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Research Article

Apple polyphenols inhibit plasma CETP activity and reduce the ratio of non-HDL to HDL cholesterol

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Previous reports demonstrated that hypocholesterolemic activity of apple was associated with its pectin and fiber. This report was to investigate the effect of apple polyphenols (AP) on blood cholesterol level and gene expression of cholesterol-regulating enzymes in Golden Syrian hamsters maintained on a 0.1% cholesterol diet. It was found that dietary supplementation of 0.3 or 0.6% of AP did not affect plasma total cholesterol (TC), but it increased HDL cholesterol (HDL-C) and decreased non-HDL-C, thus leading to a lower ratio of non-HDL-C to HDL-C. Plasma total triacylglycerol (TG) level was also significantly reduced when hamsters were fed a diet supplemented with 0.6% AP. Western blot analysis did not find any effect of AP on sterol regulatory element-binding protein 2 (SREBP-2), LDL receptor (LDLR), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and cholesterol-7α-hydroxylase (CYP7A). Most interesting was that supplementation of AP had no effect on protein abundance of plasma cholesteryl ester transport protein (CETP), but it suppressed plasma CETP activity. A series of *in vitro* assays confirmed that AP inhibited CETP in a dose dependent manner. It was concluded that AP favorably improved distribution of cholesterol in lipoproteins, most likely, by its inhibition on CETP activity.

Keywords: Apple / CETP / HDL / LDL / Polyphenolsn

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1 Introduction

Apple is the fruit of the *Malus* genus belonging to the rose family Rosaceae. Most commonly consumed is *Malus domestica* among over 7500 known individual species. Originated in Europe and mid-Asia, it is now cultivated extensively around the world. In China, both production and consumption of apples have increased substantially over the last decade. Annual production of apples accounts for 24 million tones, while its annual consumption is about 19 million tones (FAO, *U.N. FAOSTATS*, Retrieved March 5, 2007, from http://faostat.fao.org/site/340/default.aspx).

Traditionally, apples have been regarded as a healthy fruit in many cultures, as seen from the popular proverb "one apple a day keeps the doctor away." In recent years, association between apple consumption and several health benefits has been established. Epidemiological studies showed an inverse relationship between apple consumption and various cancers, including lung [1, 2], alimentary canal [3], breast, and ovary tumors [4]. Consumption of apple has also been associated inversely with cardiovascular diseases [5], diabetes [6], and hypercholesterolemia [7]. Apples are rich in phytochemicals, particularly carotenoids, flavonoids, isoflavonoids, and phenolic acids [8]. The major polyphenol compounds in apples are quercetin glycosides, procyanidin, chlorogenic acid, epicatechin, and phloretin glycosides [9, 10].

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Abbreviations: AP, apple polyphenols; **CETP,** cholesteryl ester transport protein; **CYP7A1,** cholesterol-7α-hydroxylase; **HDL-C,** HDL cholesterol; **HMG-CoA R,** 3-hydroxy-3-methylglutary-CoA reductase; **HMGR,** 3-hydroxy-3-methylglutary coenzyme A reductase; **LDL-C,** low-density lipoprotein cholesterol; **LDLR,** LDL receptor; **TC,** total cholesterol; **TG,** triacylglycerols; **SREBP-2,** sterol regulatory element-binding protein 2

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Apple possesses a hypocholesteolemic activity. However, most studies only investigated the effect of whole lyophilized apples [11, 12], apple pectin [12, 13], or apple fiber [7] on cholesterol level. There is no report to date on the hypocholesterolemic activity of apple polyphenols (AP). The present study investigated the effect of AP on blood cholesterol and cholesterol-regulating enzymes in Golden Syrian hamsters maintained on an atherogenic diet, finding that AP did not modify plasma total cholesterol (TC), but most interesting, it markedly decreased the ratio of non-high density lipoprotein cholesterol (non-HDL-C) to HDL cholesterol (HDL-C). This aroused our interest to examine the underlying mechanism of why AP could reduce the ratio of low-density lipoprotein cholesterol (LDL-C) to HDL-C.

2 Materials and methods

2.1 Isolation of AP

AP was isolated from the pomace of Red Fuji apple after juice processing. In brief, the pomace was soaked in 70% ethanol in a ratio of 1:4 (kg pomace/liter ethanol) for 1.5 h twice. The ethanol phases were pooled, filtered, and was then concentrated. After being defatted with *n*-hexane, the extract was purified through adsorption on an AB-8 macroporous resin column. Total AP was eluted using ethanol and was then freeze-dried. The final AP extract was reddish-brown and stored at -20°C. The yield was about 0.4 kg AP/100 kg apple pomace.

2.2 Characterization of AP

Individual polyphenol in AP was determined using HPLC. In brief, AP sample was injected into an HPLC column (Luna C_{18} 100A, 250×4.6 mm^2 id) and quantified on a Shimadzu LC-10AT HPLC system equipped with a UV detector at 280 nm. The elution profile was programmed at a flow rate of 1 mL/min, while the gradient mobile phase composed of 2% acetic acid (solvent A) and 0.4% acetic acid with 80% ACN (solvent B). The ratio of A to B was programmed from 10:1 to 2:8 in 80 min and then back to 10:1 in 3 min, and then was held for another 22 min.

2.3 Animals

Experiments were conducted following approval and in accordance with the guidelines set by the Animal Experimental Ethical Committee, The Chinese University of Hong Kong. Thirty-nine (113 + 6 g) male adult Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. They were randomly divided into three groups ($n = 13$) and housed individually in wire-bottomed cages at 23°C in a 12 h light–dark cycle animal room.

