrogen peroxide were from Wako Pure Chemicals (Tokyo).

Preparation of FCSRE FCSRE (Guardox™, Toyo Hakko, Japan) is a new material developed from the product of fermented rice germ and soybean extracts (Patent No. 1885507, Japan). FCSRE was manufactured as follows. First, fresh Indonesian arabican coffee beans were pulverized with a ceramic edge. Then the powder was percolated with an aqueous solution (pH adjusted to 5.5) to prepare the extracts. The extracts (20% w/v), rice bran (3.5%) and soybean (0.1%) were mixed and added with water to 100%. Thereafter, pH was adjusted to about 9 and the solution was fermented with Bacillus subtilis Natto for 18 h, with stirring and airing. At the end of fermentation, the remaining Bacillus subtilis Natto was killed by heat treatment. Solids were removed with a compressor to obtain a solution and the solution was filtered with an ultrafilter (Millipore, Bedford, MA) to remove substances with a molecular weight of 5000 or more. Then the filtrate was decolorized and deodorized with activated carbon. Finally, the solution was filtered with 5.0 μm and 0.45 μm fine filters (Toyo Roshi, Tokyo) to obtain the fermented solution, which was freeze-dried to produce the final product, FCSRE. The chemical composition of FCSRE was analyzed and is shown in Table 1.

In vitro studies

Electron spin resonance (ESR) Superoxide anion elimination activity was measured according to the method described by Chen et al. (1998). Superoxide anion was generated from a HX-XO reaction system, trapped by DMPO and the spin adduct DMPO-OH was analyzed using an ESR spectrometer. The procedure was as follows: a solution of 2.0 mM HX, 5.5 mM DETAPAC in PBS, and various concentrations of FCSRE, and 0.4 units/ml XO in phosphate buffered saline (PBS) were prepared just before use. The XOD solution was stored in an ice bath to prevent any activation of enzymes. Fifty microliters of HX, 35 μl of DETAPAC, 50 μl FCSRE, and 15 μl DMPO were added, and lastly, 50 μl XO was put into the test tube for measurement.

Hydroxy radical was generated by a Fenton reaction and the spin adduct DMPO-OH was analyzed with an ESR spectrometer. A solution of 75 μl of 1 mM FeSO₄-DETAPAC, 20 μl of 0.92 M DMPO, 50 μl of various concentrations of FCSRE and 75 μl of 0.1 mM H₂O₂ were put into the test tube and mixed. The reaction mixture was stirred, transferred to a quartz cell and placed in the cavity of the ESR spectrometer (JES-TE 2000, JEOL, Tokyo). One minute after the addition of XO or H₂O₂, samples were analyzed and the reactive intensity of signal of DMPO-OH or DMPO-OH spin adduct was measured as the ratio to the intensity of Mn²⁺ signal. ESR spectra were recorded at 37°C with a field set 335±5 mT, modulation frequency 100 kHz, modulation amplitude×0.1 mT, response time 0.1 s, sweep time 2 min, and microwave power 8.0 mW (9.416 GHz).

Preparation of LDL Blood was collected from rabbits and placed in tubes containing Na₂-EDTA at a final concentration of 0.15% (w/v). Plasma was isolated by centrifugation at 3000 rpm for 15 min at 4°C. VLDL (d=0.95~1.006) was isolated from the plasma stepwise at 150,000×g for 16 h at 16°C using the RP 55 rotor of Hitachi-angle ultracentrifugation (Hitachi, Japan). LDL (d=1.019~1.063) was isolated from the infranatant stepwise at 150,000×g for 20 h at 16°C. VLDL and LDL preparations were extensively dialyzed against a 200-fold excess volume of PBS containing 0.01% Na₂-EDTA for 48 h at 4°C in the dark. To remove EDTA, VLDL and LDL were dialyzed against a 200-fold volume of PBS without EDTA for 48 h at 4°C in the dark. After dialysis, VLDL and LDL were sterilized by passage through a 0.45 μm filter, stored at 4°C, and used within one week. Protein concentration was determined using the bichonchonic acid (BCA) protein assay reagent (Fierce, Rockford, IL).

Oxidation of LDL Isolated human LDL was diluted with PBS to a final concentration of 100 μg protein/ml. In vitro effects of FCSRE on the oxidation of LDL were examined. The oxidation was observed with the addition of 10 μM (final concentration) of freshly prepared CuSO₄ solution. Samples were incubated with or without the addition of FCSRE (0.1 mg/ml, final concentration) for up to 20 h at 37°C in a thermostatically controlled bath. Periodically, aliquots were taken and the reaction was stopped by the addition of 1 mM EDTA and 10 μM butylated hydroxytoluene (BHT). The lipid peroxidation of LDL was determined by measuring the formation of TBARS as described (Kang et al., 2000). TBARS were calculated as MDA equivalents, using freshly diluted malondialdehyde-bis-(dimethylacetal), i.e., 1,1,3,3-tetraethoxyxypropane (Aldrich Chemical, WI) as the standard.

