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Lipid Metabolism in Rats Fed Acetaminophen with Coadministration of Adzuki Bean Extract

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Abstract The effect of water extract of adzuki beans on acetaminophen-altered lipid metabolism was examined in rats. Control group of rats was fed a basal diet, another group of rats was fed 0.5% acetaminophen (APAP group), and a third group of rats was fed 0.5% acetaminophen plus 5% adzuki bean extract (ABE group) for 4 weeks. Serum total and HDL cholesterol levels in the APAP group were significantly lower than those in the control and ABE groups. Hepatic cholesterol 7α-hydroxylase and fatty acid synthase mRNA levels in the APAP and ABE groups were significantly higher and lower than in the control group, respectively. Hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA level in the APAP group was significantly lower than in the control group, whereas that in the ABE group was significantly higher than in the APAP group. These results indicate that adzuki bean extract may improve the acetaminophen-altered serum lipid metabolism in rats.

Keywords: acetaminophen, adzuki bean extract, cholesterol, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA), reductase mRNA

Introduction

Several studies have shown that different non-steroidal anti-inflammatory drugs (NSADIs), including acetyl-salicylic acid, flufenamic acid, ibuprofen, indomethacin, and troglitazone, which are commonly used as antipyretic and anti-inflammatory agents, reduce plasma cholesterol levels (1, 2). Acetaminophen (*n*-acetyl-*p*-aminophenol) is also widely used as an analgesic-antipyretic agent. However, like some lipid-lowering agents that give rise to liver abnormalities such as acute liver failure, chronic active hepatitis, cholestasis, and jaundice (3), a large dose of acetaminophen is well known to cause hepatic damage in both human subjects and laboratory animals (4-6). Such hepatic damage induced by acetaminophen is likely related to *n*-acetal-*p*-benzoquinone imine-conjugated with glutathione (7), and/or oxidative stress in the cell, ultimately leading to its demise (8).

Bean seeds are used as staple foods in many countries, and are receiving increasing attention for their health benefits (9). Some researchers reported the antioxidant activities of beans (10, 11), and Cardador-Martinez *et al.* (11) suggested that such effects are due to the presence of phytochemicals, including polyphenols in the hulls. Wu *et al.* (5) reported that adzuki bean (*Vigna angularis*, red bean) extract inhibits hepatic damage induced by a single intraperitoneal dose of acetaminophen in rats. We have also found a protective action of adzuki bean extract against acetaminophen-induced hepatic damage via a hepatic glutathione-mediated antioxidation/detoxification system in rats fed a diet containing water extract from adzuki bean hulls for 4 weeks (12). It is reported that lipid metabolic alterations can reflect the type or intensity of hepatic injury (13). Recently, Raghavendran *et al.* (14) reported that a single dose of acetaminophen increased the serum total cholesterol level. However, to the best of our knowledge, few studies have been published on the acetaminophen-altered lipid metabolism, and the mechanism of protection of adzuki bean extract against lipid metabolic alterations induced by acetaminophen in the food matrix remains unclear.

Thus, the present study was conducted to clarify the effect of lipid metabolism of adzuki bean extract on acetaminophen-induced hepatic damage, and to elucidate the mechanisms underlying its protective effect in rats.

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Materials and Methods

Materials and chemicals Adzuki beans (*Vigna angularis*) were obtained locally in the Tokachi area in Japan. Acetaminophen was purchased from Wako Co. (Osaka, Japan). Chloroform and methanol were purchased from Sigma-Aldrich Co. (Osaka, Japan).

