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Isoflavone Glycitein Diminished Plasma Cholesterol in Female Golden Syrian Hamsters

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The soybean isoflavones, daidzein, genistein, and glycitein, were hypothesized to act as cholesterol-lowering components, separate from soy protein. Pure synthetic daidzein, genistein, or glycitein (0.9 mmol/kg diet) or a casein-based control diet was fed to groups of 10 female Golden Syrian hamsters for 4 weeks. Hamsters fed glycitein had significantly lower plasma total (by 15%) and non-HDL (by 24%) cholesterol compared with those fed casein ($P < 0.05$). Daidzein and genistein's effects on these lipids did not differ from the effects of either casein or glycitein. Plasma HDL cholesterol and triglyceride concentrations were not significantly affected by dietary treatments. The percentage of urinary recovery of the ingested dose of each isoflavone was glycitein > daidzein > genistein (33.2%, 4.6%, 2.2%, respectively), with the apparent absorption of glycitein significantly greater than that of the other isoflavones. These data suggest that glycitein's greater cholesterol-lowering effect was due to its greater bioavailability, as reflected in its urinary recovery compared with that of the other isoflavones.

KEYWORDS: Daidzein; genistein; glycitein; urinary recovery; bioavailability

INTRODUCTION

Soy isoflavones are the major phytoestrogens in soybeans and soy foods. There is a great interest in soy isoflavones, daidzein, genistein, and glycitein, regarding their possible effects on plasma lipid levels. The role of soy isoflavones in lowering plasma concentrations of lipoproteins and cholesterol has been studied in animals (*1–9*) and humans (*10–16*).

Animal studies showed that isolated soy proteins containing isoflavones or alcohol extracts of soy protein rich in isoflavones were hypocholesterolemic with respect to plasma total and non-HDL cholesterol concentrations compared with controls not fed soy. Soy protein isolate (ISP) containing isoflavones (1.0 mmol/kg diet) significantly lowered plasma total and LDL + VLDL cholesterol concentrations in ovariectomized monkeys compared with isoflavone-depleted ISP (0.005 mmol/kg diet) (*7*). Balmir et al. (*1*) reported a significant reduction in plasma LDL cholesterol in male hamsters fed diets containing intact ISP, ISP with 0.6 mmol of total isoflavones/kg diet, or casein with 0.3 mmol of total isoflavones/kg diet compared with those fed casein. Several human studies also reported that intake of soy containing a large amount of isoflavones improved plasma lipid profiles. Crouse et al. (*12*) found that consumption of 25 g of isolated soy protein with 62 mg of isoflavones (~0.5 mmol/kg diet) significantly reduced the plasma total and LDL cholesterol concentrations compared with consumption of casein. The plasma LDL cholesterol concentration was significantly lessened in premenopausal women consuming 53 g of isolated soy protein with 129 mg of isoflavones (~1.0 mmol/kg diet) compared with women consuming isolated soy protein that contained 10 mg of isoflavones (~0.08 mmol/kg diet) (*13*).

Recent studies investigated the cholesterol-lowering effects of pure synthetic isoflavones or isoflavones isolated from soybean fractions (*8, 17–19*). Ovariectomized rats gavaged with the isolated pure isoflavone glucosides daidzin (25 or 50 mg/(kg body weight/day)) or glycitin (50 mg/(kg body weight/day)) showed significantly lesser plasma total cholesterol and triglyceride concentrations compared with casein-fed rats (*17*). Plasma triglyceride was significantly lessened in Sprague–Dawley rats fed a mixture of synthetic daidzein and genistein (1.4 mmol/kg diet or ~23.6 mg/(kg body weight/day)) compared with those fed casein (*18*). Hamsters fed pure synthetic daidzein (1.3 mmol/kg diet or ~16 mg/(kg body weight/day)) had significantly lesser plasma total and non-HDL cholesterol compared with those fed casein (*8*).

Isoflavone contents in urine or plasma may be considered as a biological marker of soy consumption. We hypothesized that soy isoflavones improve plasma cholesterol status and that glycitein may have greater cholesterol-lowering efficacy because glycitein is more absorbable compared with the other two purified isoflavones in hamsters, based on preliminary studies in our laboratory. To test these hypotheses, we fed pure synthetic

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daidzein, genistein, or glycitein to hamsters and then measured plasma lipid concentrations and determined the association between urinary recovery of each isoflavone and plasma lipid concentrations. Hamsters were selected as an animal model because hamsters and humans are similar in several aspects of cholesterol metabolism. Hamsters fed a diet enriched with saturated fat have greatly increased plasma total and LDL cholesterol (*20*).