Before the experiment, all the animals were allowed to stabilize by being fed a 0.1% cholesterol diet (control diet). During the following 6 wk, one group was continued to fed the control diet and the other two groups were fed one of the two experimental diets, which were similar to the control diet except that they were supplemented with 0.3% AP (0.3AP) and 0.6% AP (0.6AP) by weight, respectively. During the study, food was given daily and any uneaten food was discarded. The amount of food consumed was measured each day. The hamsters were free to access to food and distilled water, and were weighed weekly.

Blood (1 mL) was bled from the retro-orbital sinus from the animals into a heparinized capillary tube at the end of weeks 0 and 6 after food deprivation for 14 h. The blood was centrifuged at $1000 \times g$ for 10 min and the plasma was collected and stored at -20°C until analysis. After a 3 day recovery, all hamsters were sacrificed by carbon dioxide suffocation. Blood was collected from abdominal artery into a vacuum heparinized tube and centrifuged at $1000 \times g$ for 10 min, the plasma was collected and stored at -20°C until analysis. The liver, heart, and kidney were removed, washed in saline, weighed, flash frozen in liquid nitrogen, and stored at -80°C until analysis.

2.4 Diets

The control diet was prepared by mixing the following ingredients in proportion (g/kg diet): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix AIN-76, 40; viamin mix AIN-76A, 20; DL-methionine, 1; cholesterol, 1. The two experimental diets were prepared by adding 0.3 and 0.6% AP w/w into the control diet, respectively. The powered diets were mixed with a gelatin solution (20 g/L) in a ratio of 200 g diet *per* liter of solution (Table 1). Once the gelatin has set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at -20°C.

2.5 Plasma lipids measurement

Plasma TC and total triacylglycerols (TG) were quantified using commercial enzymatic kits from Thermo (Waltham, MA, USA) and Stanbio Laboratories (Boerne, TX, USA), respectively. For measurement of plasma HDL-C, LDL-C, and very LDL-C (VLDL-C) were first precipitated with phosphotungstic acid and magnesium chloride in a commercial kit (Sigma, USA). Non-HDL-C was calculated by deducting HDL-C from TC.

2.6 Western blot analysis of blood cholesterol regulating proteins and enzymes

Effect of AP supplementation on sterol regulatory element-binding protein 2 (SREBP-2), LDL receptor (LDLR), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and cholesterol-7α-hydroxylase (CYP7A) was

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Table 1. Composition (g) of the control and two experimental diets supplemented with 0.3AP and 0.6AP

	Control	0.3AP	0.6AP
Cornstarch	508	508	508
Casein	242	242	242
Lard	50	50	50
Sucrose	119	119	119
Mineral mix AIN-76	40	40	40
Vitamin mix AIN-76A	20	20	20
dl-methionine	1	1	1
Cholesterol	1	1	1
AP extract	0	3	6
Gelatin	20	20	20

investigated in the present study. Total liver protein was extracted according to the method described by Vaziri and Liang [14]. In brief, frozen hamster liver was homogenized, the extract was centrifuged, and the supernatant was collected and considered the "total protein." After the total protein was centrifuged at 35 000 rpm, the supernatant was removed and the pellet was resuspended in the same homogenizing buffer and considered the "membrane protein." Protein concentration of two fractions was determined using a protein concentration assay kit according to the manufacturer's instructions (BioRad).

For the measurement of LDLR, cholesterol-7α-hydroxylase (CYP7A1) and HMGR, 100 μg of the membrane protein was size-fractionated by 7% SDS-PAGE at 120 V for 2 h. The proteins were then transferred to a Hybond-P PVDF membrane (Amersham Pharmacia Biosciences, Uppsala, Sweden). The membrane was incubated for 1 h in blocking solution (5% nonfat milk in 1 × TBST) at 4°C and then overnight in the same solution containing 1:300 anti-LDL receptor antibody (Santa Cruz Biotechnology, CA, USA) or 1:500 anti-3-hydroxy-3-methylglutary-CoA reductase (anti-HMG-CoA R; Upstate USA, Lake Placid, NY, USA), and 1:200 anti-CYP7A1 (Santa Cruz Biotechnology) whichever appropriate. The membrane was then washed three times for 15 min in 1 × TBS and 0.1% Tween-20 and was then incubated 1 h at 4°C in diluted (1:3000) horseradish peroxidase-linked rabbit anti-goat IgG (Zymed Laboratories, San Francisco, USA) or donkey antirabbit IgG (Santa Cruz Biotechnology). The washes were repeated before the membranes were developed with ECL enhanced chemiluminescence agent (Amersham Life Science) and subjected to autoradiography for 1 – 5 min on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the computer software Photoshop® (Adobe Systems, CA, USA).

For SREBP-2, 100 μg of the membrane protein and 100 μg of the total protein aliquots were mixed and simultaneously size-fractionated on 7% SDS-PAGE at 120 V for 2 h. The remaining procedures were the same as described above except the primary antibody used was 1:300 anti-SREBP-2 antibody (Santa Cruz Biotechnology).

2.7 Determination of liver cholesterol

Cholesterol in the liver was determined as we previously described [15]. Briefly, 1 mg of stigmastanol (as an internal standard) was added into about 300 mg of liver sample. Fifteen milliliters of methanol/chloroform mixture (2:1 v/v) was used to extract lipids from the liver together with 5 mL of saline, and the chloroform/methanol phase was saved and evaporated to dryness under a nitrogen stream. The liver lipids were then mildly saponified, the cholesterol was converted into its trimethylsilyl-ether derivative before the GC analysis.

