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Hypocholesterolemic activity of hawthorn fruit is mediated by regulation of cholesterol-7α-hydroxylase and acyl CoA: cholesterol acyltransferase

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Abstract

The present study was to investigate the mechanisms by which hawthorn fruit lowers serum cholesterol in hamsters. The control group was fed a semisynthetic diet containing 0.1% cholesterol while the tested group was maintained on the same diet but supplemented with 0.5% hawthorn fruit aqueous ethanolic extract for 4 weeks. Serum total cholesterol (TC) and triacylglycerols (TG) were decreased by 10 and 13%, respectively, in hawthorn fruit group as compared with the control ($P<0.05$). Supplementation of hawthorn fruit aqueous ethanolic extract led to greater excretion of both neutral and acidic sterols. Further enzymatic assays suggest the mechanisms by which hawthorn fruit decreases serum cholesterol involve a greater excretion of bile acids mediated by up-regulation of hepatic cholesterol 7α-hydroxylase (CH) activity, and an inhibition of cholesterol absorption mediated by down-regulation of intestinal acyl CoA:cholesterol acyltransferase (ACAT) activity.

Keywords: Cholesterol; *Crataegus pinnatifida*; Hawthorn fruit; Hamster; Triglyceride

1. Introduction

Hawthorn (*Crataegus*) is widely distributed throughout the northern temperate regions of the world with approximately 280 species primarily in East Asia, Europe and North America. *Crataegus pinnatifida* and *Crataegus cuneata* are the two major species found in China where they are named as Shanzha and commonly used to cure scurvy, constipation and stomach ailment. Consumption of hawthorn fruit is also associated with long-term medicinal benefits to the cardiovascular system (Ammon & Händel, 1981a, 1981b, 1981c). The hawthorn fruit extract has been used to treat the early stages of congestive heart failure (Schussler, Holzl, & Fricke, 1995; Weihmayr & Emst, 1996) and angina pectoris (Hanack & Bruckel, 1983). The chemical composition of hawthorn fruits has been a subject of extensive studies (Huang, 1993). The major ingredients, including fructose, flavonoids, proanthocyanidins, triterpenes, organic acids, vitamins and minerals, vary with species, geographic locations and the time of harvest. However, the active compounds responsible for these beneficial effects remain unknown.

High plasma cholesterol is one of risk factors in contribution to coronary heart disease (Grundy, 1986; Neaton, Kuller, Wentworth, & Borhani, 1984). Hawthorn fruit is capable of lowering serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) in hyperlipidemic humans (Chen, Wu, Tao, Chen, & Liu, 1995; von Eiff, 1994). However, the underlying mechanism is poorly understood. It was previously reported that supplement of hawthorn fruit was associated with an increase in LDL-receptor activity of hepatic membrane in rats (Ho, Chang, & Lee, 1997). In this regard, our previous study showed that inclusion of 2% hawthorn fruit powder led to 23% lower serum TC, 51% less cholesterol accumulation in aorta, and 95% greater excretion of neutral and acidic sterols in rabbits fed a 1% cholesterol diet (Zhang, Ho, Huang, James, Lam, & Chen, 2002), suggesting that inhibition of cholesterol absorption is an alternative mechanism responsible for the hypocholesterolemic activity of hawthorn fruits.

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Inhibition of dietary cholesterol absorption is an efficient way of lowering blood cholesterol. Any dietary esterified cholesterol is hydrolyzed to the free sterol by cholesteryl esterase in or on the surface of micelles that are formed from amphipathic constituents present in the bile. Cholesterol in the micelles is transferred to the mucosal cells and 90% of the absorbed sterol is incorporated into mesenteric lymph as cholesteryl ester (Vahouny & Treadwell, 1957). Intestinal acyl CoA:cholesterol acyltransferase (ACAT) is believed to play an important role in intestinal cholesteryl esterification before cholesterol can be absorbed. Some ACAT inhibitors have been used to act as both hypocholeserolemic and antiatherosclerotic agents (Largis, Wang, DeVries, & Schaffer, 1989). Increased excretion of bile acids can also lead to a decrease of blood cholesterol. Hepatic cholesterol 7α-hydroxylase (CH) is a rate-limiting enzyme in the conversion of cholesterol to bile acids. The present study was conducted to study the effect of hawthorn fruit aqueous ethanolic extract in the diet on intestinal ACAT and hepatic CH in hamsters.