MATERIALS AND METHODS

Chemicals and Diets. The method of Chang et al. (*21*) was modified to synthesize daidzein (*22*). Boron trifluoride diethyl etherate (0.5 mL) was added to the mixture of 2,4,4′-trihydroxydeoxy benzoin (THB, 25 mg) and *N*,*N*-dimethylformamide (1 mL). The reaction mixture was heated in a microwave for 21 s, and then methanesulfonyl chloride (1 mL) was added and heated again for 67 s at low energy. Distilled water was added to the reaction mixture to obtain a yellowish precipitate. The precipitate was centrifuged, washed with distilled water, and recrystallized from methanol to give daidzein. Genistein was synthesized by the method of Chang et al. (*21*). Glycitein was synthesized according to Lang'at-Thoruwa et al. (*23*). The purity of isoflavones, determined by HPLC chromatogram peak area and Beckman Gold HPLC system peak purity software, was >97%. All chemicals for isoflavone synthesis were purchased from Sigma-Aldrich (St. Louis, MO).

All dietary ingredients except rice flour were obtained from Harlan Teklad (Madison, WI). Rice flour was purchased from Bioserv (Frenchtown, NJ). Four treatments were fed: casein as control, and casein-based diets supplemented with daidzein, genistein, or glycitein. The content of each isoflavone was ~30% less than the total molar isoflavone content equivalent to feeding 25% isolated soy protein by weight, 0.9 mmol/kg diet. The dose of isoflavones was ~13 mg/(kg body weight/day). The experimental diets contained ~37% of energy as fat, 25% casein, and 0.1% cholesterol (*8*). Rice flour was used as a carbohydrate source to prevent "wet tail" disease, a chronic diarrhea that may kill hamsters (*1*).

Animals. The use of animals and the experimental protocol were approved by the Iowa State University Animal Care Center Committee. Forty female Golden Syrian hamsters, 11–12 weeks old, were purchased from Harlan Teklad (Madison, WI) and housed individually in a temperature-controlled room (23 °C) with a 12-h light/dark cycle. Hamsters were randomly assigned to four treatments ($n = 10$/treatment) with similar body weights/group. Hamsters had free access to food and water during the 4-week experiment. Body weights were measured weekly and food intakes measured daily. At the end of the feeding period, diets were withdrawn from hamsters 16–18 h before they were euthanized under CO_2. Blood was collected by cardiac puncture and centrifuged at 5000*g* for 15 min at 4 °C to prepare plasma that was stored at −20 °C until analysis.

Plasma Lipid Analysis. Plasma total cholesterol and HDL cholesterol concentration were measured with Sigma diagnostic kits (St. Louis, MO). Plasma triglycerides were measured with Thermo Trace kits (Louisville, CO). Non-HDL cholesterol was calculated by subtraction of HDL cholesterol from total cholesterol and represented LDL + IDL + VLDL cholesterol.

Analysis of Urinary Excretion of Isoflavones. Hamsters were put in metabolic cages for 24 h to collect urine during the last 2–3 days of the feeding period. Duplicate urine samples/hamster (each sample adjusted to 5 mL) were mixed with 5 mL of sodium acetate buffer (0.2 M, pH 5.5), 50 μL of β-glucuronidase/sulfatase (H_2 type, Sigma-Aldrich, St. Louis, MO), and 50 μL of 2,4,4-trihydroxydeoxybenzoin (THB as an internal standard, 2 mg/mL) in a tube. After incubation for 20 h at 37 °C on a shaker, 9 mL of sodium phosphate buffer (10 mM, pH 7.0) was added and the solution was passed through an Extrelut QE column (EM Science, Gibbstown, NJ). The column was washed twice with 18 mL of ethyl acetate (HPLC grade) and once with 10 mL of ethyl acetate (HPLC grade). The effluent was collected in a round-bottom flask and dried in a rotary evaporator (Buchler Instrument, Fortlee, NJ), and the residues were dissolved in 9.8 mL of 20% ethanol acidified by 200 μL of 1 N HCl. Five milliliters of the ethanol-dissolved residue was slowly applied to a preconditioned Sep-Pak cartridge (C18, Waters Corp., Milford, MA). The cartridge was washed twice with 2 mL of distilled water and eluent recovered in 2 mL of 80% methanol, which was vortexed and filtered through a 0.45 μm PTFE filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis.