Animal studies

Animals and diets The protocols for animal experiments were approved by the Laboratory Animal Care Advisory Committee of Nagoya University. Male New Zealand white (NZW) rabbits (2.0~2.5 kg, Oriental Yeast, Tokyo) were housed individually at 25±1°C with a 12-h light-dark cycle. They were allowed free access to water and normal commercial rabbit diet (Oriental Yeast) for 7 days to enable them to adapt to the new environment. Then rabbits were assigned to two groups: control rabbits (n=8) were fed a diet containing 1% cholesterol for 12 weeks,

Table 1. Composition of FCSRE (g/100 g)

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Protein</td>
<td>6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Fat</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Fiber</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>34.7</td>
<td>34.8</td>
</tr>
<tr>
<td>Polyphenol</td>
<td>4.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
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</table>

Table 2. Composition of diets.

<table>
<thead>
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<th>Experiment</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>21.5</td>
</tr>
<tr>
<td>Fat</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Fiber</td>
<td>13.2</td>
<td>12.6</td>
</tr>
<tr>
<td>Minerals</td>
<td>8.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.44</td>
<td>0.45</td>
</tr>
<tr>
<td>Non-nitrogen substance</td>
<td>44.4</td>
<td>42.6</td>
</tr>
<tr>
<td>FCSRE</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>—</td>
<td>1</td>
</tr>
</tbody>
</table>

(g/100 g)
and experimental rabbits (n=8) were fed a 1% cholesterol diet with added 5% FCSRE (2 g/kg body weight/day). All of the diet ingredients were products of Oriental Yeast. The composition of the normal diet analyzed and the composition of the experimental diet calculated are shown in Table 2. Food intake of each rabbit was restricted to 100 g/day. No food was supplied for 18 h before blood collection. Blood samples were collected from the ear artery before and after 6 and 12 weeks of the experiment, and serum and plasma were isolated from the blood by centrifuge. After 12 weeks, rabbits were killed by administration of a bolus injection of pentobarbital. Liver and aorta were removed and washed three times with cold physiological saline solution. Serum and tissues were immediately stored at −80°C.

**Oxidation of LDL** LDL was isolated from rabbit plasma as described above. The oxidation of LDL (100 μg protein/ml) was initiated by the addition of 10 μM (final concentration) of freshly prepared CuSO₄ solution. The kinetics of the oxidation of LDL were determined by monitoring the formation of conjugated dienes by measuring the increase in absorbance at 234 nm on a UV spectrophotometer (Shimadzu, Tokyo). The change of absorption at 234 nm versus time was divided into three phases, i.e., a lag phase, a propagation phase, and a decomposition phase. Lag time was calculated as described (Esterbauer et al., 1989). Samples were incubated for 3 h at 37°C, then the reaction was stopped, and TBARS was measured as described (Kang et al., 2000).

**Measurement of TBARS in abdominal aorta** The abdominal aorta was used for measuring TBARS as described by Ohkawa et al. (1979). Briefly, the aorta was chilled in iced PBS and an aortic homogenate was prepared in a ratio of 1 g of wet weight to 9 ml of 1.15% KCl using a HG 30 homogenizer (Hitachi). One-tenth milliliters of aortic homogenate, 0.2 ml of 8.1% SDS (Wako), 1.5 ml of 20% acetic acid solution (pH 3.5) and 1.5 ml of 0.8% aqueous solution of TBA (Merck, Darmstadt, Germany) were mixed. The mixture was made up to 4.0 ml with distilled water and then boiled at 95°C for 60 min. After cooling with ice water, 4.0 ml n-butanol/pyridine (15:1, v/v) was added and the mixture was shaken vigorously, then centrifuged at 3000 rpm for 10 min.

**Other biochemical analyses** Serum triglycerides (TG), total cholesterol (TC), phospholipids (PL) and HDL-cholesterol (HDL-C) concentrations were determined enzymatically using commercial kits (Nippon Shouji, Osaka). Protein concentrations were determined with a BCA protein assay kit as above.