2. Materials and methods

2.1. Preparation of hawthorn fruit aqueous ethanolic extract

Dry hawthorn fruits (*C. pinnatifida*) were purchased from a local market. After the removal of seeds, the dry fruit flesh was ground into powder in a coffee grinder. Dry hawthorn fruit powder (120 g) was soaked three times with 500 ml of 80% ethanol at room temperature for 12 h in an incubation shaker. The ethanol phases were pooled, filtered and evaporated using a vacuum rotary evaporator. The resultant aqueous ethanolic extract was then weighed and saved for the diet supplement.

2.2. Diets

The hypercholesterolemic diet previously described by Sanders and Sandaradura (1992) was used with modification. The control diet was prepared by mixing the following ingredients: casein, 200 g; lard, 200 g; cornstarch, 418 g; sucrose, 100 g; AIN-76 mineral mix, 40 g; AIN-76A vitamin mix, 20 g; DL-methionine, 1 g; and cholesterol, 1 g. The ingredients were purchased from Harlan Teklad (Madison, WI, USA) except for lard, which was obtained from the local market, and DL-methionine and cholesterol, which were purchased from Sigma Chemical (St. Louis, MO, USA). The tested diet was similar to the control except that 0.5% hawthorn fruit aqueous ethanolic extract (equivalent to 2% dry hawthorn fruit powder) was added. Both the control and tested diets (1 kg) were then mixed with 300 ml of gelatin solution (20 g/l). Once the gelatin had set, the food was cut into ~20 g cubed portions and stored in a freezer at −20 °C.

2.3. Animals

Male Syrian golden hamsters (2 mo; 95–110 g, *Mesocricetus auratus,* The Chinese University of Hong Kong, Shatin, Hong Kong) were randomly divided into two groups ($n=15$) and housed in an animal room at 23 °C with 12:12-h light–dark cycles. Frozen diets were given to hamsters daily, and uneaten portion was discarded. Food intake was measured daily and body weight was recorded weekly. The hamsters were allowed access to food and tap water ad libitum. The total fecal output of each hamster was combined during entire week 2 and week 4. At the end of 4 weeks, all hamsters were killed after food deprivation for 14 h. The blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1300 × *g* for 10 min, and serum was then collected. The liver was removed, washed with saline, and stored at −80 °C for assays of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase and CH activity. The intestine was also saved; the first 10 cm from the stomach was discarded, and the next 30 cm taken for the intestinal ACAT assay. The protocol was reviewed and approved by the Committee of Animal Ethics, The Chinese University of Hong Kong.

2.4. Determination of serum TC, HDL-cholesterol and TG

Several enzymatic kits were purchased from Sigma (St. Louis, MO, USA) to measure serum TG (Catalogue Number: 336–20) and TC (Catalogue Number: 352–20). HDL-cholesterol was isolated and determined by precipitation of the apolipoprotein B-containing lipoproteins with sodium phosphotungstate-magnesium chloride using the commercial Sigma kit (HDL-C; Catalogue Number: 352–4). No attempt was made to determine the low-density lipoprotein cholesterol (LDL-C) using a formula of LDL-C = (TC) −(HDL-C) −(TG/5), which is only applicable to humans.

2.5. Determination of liver lipids

Total lipids were extracted from 300 g of liver with the addition of stigmastanol (Sigma, St. Louis, MO, USA) as an internal standard using chloroform–methanol (2:1, v/v). The lipid extracts were then saponified with 6 ml of 1 mol/l NaOH in 90% ethanol at 90 °C for 1 h, and the non-saponified substances including cholesterol were converted to their trimethylsilyl (TMS)-ether derivatives by a commercial TMS reagent (Sigma, St. Louis, MO, USA). The analysis of cholesterol TMS-ether derivative was performed in a fused silica capillary column (SAC^{TM}-5, 30 m×0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA, USA) using a Shimadzu GC-14 B gas–liquid chroma-

tograph (GLC) equipped with a flame ionization detector (Shimadzu, Kyoto, Japan) as previously described (Chan, Fong, Cheung, Huang, Ho, & Chen, 1999).