The HPLC analysis was carried out on a Hewlett-Packard 1050 Series HPLC system. This system consisted of a photodiode array detector (PDA), a quadruplicate pumping system, an autosampler, and an HP Vectra 486/66 personal computer with Chem Station 3D software (Hewlett-Packard Company, Scientific Instruments Division, Palo Alto, CA). Daidzein, genistein, glycitein, and THB were detected and quantified on an YMC-pack ODS-AM C18 reverse phase column (5 μm pore size, 25 cm × 4.6 mm, YMC, Inc., Wilmington, NC). Elution was at a flow rate of 1 mL/min at room temperature with the following solvents: 0.1% acetic acid in Milli-Q H_2O (Millipore Co., Bedford, MA) (solvent A) and methanol (solvent B). After injection of 20 μL of sample, solvent B was increased from 30% to 50% over 45 min, then held at 50% for 10 min, and decreased to 30% over 10 min. Analytes were monitored from 190 to 350 nm. Ultraviolet absorbance spectra were recorded, and area responses were integrated by Chem Station 3D Software to identify isoflavones. Recovery based on the THB internal standard was 84%, and recovery-adjusted urinary excretion of each isoflavone as a percentage of dose ingested over 24 h was reported.

Statistical Analysis. All statistical analyses were conducted with the Statistical Analysis System (SAS, Release 8.2, SAS Institute Inc., Cary, NC). Values were expressed as means ± SD. The results were analyzed by one-way analysis of variance (ANOVA). Differences between treatments were determined by a least significant difference test. An α of 0.05 was used to determine statistically significant differences.

RESULTS AND DISCUSSION

This study determined whether daidzein, genistein, or glycitein lessened plasma cholesterol in female Golden Syrian hamsters. Pure synthetic isoflavones were studied to avoid the possible confounding effects of other alcohol extractable components of soy protein such as soyasaponins. The diets were well accepted. Hamsters ate a mean of 7.5 ± 1.5 g/day over 4 weeks. The initial mean body weight was 111.6 ± 0.1 g among the groups. The final mean body weights were 127 ± 3 g, 126 ± 4 g, 128 ± 4 g, and 127 ± 4 g for the groups fed casein, daidzein, genistein, and glycitein diets, respectively. There were no significant differences in daily food intake or initial or final mean body weight among dietary treatment groups.

Hamsters fed purified synthetic glycitein had significantly lesser plasma total and non-HDL cholesterol concentrations by 15% and 24%, respectively, compared with those fed casein ($P < 0.05$). Daidzein or genistein did not differ in effects on plasma cholesterol from either glycitein or the control diet (**Table 1**).

Few studies have reported the cholesterol-lowering effects of pure isoflavones (*8, 17, 18*). Hamsters fed purified synthetic daidzein at 1.3 mmol/kg diet (or ~16 mg/(kg body weight/day)) for 10 weeks had significantly lesser plasma total and non-HDL cholesterol concentrations by 22–24% and 30–38% compared with those fed casein (*8*). In the present study, daidzein was ~30% less than in the study of Song et al. (1.3 mmol of daidzein/kg diet (*8*)), 0.9 mmol/kg diet (or ~13 mg/(kg body weight/day)) fed to the same numbers of animals. After a 4 week feeding period, daidzein lowered plasma total and non-HDL cholesterol by 7% and 14%, respectively, compared with casein (**Table 1**). Perhaps if a longer experiment was conducted, a significant reduction of plasma total and non-HDL cholesterol might be observed with 0.9 mmol of daidzein/kg diet. A mixture of pure synthetic daidzein and genistein at 1.4 mmol/kg diet (or ~23 mg/(kg body weight/day); 2:1 ratio of genistein/daidzein) fed for 21 days significantly lowered plasma triglyc-

Table 1. Plasma Cholesterol and Triglyceride Levels in Hamsters Fed Casein or Casein-Based Diets Containing Daidzein, Genistein, or Glycitein[a,b]

treatment	total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	non-HDL[c] cholesterol (mmol/L)	total cholesterol/HDL cholesterol	triglyceride (mmol/L)
casein	6.33 ± 0.32 a	3.75 ± 0.53	2.58 ± 0.31 a	1.69 ± 0.04 a	2.67 ± 0.61
daidzein	5.87 ± 0.79 ab	3.66 ± 0.58	2.22 ± 0.57 ab	1.60 ± 0.26 ab	2.22 ± 0.57
genistein	5.61 ± 0.87 ab	3.43 ± 0.64	2.19 ± 0.53 ab	1.63 ± 0.14 ab	2.40 ± 0.48
glycitein	5.34 ± 0.51 b	3.38 ± 0.86	1.95 ± 0.17 b	1.58 ± 0.03 b	2.29 ± 0.7
P value	0.018	0.249	0.025	0.048	0.109