**Extent of aortic atherosclerosis** At the end of the study period, rabbits were sacrificed by administration of a bolus injection of sodium pentobarbital. Immediately thereafter, whole aortas were removed, cleaned of excess adventitial tissues, rinsed with PBS and opened longitudinally, and separated into thoracic and abdominal aortas. After photographing, the aortas were immediately stored at −80°C for biochemical analysis. The photographs were copied onto graph paper with magnification and the atheromatous lesions were delineated. Areas of the lesions were calculated, and their percentage determined. The weight of the excised heart and liver was also measured.

**Statistical analysis** Results are presented as mean±SD. The data were tested by the Student’s t test. All analyses were performed using Stat View software version 5.0J (Abacus Concepts, Berkeley, CA). A level of p<0.05 was considered to be significant.

**Results**

**In vitro studies**

**Trapping of superoxide anion and hydroxy radical by FCSRE** ESR signals were calculated as a secondary integral area. Signals of the superoxide anion were suppressed by 86.0% and 97.2% (Fig. 1a), and of the hydroxy radical were suppressed by 35.8% and 86% (Fig. 1b), with 1 mg/ml and 10 mg/ml of FCSRE, respectively.

**In vitro effects of FCSRE on the oxidation of LDL induced by copper ion** The extent of lipid peroxidation induced by 10 μM CuSO₄ was assessed by monitoring the formation of TBARS.
The extent of human LDL lipid peroxidation induced by 10 μM CuSO₄ was assessed by monitoring the formation of TBARS (Fig. 2). TBARS formation in LDL induced by copper ion was inhibited by adding FCSRE (final concentration 0.1 mg/dl) in vitro at 2 and 4 h.

### Animal studies

**Effect on body and liver weights and plasma lipids**

There was no significant difference in body weight, liver weight, serum total cholesterol, triglycerides, phospholipids, or HDL-cholesterol between the two groups at the end of the study period.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FCSRE</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td>2.70 ± 0.16</td>
<td>2.79 ± 0.26</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>119 ± 19</td>
<td>114 ± 9</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>1052 ± 392</td>
<td>822 ± 332</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>31.6 ± 10.6</td>
<td>35.2 ± 14.7</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>145 ± 75</td>
<td>130 ± 47</td>
</tr>
<tr>
<td>PL (mg/dl)</td>
<td>842 ± 261</td>
<td>724 ± 195</td>
</tr>
<tr>
<td>VLDL-C (mg/mg protein)</td>
<td>8.86 ± 0.42</td>
<td>6.80 ± 1.65</td>
</tr>
<tr>
<td>LDL-C (mg/mg protein)</td>
<td>3.79 ± 1.14</td>
<td>3.70 ± 1.71</td>
</tr>
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</table>

There was no significant difference in body weight, liver weight, serum total cholesterol, triglycerides, phospholipids, or HDL-cholesterol between the two groups at the end of the study period.

**Effect of FCSRE administration on LDL oxidation ex vivo**

The extent of LDL lipid peroxidation induced by 10 μM CuSO₄ was assessed by monitoring the formation of conjugated dienes, using LDL isolated from the blood taken at the end of the experiment (a). Lag time was also significantly longer in the experimental group than in the control group (a, inset). TBARS formation in LDL was significantly inhibited after 3-h induction by copper ion in the FCSRE-treated group compared with the control group (b).

**Discussion**

The ESR signals of superoxide anion and hydroxy radical were effectively suppressed by FCSRE in vitro. Supplementing FCSRE in vitro also inhibited TBARS formation in LDL induced by copper ion. These results suggested that FCSRE was a promising trap for these radicals in vivo. Then we investigated the effects of FCSRE on oxidative stress and atherosclerosis in rabbits fed a high cholesterol diet. Serum total cholesterol level was slightly lower in the experimental group than in the control group, although without significance. There was no difference in LDL-cholesterol levels between the two groups; however,
VLDL-cholesterol showed a slightly lower tendency in the experimental group compared with the control group. Oxidation of LDL by copper ion, estimated by TBARS formation and lag time, was significantly inhibited in the LDL isolated from FCSRE-treated rabbits compared with that from control rabbits. There was a significant difference in the aortic area covered with atherosclerotic lesions between the FCSRE-treated and control groups. TBARS content of the abdominal aorta was also significantly more suppressed in the FCSRE-treated animals. These results suggest that FCSRE also exhibits potent antioxidative activity in vivo and may be promising for the prevention of atherosclerosis.