Preparation of adzuki bean extract For preparation of the aqueous adzuki bean extract, 24 kg of adzuki beans was crushed in a cyclomixer with distilled water, boiled at 93°C for 2 hr with gentle stirring, and adjusted to room temperature by adding cool water. Then the mixture was filtered with a 12-mesh sieve, and the residue was reextracted under the same conditions. Next, the combined filtrates were pressed (150 kg/m^2) in a cotton gauze bag. Following this, the eluted solution was lyophilized, and finally ground to powder. The yield of the aqueous extract was approximately 12% based on weight. The protein, lipid, carbohydrate, moisture, and ash contents in the adzuki bean extract were determined by the AOAC

procedure (15). The total polyphenol concentration in the adzuki bean extract was determined by the Folin-Ciocalteu method (16). The composition was as follows (g/100 g): moisture, 4.2; protein (calculated by multiplying nitrogen contents by 6.25), 17.5; lipid, 1.4; ash, 15.7; carbohydrate, 56.4; and total polyphenol, 4.8.

Animals and diets Male F344/DuCrj rats (7 weeks old) were purchased from Charles River Japan (Yokohama, Japan). All animals were housed individually in cages on a 12-hr light/dark cycle. Temperature was maintained at 23± 1°C with 60±5% relative humidity. Animals were randomly divided into 3 groups, each of 5 animals. There was no significant difference in body weight among the groups at the start of the experiment. The composition of each diet is shown in Table 1. All diets were based on the AIN-76 purified rodent diet (17). The control group was fed a basal diet, the APAP group acetaminophen (5 g/kg basal diet), and the ABE group acetaminophen (5 g/kg basal diet) plus adzuki bean extract (50 g/kg basal diet), as described previously (12). The rats were allowed free access to experimental diets and water for 4 weeks. This experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine. All animal procedures described conformed to standard principles in *Guide for the Care and Use of Laboratory Animals* (18).

Analytical procedures Blood samples (1 mL) were collected between 08:00 and 10:00 hr from the jugular veins of fasting rats once a week for 4 weeks. The samples were taken into tubes without an anticoagulant. After the samples stood at room temperature for 2 hr, serum was obtained by centrifugation at 1,500×g for 20 min. At the end of the experimental period of 4 weeks, fecal excretion during 2 days was collected, and finally the rats were anesthetized with diethyl either. Then the liver was quickly removed, washed with cold saline, dehydrated on filter paper, and weighed before freezing for storage.

Chemical analysis Serum total cholesterol and high density lipoprotein (HDL) cholesterol concentrations were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX, USA). The non-HDL cholesterol concentration was calculated as follows: Non-HDL cholesterol = total cholesterol − HDL cholesterol.

Total lipids were extracted from the liver and feces by a mixture of chloroform-methanol (2:1, v/v) (19), and neutral sterols were acetylated and analyzed by gas liquid chromatography using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm × 30 m, J&W Scientific, Folsom, CA, USA) (20).

Table 1. Experimental diets

Components (g/kg)	Dietary group[1]		
	Control	APAP	ABE
Casein	250	250	250
Corn oil	50	50	50
Mineral (AIN 76)	35	35	35
Vitamin (AIN 76)	10	10	10
Cellulose	50	50	50
Acetaminophen	-	5	5
Adzuki extract	-	-	50
Choline chloride	2	2	2
Sucrose	603	603	603

[1]APAP, acetaminophen diet group; ABE, acetaminophen plus adzuki bean extract diet group.

RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), and Southern blot analysis Hepatic total RNA was isolated by the acid guanidium/ phenol/chloroform method, using Isogen (Nippon Gene, Tokyo, Japan) (21). mRNAs encoding 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) were analyzed by semi-quantitative RT-PCR and subsequent Southern hybridization of PCR products with each inner oligonucleotide (Sigma Genosys, Ishikari, Hokkaido, Japan). The primer oligonucleotides used for PCR were as follows: for HMG-CoA reductase (sense primer, 5'-GCG TGC AAA GAC AAT CCT GGA G-3'; anti-sense primer, 5'-GTT AGA CCT TGA GAA CCC AAT G-3'); for cholesterol 7α-hydroxylase (sense primer, 5'-GCC GTC CAA GAA ATC AAG CAGT-3'; anti-sense primer, 5'-TGT GGG CAG CGA GAA CAA AGT-3'); for fatty acid synthase (sense primer, 5'-GCT GGA GCC CCT TTT TGT CTT-3'; anti-sense primer, 5'-ACC CCA GCA CTG CAG TTT TCT-3'); and for GAPDH primers of oligonucleotides (sense primer, 5'-GCCATCAACGACCC CTTCATT-3'; anti-sense primer, 5'-CGCCTGCTTCACC ACCTTCTT-3'). The expected sizes of DNA fragments amplified with these primers were 245 bp for HMG-CoA reductase, 306 bp for cholesterol 7α-hydroxylase, 682 bp for fatty acid synthase, and 702 bp for GAPDH. Blots were hybridized with an HMG-CoA reductase probe of a 54-base oligonucleotide (5'-GAT CTG TTG TGA ACC ATG TGA CTT CTG ACA AGA TGT CCT GCT GCC AAT GCT GCC-3'), cholesterol 7α-hydroxylase probe of a 54-base oligonucleotide (5'-CCC GAA GGC CTG TTT AAG TGA TGA CTC TCA GCC GCC AAG TGA CAT CAT CCA GTG-3'), fatty acid synthase probe of a 54-base oligonucleotide (5'-CTG CTC TCT GTG GAT AGG ACT GAA TGC TGT GGC CTT CTG ATA GAC TCT TCT GGA-3'), and GAPDH probe of a 54-base oligonucleotide (5'-TGA TGA CCA GCT TCC CAT TCT CAG CCT TGA CTG TGC CGT TGA ACT TGC CGT GGG-3'). The relative quantity of mRNA was estimated by densitometry scanning with X-ray film.

Statistical analysis Data are presented as means and standard deviations. The significance of differences among treatment groups was determined by ANOVA with Duncan's multiple-range test (SAS Institute, Cary, NC, USA). Student's *t*-test was used to compare the significance of differences between 2 groups, with the difference being considered significant at $p<0.05$.

Results and Discussion

Food intake, body weight gain, and liver weight In this study, there was no significant difference in body weight among the groups (Table 2). However, there was a significant difference (p<0.05) in food intake among the groups, and the food intake in the control group was the lowest. Food efficiency in the APAP group was significantly decreased (p<0.05) compared to the control group. On the other hand, there was no difference in food efficiency between the control and ABE groups. Liver weights in the APAP and ABE groups were significantly lower (p<0.05) than in the control group (Table 2).

Serum cholesterol concentration There are some reports regarding the elevation of the serum cholesterol levels of experimental animals treated acutely or repeatedly with acetaminophen (14, 22, 23). Interestingly, however, we observed that serum total cholesterol and HDL cholesterol levels in the APAP group were significantly decreased (p <0.05) compared with the control and ABE groups, and the non-HDL cholesterol level in the APAP group was also significantly decreased (p<0.05) compared to the control group throughout the feeding period (Fig. 1). Lee and Koo (24) have reported that most serum cholesterols in rats are associated with the HDL fraction; therefore the lowered serum HDL cholesterol concentration might have been an important factor in the lowered serum total cholesterol concentration in the APAP group (Fig. 1). Our data also showed that the total cholesterol level was positively correlated with the HDL cholesterol level, the correlation coefficient (r) being 0.9886 (p<0.01). Although it is unclear whether acetaminophen-induced changes in serum cholesterol concentrations after 28-day exposure are adverse, lipid metabolic alterations can be affected by the type or intensity of hepatic injury (13). Some serum lipid-lowering drugs, in particular NSADIs, have adverse effects such as hepatic failure (3). Although there were no data on lecithin cholesterol acyltransferase (LCAT) activity in this study, dietary acetaminophen in the food matrix is likely to cause a fall in the serum HDL cholesterol level due to LCAT deficiency in rats (14) because LCAT is a key enzyme in maintaining the outside composition of apoprotein (25). On the other hand, adzuki bean extract-fed rats were resistant to acetaminophen-induced changes in serum total cholesterol and HDL cholesterol concentrations. In a previous study, we reported the hepatoprotective effects of the water extract of adzuki bean hulls on acetaminophen-induced liver damage in rats (12). Therefore, it is likely that adzuki bean extract might improve the acetaminophen-altered serum lipid metabolism, which might be affected by the hepatic toxicity of acetaminophen (13).