[a] Values represent means ± SD, $n = 10$. [b] Within a column, means followed by different letters are different ($P < 0.05$). [c] Represents the VLDL + IDL + LDL fractions (by difference: total − HDL).

erides by 26% compared with the results with casein in Sprague–Dawley rats (*18*). Plasma total cholesterol and triglyceride concentrations were significantly lessened by 23–44% and by 24–46%, respectively, in ovariectomized rats fed the isolated pure isoflavone glucosides daidzin (25 or 50 mg/(kg body weight/day)) or glycitin (50 mg/(kg body weight/day)) compared with casein (*17*). Both lean and obese SHP/N-cp rats fed 20% casein plus 0.1% soybean isoflavones (3.9 mmol/kg diet) for 20 weeks had significantly lesser plasma total (by 22–25%) and LDL cholesterol (by 26–31%) levels compared with those of animals fed 20% casein (*9*). Therefore, soy isoflavones had the ability to lessen plasma lipid concentrations, separate from soy proteins in rodents. However, Fukui et al. (*24*) reported that feeding 20% casein plus a 1.6% ethanol extract containing 3.1 mmol of isoflavones/kg diet and 0.5% dietary cholesterol for 2 weeks did not lessen plasma total cholesterol compared with casein in male Sprague–Dawley rats. The high level of cholesterol fed in this study (*24*) may have overwhelmed the cholesterol-lowering efficacy of the ethanol-extractable components, isoflavones and saponins. Compared with male Golden Syrian hamsters fed 20% casein, feeding 20% casein plus 0.02% soy isoflavone (0.8 mmol/kg diet; genistein/daidzein/glycitein = 1.4:1:0.2) and 0.08% dietary cholesterol for 5 weeks did not significantly lower plasma total and VLDL + LDL cholesterol levels (*25*). This study was similar to the present study in total isoflavone dose. In general, it seems that isoflavone doses greater than ~1.3 mmol/kg diet may be needed to observe significant cholesterol lowering by isoflavones in rodents fed diets that simulate a typical U.S. diet in total and saturated fat and cholesterol content.

The mechanisms of soy isoflavones in lowering plasma cholesterol levels are not clear. Soy isoflavones are estrogen-like and bind estrogen receptors, especially ER-β. Glycitein was more estrogenic than genistein when given by gavage to immature female mice over 4 days, causing uterine enlargement (*26*). The loss of estrogen increased plasma concentrations of total cholesterol, LDL cholesterol, and triglycerides and decreased HDL cholesterol in women (*27*, *28*). Jensen (*27*) investigated the changes of plasma lipid concentrations after natural menopause in 170 premenopausal women. After menopause, women had significantly increased plasma total cholesterol (by 6%) and LDL cholesterol (by 8%) and decreased HDL cholesterol (by 7%). Angelin et al. (*29*) obtained liver biopsies from men treated with estrogen or nonestrogenic drugs for prostatic carcinoma (control). Hepatic LDL receptor expression was increased in estrogen-treated men compared with controls. Although soy isoflavones may affect plasma lipid concentrations through their estrogenic activity, the greater cholesterol-lowering efficacy of glycitein than that of daidzein or genistein was due mostly to bioavailability differences. The potential mechanisms of isoflavones to reduce plasma lipid concentrations should be further investigated.

Table 2. Percentage of Urinary Isoflavone Recovery of Dose Ingested over 24 h by Hamsters Fed Diets Containing Daidzein, Genistein, or Glycitein[a,b]

	daidzein ($n = 10$)	genistein ($n = 8$)	glycitein ($n = 7$)
urinary recovery (% ingested dose)	4.6 ± 1.8 b	2.2 ± 1.7 b	33.2 ± 25.7 a

[a] Values represent means ± SD. [b] Within a column, means followed by different letters are different ($P = 0.0098$).