2.8 Determination of fecal neutral and acidic sterols

Total feces in weeks 4 and 5 was only collected to analyze the total neutral and acidic sterol excretion, because the hamsters were maintained on the diet for 5 wk and the fecal composition was relatively constant [15]. Stigmasterol and hyodeoxycholic acid (0.5 mg each) were used as internal standards for quantification of fecal neutral and acidic sterols, respectively. In brief, 300 mg dried fecal samples were mildly hydrolyzed and extracted with cyclohexane. The neutral sterols in cyclohexane phase was dried and converted into their trimethylsilyl derivatives. The bottom aqueous layer left after cyclohexane extraction was saponified, extracted, and similarly converted into their trimethylsilyl derivatives. Both neutral and acidic sterol trimethylsilyl derivatives were then subjected to the GC analysis.

2.9 Plasma CETP activity measurement and immunoreactive mass by Western blotting

Plasma CETP activity of the hamsters was measured using a commercial kit (Amersham Pharmacia Biosciences, NJ, USA). In brief, 10 μL of ^{3}H-HDL, biotinylated LDL, and assay buffer was mixed with 5 μL of hamster plasma samples and the mixture was incubated at 37°C for 16 h. A stop solution containing SPA-beads that specifically bound to biotinylated LDL was then added to detect [^{3}H] radiation emitted from LDL. The reaction mixture was read on a Beckman LS6500 scintillation counter (Beckman, Fullerton, CA, USA).

Effect of AP on *in vitro* CETP enzyme activity was studied. 10 μL of ^{3}H-HDL, biotinylated LDL, and assay buffer was mixed with 10 μL of AP aqueous solutions of varying concentrations. The reaction was started by adding 10 μL of a partially purified human CETP and the mixture was incubated at 37°C for 16 h. After that, a stop solution containing SPA-beads that specifically bound to biotinylated LDL was added to detect ^{3}H radiation emitted from LDL.

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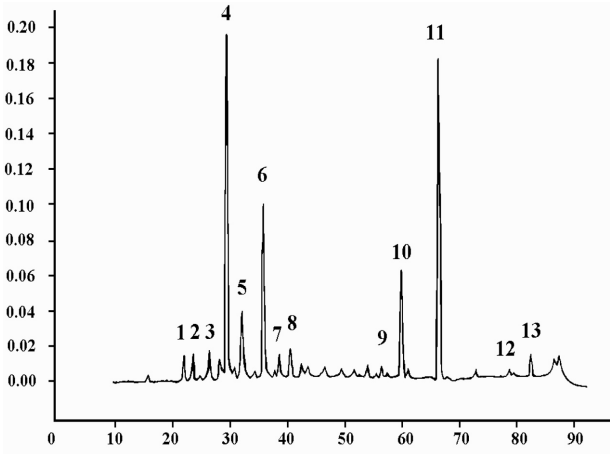

Figure 1. HPLC chromatogram of AP isolated from Red Fuji apple pomace after juice processing. Identification of peaks: 3, catechin; 4, chlorogenic acid; 5, proanthocyanidin B2, 6, epicatechin; 9, rutin; 11, phloridzin; 12, quercetin; 13, phloretin; 1, 2, 7, 8, 10 = unknown.

The reaction mixture was read on a Beckman LS6500 scintillation counter.

For measurement of CETP immunoreactive mass, plasma was diluted in a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 M sucrose, and protease inhibitor cocktail pellet (Complete®, Roche, Mannheim, Germany). The diluted sample (0.01 μL) was size-separated on 7% SDS-PAGE at 120 V for 2 h. After electrophoresis, the proteins were transferred on a Hybond-P PVDF membrane (Amersham Pharmacia Biosciences) with a semidry transfer cell (BioRad) at 15 V for 1 h. The membrane was blocked in a blocking solution (1 × Tris-buffered saline, 0.1% Tween-20, and 5% nonfat milk) at 4°C for 1 h and then overnight at 4°C in the same solution containing 1:600 anti-CETP antibody (Abcam plc, Cambridge, UK). The membrane was then washed three times for 10 min and then incubated in the blocking solution containing 1:3000 horseradish peroxidase-linked anti-rabbit IgG (Abcam plc) at 4°C for 1 h. The washes were repeated before the membranes were developed with ECL chemiluminescent agent (Amersham Life Science) and subjected to autoradiography for 1 – 5 min on SuperRX medical X-ray film (Fuji). Band size and OD were quantified using the computer software Photoshop® (Adobe Systems).

2.10 Statistics

Data were expressed as mean ± SD. Student's *t*-test was used for statistical evaluation of differences between groups (SigmaStat version 2.01, SigmaStat Advisory Statistical Software, MO, USA), and two-way analysis of variance (ANOVA) was used for statistical evaluation of differences among the three groups (SPSS version 11.0, Statistical Package for the Social Sciences software, SPSS, Chicago, USA). $p < 0.05$ was considered statistically significant.

Table 2. Body weight gain, food intake, and organ weights in hamsters fed the control, and two experimental diets supplemented with 0.3AP and 0.6AP

	Control	0.3AP	0.6AP
Initial body weight (g)	113.5 ± 5.5	115.8 ± 4.5	111.2 ± 7.2
Final body weight (g)	120.8 ± 10.0	120.8 ± 7.7	114.2 ± 7.9
Food intake (g/day)	10.3 ± 1.4	10.2 ± 1.4	9.7 ± 1.4
Absolute organ weight (g)			
Liver	6.59 ± 0.86[a,b]	7.00 ± 0.76[a]	6.29 ± 0.51[b]
Kidneys	1.12 ± 0.11	1.18 ± 0.07	1.12 ± 0.08
Heart	0.55 ± 0.10[a]	0.53 ± 0.09[a,b]	0.48 ± 0.06[b]
Relative organ weight (g/100 g body weight)			
Liver	5.45 ± 0.71	5.79 ± 0.62	5.51 ± 0.44
Kidneys	0.93 ± 0.09	0.97 ± 0.06	0.98 ± 0.07
Heart	0.46 ± 0.08	0.44 ± 0.07	0.42 ± 0.05

Values are expressed as mean ± SD, $n = 13$.
a, b) Means at the same row with different superscripts differ significantly at $p < 0.05$.