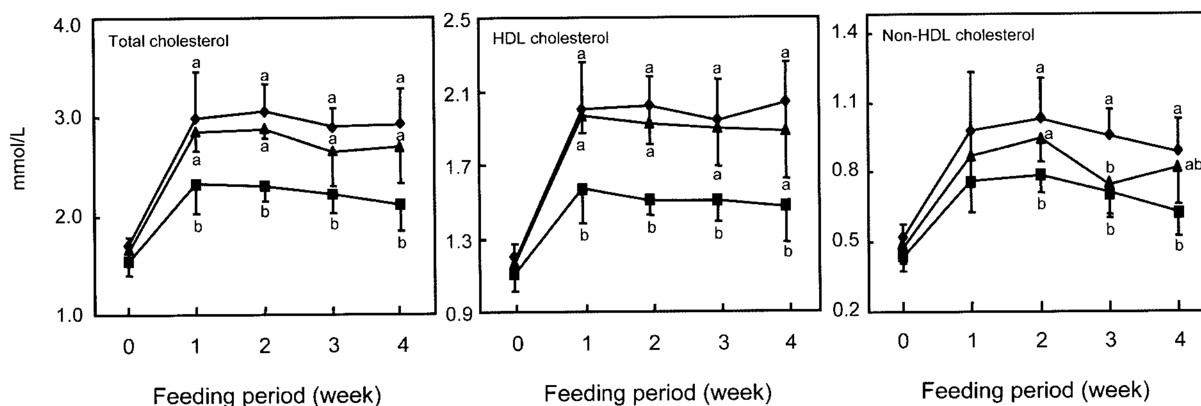

Fig 1. Serum cholesterol and triglyceride concentrations in rats. The control group (◆) was fed a basal diet, the APAP group (■) acetaminophen (5 g/kg basal diet), and the ABE group (▲) acetaminophen (5 g/kg basal diet) plus adzuki bean extract (50 g/kg basal diet) for 4 weeks. Mean values with different letters (a, b) are significantly different at p<0.05, as determined by analysis of variance with Duncan's multiple-range test.

Table 2. Body weight, food intake, and liver weight in rats

	Dietary gorup[1)]		
	Control	APAP	ABE
Initial body weight (g)	150.5±3.3[a]	148.2±3.5[a]	149.3±2.6[a]
Body weight gain (g/4 week)	74.1±6.1[a]	70.3±9.5[a]	80.±8.1[a]
Food intake (g/day)	13.1±0.8[c]	14.2±0.3[b]	15.3±0.6[a]
Food efficiency (body gain/food intakes)	0.201±0.010[a]	0.177±0.021[b]	0.187±0.019[ab]
Liver weight (wet g/100 g body weight)	4.51±0.10[a]	3.70±0.32[b]	3.85±0.28[b]

[1)]APAP, acetaminophen diet group; ABE, acetaminophen plus adzuki bean extract diet group.
Values are expressed as mean±SD for 5 rats; Mean values within each row with different superscript letters are significantly different at p<0.05, as determined by analysis of variance with Duncan's multiple-range test.