The effects of soy isoflavones on plasma HDL cholesterol and triglyceride concentrations are not consistent. Kawakami et al. (*30*) reported that feeding 20% casein plus 0.37% isoflavone aglycone-rich powder containing 3.8 mmol of isoflavones/kg diet and 0.3% dietary cholesterol for 40 days significantly increased HDL cholesterol and reduced triglycerides compared with casein in male Sprague–Dawley rats. Compared with rats fed casein, the plasma triglyceride concentration was significantly decreased (26%) in rats fed a diet containing a mixture of pure synthetic daidzein and genistein (*18*). However, the isoflavone-poor soy protein isolate gave a significantly lower triglyceride level by 48% compared with the case of male Sprague–Dawley rats fed casein (*31*). Ethanol-extracted isolated soy protein significantly lowered triglycerides and increased HDL cholesterol concentrations compared with casein in female hamsters. Female hamsters fed daidzein had no difference in plasma HDL cholesterol compared with those fed casein (*8*). In our study, there were no significant differences in plasma HDL cholesterol and triglyceride concentrations among different dietary treatments (**Table 1**). These results were consistent with those of other studies (*1*, *6*, *9*). Balmir et al. (*1*) reported that plasma HDL cholesterol and triglyceride concentrations were not significantly affected by dietary treatments (casein, casein with ~0.3 mmol of total isoflavones/kg diet, casein with ~0.6 mmol of total isoflavones/kg diet, isolated soy protein (ISP), and ISP with alcohol extract) in hamsters. Further studies are needed to investigate the mechanism or effect of soy isoflavones on plasma HDL cholesterol and triglycerides concentrations.

The percentage of urinary recovery of the ingested dose of each isoflavone was greater in hamsters fed glycitein than in those fed the other two isoflavones (**Table 2**). The percentage of urinary recovery of the ingested dose of each isoflavone was glycitein > daidzein > genistein in this study. Seemingly, glycitein was absorbed to a greater extent than was daidzein or genistein. A previous study (*32*) in our laboratory showed that urinary isoflavone recovery was greater for glycitein than for daidzein or genistein in hamsters fed Novasoy containing 1.18 or 1.77 mmol of total isoflavones/kg diet. Glycitein was more bioavailable than daidzein and genistein, as reflected in the urinary recovery of glycitein (52%) compared with those of daidzein (17%) and genistein (18%). The average 24 h urinary excretion of glycitein (~43%) was significantly greater than that

of daidzein or genistein (~13%) in male Golden Syrian hamsters, indicating that the bioavailability of glycitein was greater than that of the other isoflavones (*33*). One hamster fed glycitein produced daidzein in urine in our study. We excluded this animal from the urinary isoflavone recovery data, but the conversion of glycitein to daidzein has been documented previously (*34*). Differing from hamsters, the urinary recoveries in humans of daidzein, glycitein, and genistein were ~52%, ~47%, and ~37%, respectively, in a study providing single doses of soymilk or soygerm (glycitein-rich) to seven men and seven women (all moderate in vitro fecal isoflavone degraders in order to lessen interindividual variability in isoflavone uptake) (*35*). This interspecies difference may be due to differences in isoflavone metabolism and degradation by intestinal microbes. Women who showed a lesser fecal anaerobic disappearance rate of genistein and a faster gut transit time had greater genistein bioavailability, as reflected in urinary recovery of genistein compared with women who had a high genistein disappearance rate and a long gut transit time (*36*). Three different in vitro anaerobic fecal isoflavone disappearance phenotypes were shown in humans and a negative correlation between plasma isoflavone concentrations and fecal isoflavone disappearance rate in eight young adult men (*37*). These findings indicated that the variability in metabolic response to isoflavones may be due to the relative ability of gut microbes to degrade isoflavones, perhaps accounting for differences among species and isoflavones in apparent isoflavone absorption. The far greater apparent absorption of glycitein than of daidzein or genistein in hamsters suggests that this model is not ideal for understanding the human health effects of glycitein or for understanding the bearing of the structure/activity relationships among the three isoflavones on cholesterol status. For comparing structure/activity relationships between daidzein and genistein independent of bioavailability, hamsters would be useful because these isoflavones have similar apparent absorption in this animal model. This is not the case for most humans, who show greater absorption of daidzein than of genistein, but some individuals show similar absorption of daidzein and genistein (*38*).

In conclusion, this study demonstrated that 0.9 mmol of glycitein/kg diet lessened plasma cholesterol in female hamsters compared with a casein control diet, an effect not observed with purified daidzein or genistein at the same doses, and associated with the far greater apparent absorption of glycitein than of the other isoflavones.

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