It is difficult to determine which ingredient(s) is(are) contributing to its efficacy. However, various substances which may be contained in FCSRE have been reported to be beneficial. These include inositol-phosphates (Jariwalla, 2001), isoflavones, phytic acid, polyphenols and polyphenol glycosides, peptides and amino acids, and minerals. Inositol phosphates include IP$_1$, IP$_2$, IP$_3$, IP$_4$, IP$_5$, and IP$_6$, which may have protective effects on lipid peroxidation (Miyamoto et al., 2000). Polyphenols and polyphenol glycosides include daizine, genistin, and chlorogenic acid (Iwashashi, 2000). The effects shown in this study may be the integral results of these substances and may not be attributable to a sole or specific substance(s).

FCSRE contains a large amount of polyphenols, particularly chlorogenic acid. Chlorogenic acid is an ester of caffeic acid with quinic acid, which is found naturally in various plants such as coffee beans, tea leaves and blueberries. It has been reported that chlorogenic acid effectively inhibited the iron-induced lipid peroxidation of bovine liver microsomes in a concentration-dependent manner (Kono et al., 1998). In the Fenton-type reaction, chlorogenic acid inhibited the production of the hydroxyl radical by iron-EDTA or iron-ADP, while iron plus chlorogenic acid did not generate the hydroxyl radical. The ferric complex with chlorogenic acid was in the ferric high-spin state near rhombicity, and had no radical scavenging activity. The results indicate that chlorogenic acid prevented the formation of the hydroxyl radical by forming a chelate with iron whose complex cannot catalyze the Fenton-type reaction. It should also be pointed out that, unlike most antioxidants, the one-electron oxidation product of chlorogenic acid formed by the reaction with free radicals is rapidly broken down to further products, which cannot generate any free radicals.

Diet-induced atherosclerosis has been shown to be reduced in animals fed soy protein-based diets compared with those fed animal protein-based diets (Damasceno et al., 2000). There is also substantial evidence supporting the existence of lipoprotein-independent pathways by which soy inhibits atherosclerosis. Soy isoflavones may directly inhibit atherosclerosis through estrogen receptor-mediated effects on tyrosine kinase activity, macrophage cytokine expression, the migration or proliferation of arterial smooth muscle cells, endothelial-dependent arterial dilation, arterial elasticity and platelet aggregation or activation (Anthony, 2000). Soy isoflavones also have potent antioxidant activity and may therefore inhibit atherosclerosis progression by reducing LDL oxidation (Yamakoshi et al., 2000). Aortic cholesterol content is reduced by intact and alcohol-washed (isoflavone-deficient) soy protein isolate in atherosclerosis-susceptible (LDL receptor$^{−/−}$ + apoB transgenic and apoE$^{−/−}$) mice (Adams et al., 2002). The effect is enhanced in mice fed intact soy protein relative to those fed alcohol-washed soy protein, and was enhanced in LDL receptor$^{−/−}$ + apoB transgenic mice relative to apoE$^{−/−}$ mice. Inhibitory effects of soy protein on atherosclerosis were independent of plasma lipoproteins in both types of mice. These results suggest that isoflavones are mainly involved in the anti-atherosclerotic effect of soy protein isolate, but additional factor(s) may also be involved. However, in the present study, only a small amount of soybean is contained in the materials for FCSRE, and the significance of these substances are unclear.

Although we did not determine which ingredient(s) is(are) really involved in its efficacy, antioxidative compounds have been shown to be produced by fermentation. A fermented soybean product, tempeh has been known to be very stable to rancidity development, and the antioxidative 6,7,4′-trihydroxyisoflavone was isolated and identified. Two potent antioxidative isoflavones, 8-hydroxydaidzein and 8-hydroxygenisteen, were isolated from soybeans fermented with Aspergillus saitoi (Esaki et al., 1998). These isoflavones, which have an o-dihydroxy structure between the 7- and 8-position, exhibited significantly stronger antioxidative activity than daidzein and genistein in both oil and lipid/aqueous systems; they are also the principal antioxidative isoflavones in potent antioxidative soybeans fermented with A. saitoi. Dried tempeh is known to be significantly stable to lipid oxidation compared with unfermented soybeans. An antioxidant, 3-hydroxyanthranilic acid (HAA), isolated from the methanol extract of tempeh was effective in preventing autoxidation of soybean oil and soybean powder (Esaki et al., 1996). HAA was not found in unfermented soybeans, but was produced during the incubation with Rhizopus oligosporus. It will be necessary to identify the substance(s) involved in the antiatherogenic effects of FCSRE, particularly those produced by fermentation.

In conclusion, although FCSRE is a mixture of multiple compounds and we did not determine which ingredient(s) is(are) involved in its efficacy, it may be a promising material to prevent or inhibit atherosclerotic diseases in humans.

References