3 Results

3.1 Polyphenol content in AP

A typical HPLC chromatogram is shown in Fig. 1. The peaks were identified according to the retention times of standards. The AP extract contained chlorogenic acid most (16.4%) followed by phloretin (6.2%), proanthocyanidin B2 (4.3%), epicatechin (2.6%), catechin (1. 2%), phloretin (0.3%), rutin (0.2%), and quercetin (0.1%). Under the present experimental conditions, other phenolic compounds and proanthocyanidins (dimmer, trimer, and tetramer) were not resolved and therefore unquantified.

3.2 Body weight gain, food intake, and organ weights

The data on body weight, absolute organ weight, relative organ weight, and food intake are shown in Table 2. No significant differences in body weight and food intake were observed among the control, 0.3AP and 0.6AP groups. Liver and kidney weights of 0.3AP and 0.6AP groups were not significantly different from that of the control. However, the absolute heart weight of the 0.6AP group was significantly lower than that of the control group. But the difference became insignificant when the relative organ weights were compared.

3.3 Effect of AP supplementation on lipoproteins

There was no difference in plasma lipoprotein profiles among the three groups at week 0 (Table 3). At the end of week 6, no significant difference in plasma TC was observed among the three groups. However, plasma HDL-C in AP-fed hamsters was found significantly higher than that

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Table 3. Changes in serum TC, total TG, HDL-C, non-HDL-C, and non-HDL-C/HDL-C in hamsters fed the control diet, and two experimental diets supplemented with 0.3AP and 0.6AP

	Control	0.3AP	0.6AP
Week 0			
TC	212.4 ± 28.8	219.9 ± 42.0	213.7 ± 36.5
HDL-C	105.7 ± 22.8	101.8 ± 12.2	101.1 ± 11.2
Non-HDL-C	101.1 ± 20.8	112.8 ± 29.1	112.6 ± 35.1
Non-HDL-C/HDL-C	1.01 ± 0.37	1.10 ± 0.21	1.12 ± 0.35
Triglycerides	108.3 ± 40.4	105.7 ± 58.4	102.6 ± 45.6
Week 6			
TC	199 ± 28	197 ± 26	184 ± 12
HDL-C	109 ± 18[a)]	125 ± 15[b)]	127 ± 13[b)]
Non-HDL-C	90 ± 13[a)]	72 ± 15[b)]	57 ± 11[c)]
Non-HDL-C/HDL-C	0.84 ± 0.12[a)]	0.58 ± 0.10[b)]	0.46 ± 0.11[c)]
Triglycerides	144 ± 31[a)]	142 ± 45[a)]	98 ± 28[b)]

Values are expressed as mean ± SD, $n = 13$.
a–c) Means at the same row with different superscripts differ significantly at $p < 0.05$.

Table 4. Changes in fecal output of individual neutral and acidic sterols in hamsters fed the control, and two experimental diets supplemented with 0.3AP and 0.6AP

	Control	0.3AP	0.6AP
Neutral sterols			
Coprostanol	0.84 ± 0.16[a)]	0.58 ± 0.13[b)]	0.43 ± 0.15[b)]
Coprostanone	0.03 ± 0.01[a)]	0.02 ± 0.01[b)]	0.02 ± 0.01[b)]
Cholesterol	0.26 ± 0.15	0.20 ± 0.11	0.31 ± 0.11
Dihydrocholesterol	0.22 ± 0.04[a)]	0.17 ± 0.02[b)]	0.17 ± 0.03[b)]
Total	1.35 ± 0.33[a)]	0.98 ± 0.17[b)]	0.93 ± 0.17[b)]
Acidic sterols			
Lithocholic acid	1.19 ± 0.65[b)]	2.81 ± 1.25[a)]	3.37 ± 1.15[a)]
Deoxycholic acid	0.18 ± 0.15[b)]	0.69 ± 0.45[a)]	0.81 ± 0.33[a)]
Chenodeoxycholic acid	0.29 ± 0.14[b)]	0.56 ± 0.22[a)]	0.65 ± 0.15[a)]
Cholic acid	0.43 ± 0.24[b)]	1.04 ± 0.68[a)]	1.22 ± 0.34[a)]
Total	1.76 ± 0.84[b)]	4.63 ± 1.25[a)]	5.62 ± 1.56[a)]

Values are expressed as mean ± SD, $n = 13$.
a, b) Means at the same row with different superscripts differ significantly at $p < 0.05$.

of the control (Table 3). To be specific, HDL-C of 0.3AP and 0.6AP groups was 14.7 and 16.5% higher than that of the control, respectively. In contrast, non-HDL-C in 0.3AP and 0.6AP groups was significantly lower than that in the control by 20.0 and 36.7%, respectively, leading to a smaller ratio of non-HDL-C to HDL-C in the AP fed groups than in the control (Table 3). Plasma TG in 0.3AP group was not significantly different from the control, but that in 0.6AP group was significantly lower than the control by 31.9% (Table 3).

3.4 Fecal neutral and acidic sterol output and cholesterol balance

Concentration of individual neutral and acidic sterols in the feces of the hamsters at week 5 is shown in Table 4. In general, the two experimental groups excreted lesser amount of neutral sterols than the control hamsters except for cholesterol. In contrast, the two experimental groups excreted greater amount of individual acidic sterols than the control group.