mRNA Expression To investigate gene expression changes after treatment with acetaminophen and adzuki bean extract, we analyzed mRNAs with Southern blotting. Expression of key enzymes in cholesterol metabolism, HMG-CoA reductase and cholesterol 7α-hydroxylase, in the APAP group were downregulated and upregulated more than those in the control group, respectively (Fig. 2). Furthermore, the hepatic fatty acid synthase mRNA levels in the APAP and ABE groups were significantly decreased ($p<0.05$) compared to the control group (Fig. 2). Those changes are interesting, because Heinloth *et al.* (26) similarly observed that gene expression related to cholesterol synthesis and fatty acid synthesis was downregulated in rats treated with various acetaminophen dosages. Therefore, the decrease in the hepatic HMG-CoA reductase mRNA, and the increase in the hepatic cholesterol 7α-hydroxylase and fatty acid synthase mRNAs due to acetaminophen-induced hepatic damage might suggest an altered lipid metabolism in the condition of 0.5% acetaminophen-administration in rats for 4 weeks. On the other hand, the hepatic HMG-CoA reductase mRNA level in the ABE group was significantly higher ($p<0.05$) than in the APAP group (Fig. 2). Lund *et al.* (27) and Moundras *et al.* (28), using viscous fibers, have reported that dietary fiber increases hepatic HMG-CoA reductase activity. Our findings suggest that some soluble fibers in adzuki bean extract might increase hepatic HMG-CoA reductase mRNA against acetaminophen, thereby probably increasing the serum cholesterol concentration. Therefore, it is considered that dietary adzuki bean extract in the food matrix might be helpful to potentialize the development of lipid metabolism against acetaminophen through inhibiting the serum cholesterol concentration and synthesis of cholesterol in rats.

Hepatic and fecal lipid concentrations There was no significant difference in the liver cholesterol level among the groups, but the liver total lipid levels in the APAP and ABE groups were significantly higher ($p<0.05$) than in the control group. The fecal cholesterol concentration in the APAP group was significantly greater ($p<0.05$) than those in the control and ABE groups (Table 3). Recently, Raghavendran *et al.* (14) observed that a single dose of acetaminophen significantly increased the hepatic total lipid level, and histological fatty and inflammatory infiltration of liver sections in rats. In this study, we also found that dietary acetaminophen increased the liver total lipid concentration (Table 3). This suggests that acetaminophen causes hepatocyte microvesicular lipid degeneration with necrosis (29), and that the function of hepatocytes related to lipid metabolism in acetaminophen-treated rats might thereby have become impaired. Furthermore, such adverse actions of acetaminophen on lipid metabolism might result in the reduction of fatty acid synthesis through the expression of mRNA in the liver (Fig. 2) to maintain the homeostasis in rats fed