Liver triacylglycerols (TG), phospholipids (PL) and free fatty acids (FFA) were measured as previously described (Chen & Cunnane, 1992). Lipid classes in an aliquot of liver lipid extract were separated by neutral lipid thin-layer chromatography (TLC; 20×20 cm plate pre-coated with 250 μm silica gel 60A, Macherey-Nagel, Durenm, Germany) using a developing solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). TG, PL and FFA bands were recovered from the TLC plate and converted to fatty acid methyl esters, which was then analyzed in a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA, USA) equipped with a SP-2560 flexible fused silica capillary column (100×0.25 mm, i.d., 20 μm film thicknes, Supelco, Inc., Bellefonte, PA). The liver TG, PL and FFA were quantified according to the amounts of triheptadecanoin, L-α-phosphotidylcholine diheptadecanoyl and heptadecanoic acid (internal standards) added during the extraction.

2.6. Quantification of fecal neutral and acidic sterols

Individual fecal neutral and acidic sterols were quantified as previously described (Chan et al., 1999). In brief, stigmasterol (0.3 mg) as an internal standard for neutral sterols was added to a fecal sample (300 mg). The sample was then saponified using 9 ml of 1 mol/l NaOH in 90% ethanol containing 0.3 mg hyodeoxycholic acid as an internal standard for acidic sterols (Sigma, St. Louis, MO, USA). The total neutral sterols were extracted using 8 ml of cyclohexane and were then converted to their corresponding TMS-ether derivatives for GLC analysis.

After the cyclohexane extraction, 1 ml of 10 mol/l NaOH was added to the remaining aqueous layer and heated at 120 °C for 3 h. After cooling down, 1 ml of distilled water and 3 ml of 3 N HCl were added followed by extraction with 7 ml of diethyl ether twice. The diethyl ether layers were then pooled followed by adding 2 ml of methanol, 2 ml of dimethoxypropane and 40 μl of concentrated HCl (12 mol/l). After standing overnight at room temperature, the solvents were dried down and the acidic sterols were similarly converted to their TMS-ether derivatives at 60 °C for GLC analysis.

2.7. Liver HMG-CoA reductase, CH and intestinal ACAT

The liver microsomes were isolated according to the method of Erickson, Copper, Matsui and Gould (1977). The activity of liver HMG-CoA reductase (EC.1.1.1.34) was measured as previously described by Edwards, Lemongello, and Fogelman (1979). The activity of liver CH (EC1.14.13.17) was measured using the method of Souidi, Parquet, and Lutton (1998). The activity of intestinal ACAT (EC 2.3.1.26) was measured according to the method described by Stange, Sucking, and Dietschy (1983) and modified by Chautan, Termine, Nalbone, and Lafont (1988).

2.8. Statistical analysis

Data are expressed as mean±S.D. The analysis of variance (ANOVA) was used where applicable for statistical evaluation of significant differences between the control and tested group using Sigmastat (Jandel Scientific Software, San Rafael, CA, USA). Differences were considered significant when $P<0.05$.

3. Results and discussion

3.1. Body weight and food intake

The body weight and food intake for the two groups of hamsters are shown in Table 1. No significant differences in body weight and food intake were observed between the control group and hawthorn-supplemented group.

3.2. Serum lipids

Significant reductions in the serum TC by 10% and TG by 13% were observed in the hawthorn group compared with the control hamsters ($P<0.05$, Fig. 1). However, supplementation of hawthorn fruit aqueous ethanolic extract had no effect on the serum HDL-C level (Fig. 1).