Total intake of cholesterol was compared with its excretion in neutral and acidic sterols (Table 5). Net cholesterol equivalent retained was calculated by difference between intake and excretion of both neutral and acidic sterols. It was found that net cholesterol retention was the most in the control followed by 0.3AP, and 0.6AP in a decreasing order. The apparent cholesterol absorption was calculated in an equation [(cholesterol intake – excretion of neutral and acidic sterols)/cholesterol intake]. It appeared that AP decreased cholesterol absorption in a dose-dependent manner (Table 5).

Table 5. Cholesterol balance in hamsters fed the control diet, and two experimental diets supplemented with 0.3AP and 0.6AP

	Control	0.3AP	0.6AP
Cholesterol intake (mg/day)	16.2 ± 0.8	16.3 ± 1.8	15.8 ± 0.7
Fecal total neutral sterol output (mg/day)	2.0 ± 1.5[a)]	1.0 ± 0.2[b)]	0.9 ± 0.2[b)]
Fecal total acidic sterol output (mg/day)	1.9 ± 1.0[a)]	4.6 ± 1.9[b)]	5.6 ± 1.6[b)]
Net cholesterol equivalent retained (mg/day)	12.3 ± 2.3[a)]	11.2 ± 2.6[b)]	9.3 ± 1.6[c)]
Apparent cholesterol absorption (% intake)	75.9 ± 12.4[a)]	66.3 ± 12.4[b)]	58.6 ± 9.7[c)]

Values are expressed as mean ± SD, $n = 13$.
a–c) Means at the same row with different superscripts differ significantly at $p < 0.05$. Apparent cholesterol absorption = [(cholesterol intake - excretion of neutral and acidic sterols)/cholesterol intake].

3.5 Effect of AP supplementation on liver cholesterol level

Liver cholesterol content in the control, 0.3AP, and 0.6AP groups was 19.5 ± 3.0, 18.6 ± 2.9, and 21.0 ± 3.1 mg/g, respectively. The statistical analysis did not find any difference in liver cholesterol content among the three groups.

3.6 Western blot analysis of SREBP-2, LDLR, HMGR, and CYP7A1

Western blot analysis of SREBP-2, LDLR, HMGR, and CYP7A1 is shown in Fig. 2. No significant difference in immunoreactive mass of these four proteins was observed among the three groups.

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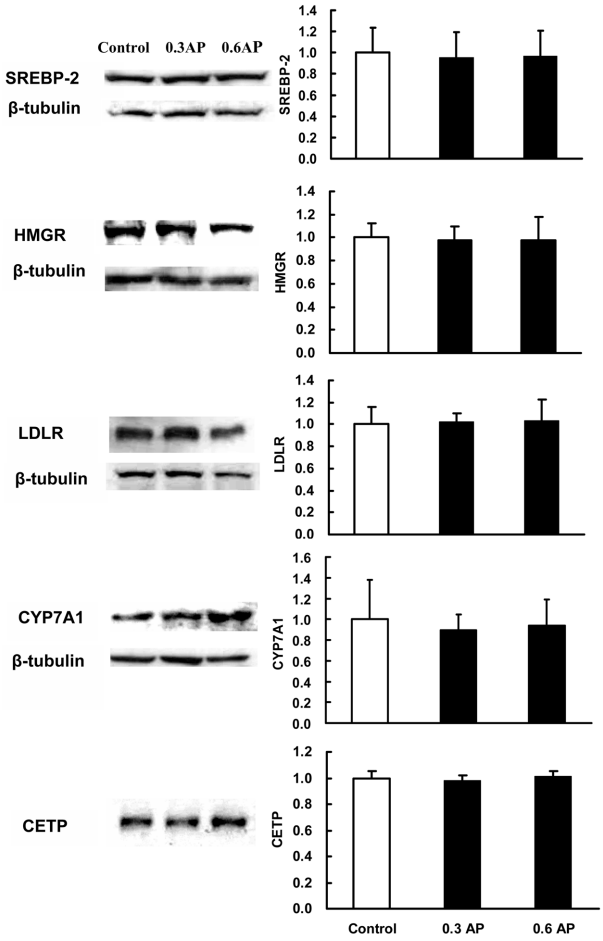

Figure 2. Effect of AP on the relative immunoreactive mass of hepatic SREBP-2, HMGR, LDLR, and CYP7A1 as determined by Western blot analysis. Data were normalized with β-tubulin so that value of the control group was regarded as 1.0. Values were expressed as means ± SD, $n = 13$.

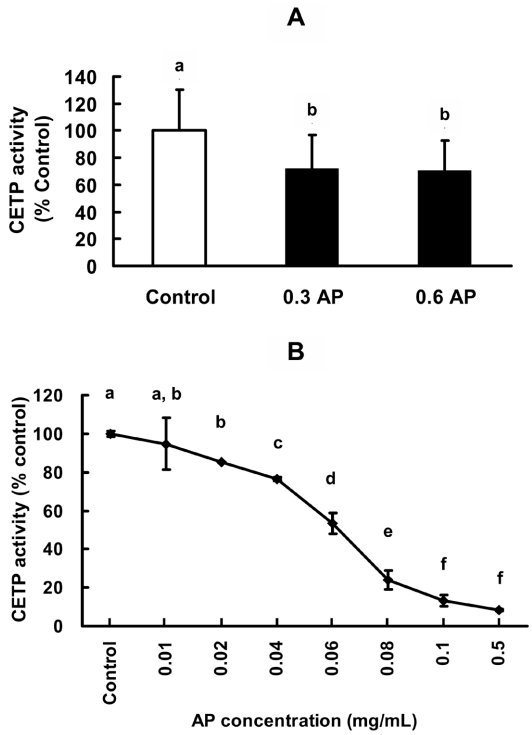

Figure 3. (A) Plasma CETP activity in the hamsters fed the control and AP-supplemented diets. Data were normalized so that CETP activity of the control group was regarded as 100%. Values were expressed as means ± SD ($n = 13$). Means with different superscript letters (a, b) differed significantly at $p < 0.05$. (B) Concentration–response curves of AP on *in vitro* CETP activity. Values were expressed as means ± SD ($n = 3$). Values with different letters (a–f) were significantly different from that of the control at $p < 0.05$.