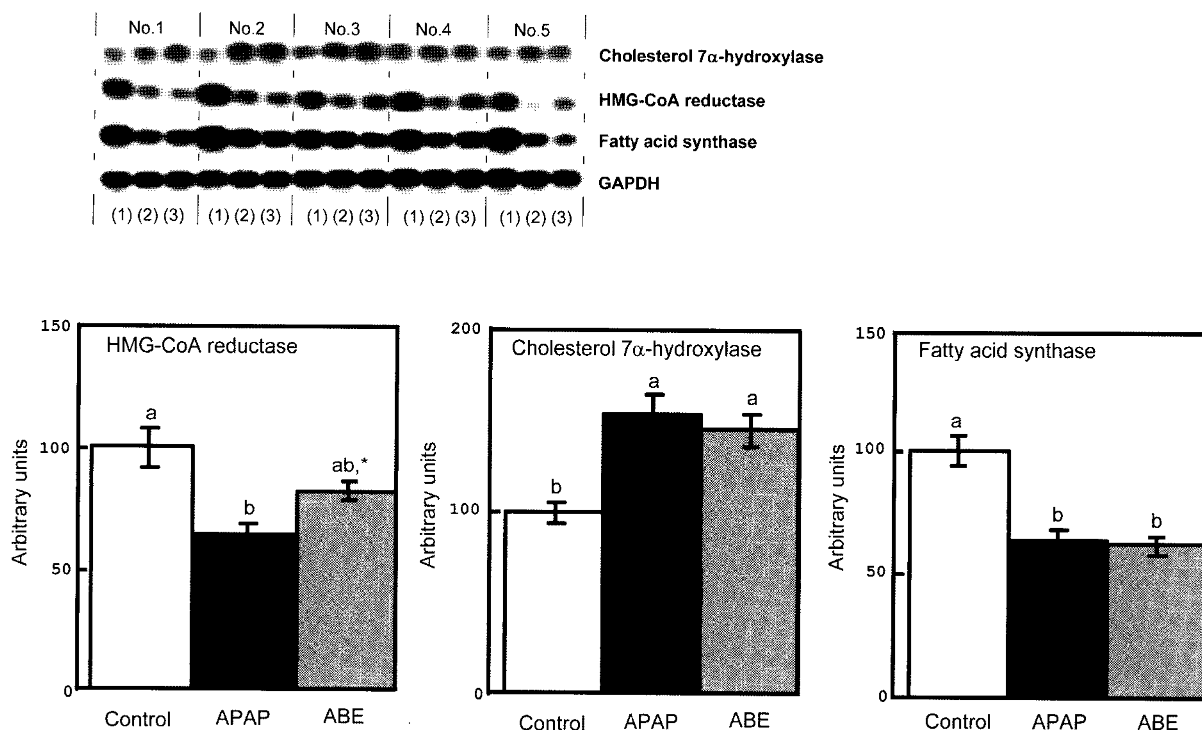

Fig 2. Southern blot analysis of liver HMG-CoA reductase mRNA and cholesterol 7α-hydroxylase mRNA expression in rats. The control group (1) was fed a basal diet, the APAP group (2) acetaminophen (5 g/kg basal diet), and the ABE group (3) acetaminophen (5 g/kg basal diet) plus adzuki bean extract (50 g/kg basal diet) for 4 weeks. Mean values with different letters (a, b) are significantly different at $p<0.05$, as determined by analysis of variance with Duncan's multiple-range test. *$p<0.05$ vs. control, as determined by analysis using Student's *t*-test. The values for HMG-CoA reductase and cholesterol 7α-hydroxylase are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in all groups.

Table 3. Hepatic total lipids and cholesterol concentrations, and fecal cholesterol concentrations in rats[1)]

	Dietary group[2)]		
	Control	APAP	ABE
Liver total lipids (mg/wet g)	35.7±10.4[a]	57.3±7.5[b]	52.5±7.5[b]
Liver cholesterol (mmol/wet g)	1.86±0.25[a]	1.57±0.33[a]	1.49±0.25[a]
Fecal cholesterol (mmol/wet g)	2.07±0.47[a]	4.41±4.62[b]	1.12±0.24[a]

[1)]Values are expressed as mean±SD for 5 rats; Mean values within each row with different superscript letters are significantly different at $p<0.05$, as determined by analysis of variance with Duncan's multiple-range test.
[2)]APAP, acetaminophen diet group; ABE, acetaminophen plus adzuki bean extract diet group.

acetaminophen for 4 weeks. On the other hand, the lowered serum cholesterol concentrations in the APAP group might also be attributable to inhibition of intestinal absorption of neutral steroids, resulting in greater fecal steroid excretion. This might be explained by the observations that the APAP group has significantly increased fecal cholesterol excretion (Table 3) and hepatic cholesterol 7α-hydroxylase mRNA expression (Fig. 2). However, the fecal lipid concentration in the ABE group was not higher than in the APAP group. It might be that some bioactive components in adzuki bean extract improve the acetaminophen-altered lipid metabolism by reducing the absorption of neutral steroids from the small intestine in rats fed cholesterol-free diets. However, we could not clarify the mechanism in this study.

In conclusion, in the present study administration of acetaminophen lowered serum total cholesterol and HDL cholesterol concentrations by decreasing hepatic HMG-CoA reductase mRNA, and increasing hepatic cholesterol 7α-hydroxylase mRNA and the fecal cholesterol concentration. Furthermore, these results indicate that adzuki bean extract may improve the acetaminophen-altered serum lipid metabolism in rats.

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