3.3. Liver lipids

The effects of dietary hawthorn fruit aqueous ethanolic extract on liver TG, PL, FFA and cholesterol are shown in Table 2. The hawthorn-supplemented group had liver FFA significantly lower than the control group ($P<0.01$). No significant differences in liver cholesterol, TG and PL were observed between the control group and hawthorn-supplemented group (Table 2).

Table 1
Changes in body weight and food intake in hamsters supplemented with hawthorn fruit aqueous ethanolic extract[a]

	Control ($n=15$)	Hawthorn ($n=15$)
Initial body wt (g)	101.7±4.5	101.3±3.5
Final body wt (g)	123.7±8.8	122.0±7.3
Food intake (g/day)	7.9±0.6	7.6±0.8

[a] Values are means±S.D.; numbers in parentheses indicate the number of hamsters used.

Table 2
Effects of supplementation of 0.5% hawthorn fruit aqueous ethanolic extract in diet on liver lipid concentrations in hamsters[a]

	Control ($n=15$)	Hawthorn ($n=15$)
TG (mg/g)	6.3±2.9	7.2±2.1
PL (mg/g)	32.0±1.4	31.3±1.7
FFA (mg/g)	3.3±0.5	2.8±0.4*
Cholesterol (mg/g)	30.1±3.6	28.7±4.0

[a] TG, triacylglycerols; PL, phospholipids; FFA, free fatty acids. Values are means±S.D.; numbers in parentheses indicate the number of hamsters used.
* Differ significantly from the control at $P<0.01$.

3.4. Fecal neutral and acidic sterols

The hawthorn-supplemented group had higher fecal excretions of neutral sterols per se during week 4 compared with the control group ($P<0.05$, Table 3). However, the fecal excretion of total neutral sterols exhibited no significant difference between the two groups in week 2. The output of total fecal bile acids in the hawthorn-supplemented group was also not different from that in the control group during week 2 but it was greater ($P<0.05$) during week 4 (Table 3). Among the bile acids, lithocholic acid, deoxycholic acid and chenodeoxycholic acid were significantly higher in the feces of hamsters fed the diet supplemented with hawthorn aqueous ethanolic extract, whereas the concentration of cholic acid did not differ between the two groups.

3.5. HMG-CoA reductase, CH and ACAT

The activity of liver HMG-CoA reductase in the hawthorn-supplemented group was not different from that of the control group. However, the activity of liver CH in the hawthorn-supplemented group was significantly increased compared with that for the control group ($P<0.01$, Table 4). The activity of intestinal ACAT in the hawthorn-supplemented group was significantly decreased compared with that of the control group ($P<0.05$, Table 4).

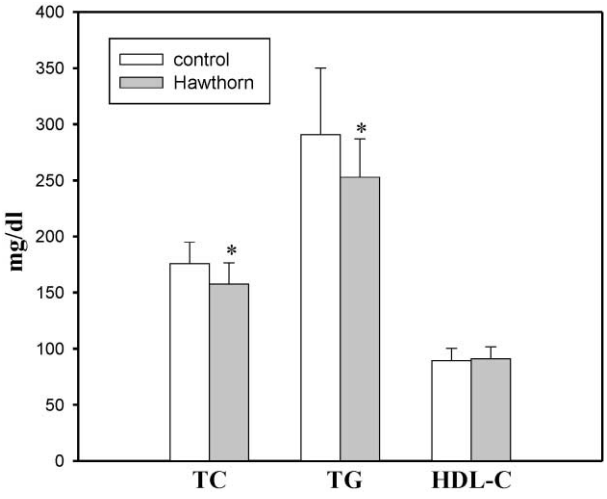

Fig. 1. Effects of supplementation of 0.5% hawthorn fruit ethanol extract (equal 2% dried fruit powder) in diet on serum total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) in hamsters. Values are means±S.D., $n=15$. *Differs significantly at $P<0.05$.