3.7 Effect of AP supplementation on CETP activity

Plasma CETP activity in hamsters fed the AP diets was significantly lower than that of the control. To be specific, CETP activity in 0.3AP and 0.6AP groups was 71.7 and 70.1% of the control (Fig. 3). However, Western blot analysis found no difference in plasma CETP protein *per* milliliter of plasma among the three groups (Fig. 2), indicating AP was an inhibitor on CETP. To prove this, effect of varying concentrations of AP on CETP activity was carried out *in vitro*. As shown in Fig. 3B, transfer of ^{3}H-cholesterol from HDL to LDL decreased as AP concentration increased. AP exhibited a dose-dependent inhibition on CETP activity.

4 Discussion

The present study was the first time to demonstrate that supplementation of AP did not affect plasma TC, but it decreased non-HDL-C and concomitantly increased HDL-C, leading favorably to a substantial decrease in non-HDL-C/HDL-C ratio (Table 3). In this regard, non-HDL-C/HDL-C in the control group was 0.84, whereas in 0.3AP and 0.6AP groups, it was reduced to 0.58 and 0.46, respectively. The present result was agreement with that of Nakazato *et al*. [16], who reported that AP had no hypocholesterolemic activity in Wistar rats. Unfortunately, they did not measure cholesterol distribution in different lipoprotein classes. Consumption of apple fiber had been shown to reduce blood cholesterol in humans [7] and rats [12, 13]. Together with available reports in the literatures, apple contains at least two types of functional ingredients that could modify favorably plasma lipoproteins with fiber being mainly to reduce plasma TC while polyphenols being to model the ratio of LDL to HDL-C.

Cholesterol homeostasis is maintained delicately in several regulatory points: intestinal cholesterol absorption, hepatic LDL cholesterol uptake, hepatic cholesterol *de novo* synthesis, hepatic conversion to bile acids and cholesteryl ester transfer between lipoprotein classes in the blood (Fig. 4). ACAT-2 is an important factor in intestinal choles-

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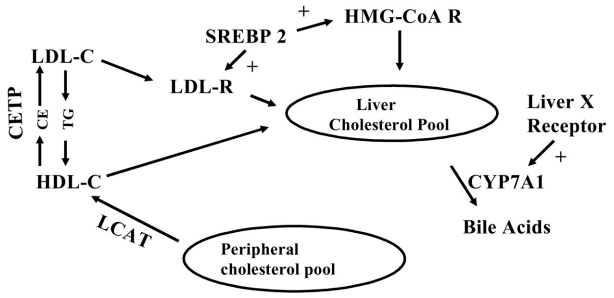

Figure 4. Roles of CETP, lecithin-cholesterol acyltransferase (LCAT), LDL-R, SREBP-2, HMG-CoA R, and CYP7A1 in cholesterol metabolism. +, Up-regulation.

terol absorption [17, 18]. SREBP-2 has been shown to play an important regulatory role in cholesterol synthesis [19]. SREBP-2 binds to the sterol regulatory element on the chromosome, turning on the expression of a number of cholesterol-generating genes, including HMGR and LDLR [20]. HMG-CoA R is the rate-limiting enzyme in cholesterol synthesis [21]. LDL receptor is responsible for LDL cholesterol removal from blood whereas CYP7A1 is the rate-limiting enzyme in bile acid synthesis for cholesterol elimination [22, 23]. Initially, we had targeted these proteins to examine if there were any interactions of AP with their gene expression. The present study did not find any evidence that AP feeding could affect SREBP-2, LDL-R, HMGR, and CYP7A1 (Fig. 2).

The major finding was that AP had no effect on serum TC but they significantly decreased the ratio of non-HDL-C (mainly LDL-C) to HDL-C, arousing our interest to study the effect of AP on CETP activity. CETP is a plasma protein that mediates the movement of cholesteryl esters from HDL to LDL or VLDL in an exchange of equivalent triglycerides. This process is an essential part of the reversal cholesterol transport [24]. The present study demonstrated that AP supplementation reduced CETP activity both *in vitro* and *in vivo*. As shown in Fig. 3, AP acted as an inhibitor to CETP *in vitro* and such property was confirmed *in vivo*, finding that AP-fed hamsters had CETP activity significantly lower than that of the control group (Fig. 3). Western blotting showed no difference in plasma CETP immunoreactive mass between the AP-fed and control hamsters, suggesting that AP did not affect expression and regulate translation of CETP gene but instead it inhibited its enzyme activity in plasma. The present study is in agreement with those of Kothari *et al*. [25] and Rittershaus *et al*. [26] who showed that inhibition of CETP activity was associated with an increase in blood HDL-C level. In recent years, inhibiting CETP activity as a strategy of raising blood HDL-C has become a popular investigation topic [27 – 30]. In this regard, AP-fed groups had lower non-HDL-C to HDL-C ratio, and modified lipoprotein profile favorably against atherosclerosis.