The present study confirmed that hawthorn fruit possesses a hypolipidemic activity. Supplementation of 0.5% hawthorn fruit aqueous ethanolic extract could significantly lower serum TC and TG with HDL being unchanged in hamsters. The results are consistent with that observed for humans (Chen et al., 1995) and rabbits (Zhang et al., 2002). It is unlikely that the hypocholesterolemic effect is due to dietary fibre present in hawthorn fruit because the present study did not supplement with the whole fruit but only aqueous ethanolic extract in which the fibre was excluded. The present

Table 3
Effects of supplementation of 0.5% hawthorn fruit aqueous ethanolic extract on fecal output of neutral and acidic sterols in hamsters[a]

	Week 2		Week 4	
	Control ($n=15$)	Hawthorn ($n=15$)	Control ($n=15$)	Hawthorn ($n=15$)
Coprostanol	2.79±1.37	2.84±1.12	5.10±2.00	7.36±2.54*
Coprostanone	0.15±0.05	0.18±0.03	0.16±0.03	0.26±0.09*
Cholesterol	1.16±0.61	1.42±0.50	2.07±0.96	2.38±0.94
Dihydrocholesterol	0.53±0.22	0.57±0.16	0.99±0.22	1.19±0.22*
Campesterol	0.21±0.06*	0.36±0.07*	0.28±0.04	0.57±0.10*
Total neutral sterols	4.83±1.13	5.36±1.01	8.61±1.37	11.77±2.00*
Lithocholic acid	1.13±0.31	1.21±0.26	1.35±0.42	1.74±0.26*
Deoxycholic acid	1.12±0.28	1.24±0.26	1.40±0.48	2.20±0.48*
Chenodeoxycholic acid	0.17±0.08	0.22±0.12	0.25±0.16	0.40±0.27*
Cholic acid	0.19±0.10	0.23±0.10	0.28±0.15	0.42±0.21*
Total acidic sterols	2.60±0.69	2.90±0.62	3.28±0.96	4.75±0.98*

[a] Values are means±S.D.
* Differ significantly from the control for a given time at $P<0.05$.

Table 4
The activities of liver 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, liver cholesterol 7α-hydroxylase (CH) and intestinal acyl CoA:cholesterol acyltransferase (ACAT) in hamsters fed a high fat and cholesterol diet with or without supplementation of hawthorn fruit aqueous ethanolic extract[a]

	HMG-CoA reductase	CH	ACAT
	pm/ min · mg protein	pm/ min · mg protein	nm/ min · mg protein
	Liver	Liver	Intestine
Control ($n=15$)	6.60±2.46	53.0±29.2d	1.03±0.28a
Hawthorn ($n=15$)	6.40±2.49	148.9±57.2c	0.78±0.20b

[a] Values are means±S.D. Means at a column with different letters differ significantly, ab$P<0.05$, cd$P<0.01$.

study indicates that hawthorn fruit contains unknown active ingredient(s) that may influence favorably cholesterol metabolism.

The mechanism by which hawthorn fruit decreases serum cholesterols remains unclear. Serum cholesterol can be lowered at several metabolic points including decreased synthesis, activation of LDL receptors, inhibition on absorption of dietary cholesterol, and conversion of cholesterol to bile acids. Rajendran, Deepalakshmi, Parasakthy, Devarji, and Niranjali Devaraji (1996) found that there was a significant increase in binding LDL to liver plasma membrane in vitro when hawthorn ethanol extract (*Crataegus oxyacentha*) was administered to rats fed an atherogenic diet. Similar effect was found in the study by Ho et al., (1997), who investigated effect of hawthorn extract at LDL receptor level in HepG2 cells and found that the extract could up-regulate LDL receptors.

The decrease in cholesterol biosynthesis would also lead to a lower blood cholesterol level. The results demonstrated that the extract had no action on HMG-CoA reductase, suggesting that inhibition of cholesterol synthesis is unlikely part of the hypocholesterolemic action of hawthorn fruit. The observation is in agreement with that observed in rabbits fed a diet containing 2% hawthorn fruit powder derived from *C. pinnatifida* (Zhang et al., 2002)

The inhibition of cholesterol absorption in the intestine could also reduce serum cholesterol. It is known that cholesterol in the large intestine is partially degraded and converted to other metabolites by intestinal microflora (Midtvedt & Midtvedt, 1993). The amount of cholesterol and bile acids degraded and converted during the passage through the large intestine cannot be quantified in this study but the total sterols and some metabolites were measured. The hawthorn group excreted slightly greater amount of fecal cholesterol but the difference between the two groups was not significant. However, higher excretion of total fecal sterols and coprostanol, a metabolite of intestinal microflora, was observed in hawthorn-supplemented group (Table 3). It was possible that greater amount of dietary cholesterol entered tzzhe large intestine but it was degraded and converted to its metabolites, leading to a significant increased excretion of fecal coprostanol and total neutral sterol. To study further the effect of hawthorn fruit supplementation on the absorption of cholesterol, ACAT activity in the intestine was measured. Intestinal ACAT may play a key role in the absorption of cholesterol by esterification of cholesterol prior to absorption (Wrenn, Parks, Immermann, & Rudel, 1995). The data demonstrated that supplementation of hawthorn fruit aqueous ethanolic extract was associated with a lower intestinal ACAT activity, suggesting that inhibition on absorption of dietary cholesterol is at least partly responsible for the hypocholesterolemic activity of hawthorn fruit.

Greater excretion of bile acids could also lead to a lower level of serum cholesterol. The present results showed that fecal excretion of both primary (cholic and chenodeoxycholic) and secondary (lithocholic and deoxycholic) bile acids were higher in the hawthorn group compared with that in the control group in week 4. The liver CH removes cholesterol and produces 7α-hydroxycholesterol, which is the first step on the metabolic conversion of cholesterol to bile acids and CH is generally recognized as a rate-limiting enzyme for the entire pathway (Myrant & Mitropoulos, 1977). Inclusion of hawthorn aqueous ethanolic extract in the diet significantly increased the liver CH activity, indicating that the increased excretion of bile acids is mediated by up-regulation of this enzyme.

Excretion of neutral and acidic sterols is a function of dietary cholesterol. It was noteworthy that excretion of cholesterol and bile acids was greater in week 4 than that in week 2 within each group. The observation is in agreement with that of Chan et al., (1999), who found that excretion of both cholesterol and bile acids was gradually increased during the first 4 weeks when hamsters were switched from a low-cholesterol chow diet to a 0.1% cholesterol diet. Perhaps, saturation of cholesterol absorption was slowly reached after hamsters were placed on a high-cholesterol diet, leading to a greater amount of fecal cholesterol in week 4.

Up-regulation of LDL-receptors is probably an alternative mechanism responsible for the hypocholesterolemic activity of hawthorn fruits. In the study by Rajendran et al. (1996), supplementation of 0.5 ml alcholic extract per 100 g body weight per day for 6 weeks was associated with a significant increase of hepatic LDL-receptor activity, resulting in greater influx of plasma cholesterol into the liver. The hypothesis is also supported by the study of Ho, Chang, and Lee (1997), who investigated the effect of hawthorn fruit extract on HepG2 cells and demonstrated a significantly increased LDL-receptor activity.

4. Conclusion

The reduction in serum TC by dietary hawthorn fruit is a complex process that involves multifaceted interactions of cholesterol metabolism. In addition to the activation of LDL receptors, hypocholesterolemic properties of hawthorn fruit are possibly mediated simultaneously by down-regulation of intestinal ACAT and up-regulation of hepatic CH, leading a greater excretion of both neutral and acidic sterols.

The following aspects need to be addressed in further investigations. Firstly, the inclusion of hawthorn aqueous ethanolic extract in the diet reduced not only the level of serum cholesterol but also the concentration of serum TG. It remains unknown if the reduction of serum TG is associated with a lower activity of hepatic fatty acid synthase and/or a reduced absorption of dietary fat. Secondly, hawthorn fruit contains flavonoids, triterpenoids, tartaric acid, citric acid, glucosides and saponin but little is known about the active ingredients that exert the beneficial effects. Thirdly, no data are available on the biological activity of hawthorn fruit in relation to its composition, genetic variation and amount of consumption.

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