It is noteworthy that supplementation of AP in the diet caused no change in cholesterol excretion but it led to a lesser excretion of other neutral sterols. Mostly interesting was that AP supplementation increased markedly excretion of fecal bile acids by two- to three-fold. The present result is in agreement with that of Aprikian *et al*. [31], who found that polypenol-rich freeze-dried apple could increase the pool of bile acids in small intestine and had a greater fecal bile acid excretion in Zucker rats. In addition to its polyphenols, pectin and fiber in apple are also able to increase the bile acid excretion [12, 13, 32]. In this regard, other plant polyphenols including grape seed and tea leaves have also been shown to enhance the fecal bile acid excretion when they are incorporated into diets [15, 33]. It is conceivable that greater bile acid excretion associated with plant polyphenols may be related to their binding with bile acids during passage through small intestine [34]. However, the molecular mechanism concerning the interaction of polyphenols with bile acids and cholesterol in intestine remains poorly understood.

The present study was not in attempt to study the bioavailability and metabolism of AP in hamsters. Data on bioavailability of polyphenols in whole apple are scarce but information on bioavailability of some individual apply polyphenols is available in the literature [8]. In this regard, chlorogenic acid, the major component in AP, was found to have 33% absorption in humans [35]. Phloretin is another major polyphenol in apple and was absorbed rapidly in the intestine and quickly eliminated in urine when it was administered as the alycone or as the glycoside [36]. Another study found that bioavailability of quercetin in apple could reach 30% relative to onion [37]. Catechin and epicatechin, rich in both apple and tea, was also found to be absorbable in the gastrointerstinal tract [38]. We had analyzed the concentration of these active compounds in serum and did not detect any of these active ingredients. The possible reason was that these compounds were already metabolized and excreted in urine after overnight fasting. It deems necessary therefore to carry out additional experiments to study pharmacokinetic behaviors of AP and prove their anti-CETP activity *in vivo*.

There were no differences in relative organ weights among the three groups (Table 2). AP may not probably cause any toxic effect because apple is one of the common fruits consumed by humans. There has been no report to demonstrate that consumption of apple is associated with any toxicity. It is however unclear if these polyphenols isolated from the apple in a concentrated form have any toxic effect. In addition, it is also unknown if AP decreases blood cholesterol level in a normal low-cholesterol diet. We currently focus on further chemical characterization of AP and investigate the active ingredient(s) which is responsible for anti-CETP activity and study the effect of AP on blood cholesterol level and atherosclerosis using rabbits as a model.

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In conclusion, the present investigated interaction of AP with proteins/enzymes involved in blood cholesterol partition between HDL and LDL lipoproteins. AP reduced plasma CETP activity both *in vivo* and *in intro* but it could not affect SREBP-2, LDL-R, HMGR, and CYP7A1, at both transcriptional and translational levels. Currently, we are studying if other fruit polyphenols affect CETP in a same way as AP, and how AP reduces plasma CETP activity. Human diet is rich in polyphenols. The present study is the first to observe that AP favorably modifies the ratio of non-HDL-C to HDL-C. A better understanding of its biochemical mechanism deems necessary.

The authors have declared no conflict of interest.

5 References

[1] Feskanich, D., Ziegler, R. G., Michaud, D. S., Giovannucci, E. L., *et al*., Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women, *J. Natl. Cancer Inst*. 2000, *92*, 1812 – 1823.

[2] Le Marchand, L., Murphy, S. P., Hankin, J. H., Wilkens, L. R., Kolonel, L. N., Intake of flavonoids and lung cancer, *J. Natl. Cancer Inst*. 2000, *92*, 154 – 160.

[3] Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B., Kromhout, D., Dietary flavonoids and cancer risk in the zutphen elderly study, *Nutr. Cancer* 1994, *22*, 175 – 184.

[4] Gallus, S., Talamini, R., Giacosa, A., Montella, M., *et al*., Does an apple a day keep the oncologist away? *Ann. Oncol.* 2005, *16*, 1841 – 1844.

[5] Sesso, H. D., Gaziano, J. M., Liu, S., Buring, J. E., Flavonoid intake and the risk of cardiovascular disease in women, *Am. J. Clin. Nutr*. 2003, *77*, 1400 – 1408.

[6] Song, Y., Manson, J. E., Buring, J. E., Sesso, H. D., Liu, S., Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: A prospective study and cross-sectional analysis, *J. Am. Coll. Nutr*. 2005, *24*, 376 – 384.

[7] Mee, K. A., Gee, D. L., Apple fiber and gum arabic lowers total and low-density lipoprotein cholesterol levels in men with mild hypercholesterolemia, *J. Am. Diet. Assoc*. 1997, *97*, 422 – 424.

[8] Boyer, J., Liu, R. H., Apple phytochemicals and their health benefits, *Nutrition* 2004, *3*, 5.

[9] Lee, K. W., Kim, Y. J., Kim, D. O., Lee, H. J., Lee, C. Y., Major phenolics in apple and their contribution to the total antioxidant capacity, *J. Agric. Food Chem*. 2003, *51*, 6516 – 6520.

[10] Wolfe, K., Wu, X., Liu, R. H., Antioxidant activity of apple peels, *J. Agric. Food Chem*. 2003, *51*, 609 – 614.

[11] Karvinen, E., Miettinen, M., Effect of apple and pectin diets on serum and liver cholesterol in rats, *Acta Physiol. Scand.* 1968, *72*, 62 – 64.

[12] Aprikian, O., Duclos, V., Guyot, S., Besson, C., *et al*., Apple pectin and a polyphenol-rich apple concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats, *J. Nutr*. 2003, *133*, 1860 – 1865.

[13] Gonzalez, M., Rivas, C., Caride, B., Lamas, M. A., Taboada, M. C., Effects of orange and apple pectin on cholesterol concentration in serum, liver and faeces, *J. Physiol. Biochem*. 1998, *54*, 99 – 104.

[14] Vaziri, N. D., Liang, K. H., Down-regulation of hepatic LDL receptor expression in experimental nephrosis, *Kidney Int.* 1996, *50*, 887 – 893.

[15] Chan, P. T., Fong, W. P., Cheung, Y. L., Huang, Y., *et al*., Jasmine green epicatechins are hypolipidemic in hamsters fed a high fat diet, *J. Nutr.* 1999, *129*, 1094 – 1101.

[16] Nakazato, K., Song, H., Waga, T., Effects of dietary apple polyphenol on adipose tissues weights in wistar rats, *Jpn. Assoc. Lab. Anim. Sci*. 2006, *55*, 383 – 389.

[17] Parini, P., Davis, M., Lada, A. T., Erickson, S. K., *et al*., ACAT2 is localized to hepatocytes and is the major cholesterol-esterifying enzyme in human liver, *Circulation* 2004, *110*, 2017 – 2023.

[18] Repa, J. J., Buhman, K. K., Farese, R. V., Jr., Dietschy, J. M., Turley, S. D., ACAT2 deficiency limits cholesterol absorption in the cholesterol-fed mouse: Impact on hepatic cholesterol homeostasis, *Hepatology (Baltimore, Md.)* 2004, *40*, 1088 – 1097.

[19] Eberlé, D., Hegarty, B., Bossard, P., Ferré, P., Foufelle, F., SREBP transcription factors: Master regulators of lipid homeostasis, *Biochimie* 2004, *86*, 839 – 848.

[20] Sakakura, Y., Shimano, H., Sone, H., Takahashi, A., *et al*., Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis, *Biochem. Biophys. Res. Commun*. 2001, *286*, 176 – 183.

[21] Goldstein, J. L., Brown, M. S., Regulation of the mevalonate pathway, *Nature* 1990, *343*, 425 – 430.

[22] Issandou, M., Pharmacological regulation of low density lipoprotein receptor expression: Current status and future developments, *Pharmacol. Ther*. 2006, *111*, 424 – 433.

[23] Norlin, M., Wikvall, K., Enzymes in the conversion of cholesterol into bile acids, *Curr. Mol. Med*. 2007, *7*, 199 – 218.

[24] Shah, P. K., Inhibition of CETP as a novel therapeutic strategy for reducing the risk of atherosclerotic disease, *Eur. Heart J.* 2007, *28*, 5 – 12.

[25] Kothari, H. V., Poirier, K. J., Lee, W. H., Satoh, Y., Inhibition of cholesterol ester transfer protein CGS 25159 and changes in lipoproteins in hamsters, *Atherosclerosis* 1997, *128*, 59 – 66.

[26] Rittershaus, C. W., Miller, D. P., Thomas, L. J., Picard, M. D., *et al*., Vaccine-induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in a rabbit model of atherosclerosis, *Arteriosc. Thromb. Vasc. Biol.* 2000, *20*, 2106 – 2112.

[27] Barter, P. J., Kastelein, J. J., Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease, *J. Am. Coll. Cardiol*. 2006, *47*, 492 – 499.

[28] Doggrell, S. A., Inhibitors of cholesteryl ester transfer protein – a new approach to coronary artery disease. *Expert Opin. Invest. Drugs* 2006, *15*, 99 – 106.

[29] Dullaart, R. P., Dallinga-Thie, G. M., Wolffenbuttel, B. H., van Tol, A., CETP inhibition in cardiovascular risk management: A critical appraisal, *Eur. J. Clin. Invest*. 2007, *37*, 90 – 98.

[30] Okamoto, H., Miyai, A., Sasase, T., Furukawa, N., *et al*., Cholesteryl ester transfer protein promotes the formation of cholesterol-rich remnant like lipoprotein particles in human plasma, *Intl. J. Clin. Chem*. 2007, *375*, 92 – 98.

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[31] Aprikian, O., Busserolles, J., Manach, C., Mazur, A., *et al*., Lyophilized apple counteracts the development of hypercholesteoleimia oxidative stress, and renal dysfunction in obese zucker rats, *J. Nutr.* 1002, *132*, 1969 – 1976.

[32] Sembris, S., Dongowski, G., Mehrlaender, K., Will, F., Dietrich, H., Dietary fibre-rich colloids from apple pomace extraction juice don affect food intake and blood serum lipids level, but enhance fecal excretion of steroids in rats, *J. Nutr. Biochem*. 2004, *15*, 296 – 302.

[33] Nakamura, Y., Tonogai, Y., Effects of grape seed polyphenols on serum and hepatic lipid contents and fecal steroid excretion in normal and hypercholeterolemic rats, *J. Health Sci*. 2002, *48*, 570 – 578.

[34] Kahle, K., Kraus, M., Scheppach, W., Achermann, M., *et al*., Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after injection, *Mol. Nutr. Food Res*. 2006, *50*, 418 – 423.

[35] Olthof, M. R., Hollman, P. C., Katan, M. B., Chlorogenic acid and caffeic acid are absorbed in humans, *J. Nutr*. 2001, *131*, 66 – 71.

[36] Crespy, V., Aprikian, O., Morand, C., Besson, C., *et al*., Bioavailability of phloretin and phloridzin in rats, *J. Nutr*. 2002, *132*, 3227 – 3230.

[37] Hollman, P. C. H., van Trijp, J. M. P., Buysman, M. N. C. P., *et al*., Relative bioavailability of the antioxidant flavonoid quercein from various foods in man, *FEBS Lett*. 1997, *418*, 152 – 156.

[38] Spencer, J. P., Metabolism of tea flavonoids in the gastrointestinal tract, *J. Nutr*. 2003, *133*, 3255S – 3261S.

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