A BRIEF COMMUNICATION

Subcutaneously Administrated Genistein and Daidzein Decrease Serum Cholesterol and Increase Triglyceride Levels in Male Middle-Aged Rats

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Nutritional supplements containing soybean phytoestrogens, the isoflavones genistein (G) and daidzein (D), are increasingly used as alternative therapy for osteoporosis, cancer, and cardiovascular and other diseases with a frequency that increases with advancing age. In this study we examined the effects of subcutaneous administration of either G or D on serum lipid levels in orchidectomized (Orx) and intact (IA) middle-aged male rats, which are experimental models of andropause. Sixteen-month-old Wistar rats were treated with 10 mg/kg and 30mg/kg of either G or D. The control groups received testosterone, estradiol, or vehicle for 3 weeks, after which the total serum cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), and total triglycerides (TT) were measured. Compared with the matching vehicle-treated controls, the higher doses of G and D and testosterone treatment significantly ($P < 0.05$) lowered the TC and lipoprotein cholesterol levels. The greatest effect was observed regarding LDL-C in both Orx and IA males after G and D treatments, in which LDL-C decreased by more than 30%. The lower isoflavone doses induced a significant cholesterol-lowering effect ($P < 0.05$) only in the Orx group. Like the estradiol treatment, the higher doses of G and D increased the TT levels in both rat models by more than 50% ($P < 0.05$). The lower doses of isoflavones increased TT only in the Orx group. In male middle-aged rats, injections of higher doses of G and D decreased the serum cholesterol levels, as did testosterone injection, and brought about an increase in serum triglycerides similar to that observed after estradiol treatment. Exp Biol Med 232:1222–1227, 2007

Key words: isoflavones; sex steroids; cholesterol; triglycerides; rats; middle age

Introduction

The sex hormones testosterone and estrogen play an important but still poorly understood role in age-related male physiology and pathology (1). Based mostly on the observation that men are twice as likely to die of coronary heart disease then premenopausal women, it was previously assumed that androgens exert a negative effect on the male cardiovascular system. More recent research claims the opposite. Partial androgen deficiency in aging men during andropause and in young men with hypogonadism is associated with an increased incidence of coronary artery disease (2). Endogenous estrogens, produced by aromatase activity in numerous male tissues, including the testis and liver, may also alter the serum lipid levels and contribute to

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cardiovascular disease development in men (3). The estrogens exert their effects through specific α and β estrogen receptors (ERs), which have been detected in different male tissues (4). This finding has finally changed the classical view of the estrogens as exclusively female hormones.

The soy isoflavones genistein (G) and daidzein (D) are structurally similar to estradiol-17β. They bind with a weaker potency to ERs in both humans and animals, preferably to ERβ (5). Aside from exerting a mild estrogenic/antiestrogenic effect, the isoflavones have antioxidative and antiproliferative activities *in vitro* and *in vivo* (6, 7). The assumption that the isoflavones are the main bioactive components in soy food is connected to their putative favorable effects in preventing cardiovascular disease, osteoporosis, and cancer (8). Thus, the isoflavones are becoming increasingly popular in the West and are frequently consumed in the purified form, as nutritional supplements or herbal preparations.

It is well documented that dietary soy protein decreases the serum total and LDL cholesterol concentrations in humans (9, 10) and various animal models (11–15). Removal of the isoflavone-containing fraction from soy protein resulted in a loss of its beneficial effect on the serum lipid profile and atherosclerosis progression (16, 17). Some authors reported that ingested purified isoflavones exert lipid-lowering effects (18–21). However, others showed minimal effects of isolated isoflavones on blood lipid levels (14, 15, 22), supporting the hypothesis that soy protein or components other than the isoflavones are responsible for lipid-lowering effects of soy.

The aim of this study was to examine the effects of subcutaneous administration of phytoestrogens G and D as potential natural hypolipidemic agents in soy on serum lipid levels, the parameters of atherosclerosis progression, in the male middle-aged rat model of andropause. The endogenous sex steroids might affect the obtained results, and therefore comparison between Orx and intact animals, as well as between isoflavone and sex steroid treatments, was expected to provide more proper evaluation of the obtained results.

Materials and Methods

Animals and Diets. Male middle-aged (15–16 months old) Wistar rats weighing about 650 ± 50 g, were housed in the experimental animal unit of the Institute for Biological Research in Belgrade and were maintained under standard laboratory conditions of room temperature (22°C ± 2°C) and lighting (12:12-hr light:dark cycle). The animals were fed a soy-free diet (23), which was prepared in cooperation with PKB INSHRA (Belgrade, Serbia) and the Department of Food, School of Veterinary Medicine, Belgrade, Serbia, with corn oil serving as the main source of fat. The food contained (per 100 g): casein, 20.3; cornstarch, 45; sucrose, 20; corn oil, 5.2; fiber 3.7; vitamin/mineral mix (Ca-P deficient), 1.5; calcium phosphate dibasic, 1.8; calcium carbonate, 1; and DL-methionine 1.5. Casein and crystalline cellulose were purchased from Alfa Aesar, Johnson Matthey (Karlsruhe, Germany). DL-methionine was obtained from the Sigma Chemical Co. (St. Louis, MO). All other ingredients were from PKB INSHRA. Food and water were consumed *ad libitum*.

Experimental Protocol. The work was approved by the Local Animal Ethics Committee, whose guidelines conform to the recommendations given in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23).

At 15 months, the rats were randomly divided into orchidectomized (Orx; $n = 56$) and intact (IA; $n = 56$) groups. Orchidectomy was performed under ketamine anesthesia (15 mg/kg body wt). Sham operations were performed on animals from the IA group (SO; $n = 8$).

Two weeks after the surgery, both Orx and IA animals were divided into groups ($n = 8$) according to the received treatments. Either G or D (10 or 30 mg/kg body wt) was injected subcutaneously (sc) to orchidectomized (Orx G1 and Orx G2; Orx D1 and Orx D2, respectively) and intact (IA G1 and IA G2; IA D1 and IA D2, respectively) rats. G and D were obtained from Nutraceutica Monterenzio (Bologna, Italy).

The testosterone-treated orchidectomized (Orx T) and intact (IA T) groups were injected sc with 5 mg/kg body wt testosterone-propionate (Fluka Chemie, Buchs, Switzerland). Estradiol-treated orchidectomized (Orx E) and intact (IA E) groups were injected sc with 0.6 mg/kg body wt estradiol-dipropionate (ICN Galenika Pharmaceuticals, Belgrade, Serbia). G, D, T, and E were dissolved in a minimal volume of absolute ethanol and mixed with sterile olive oil. All substances were administered daily (except on Sundays) for 3 weeks. Rats in the control SO, Orx, and IA groups were sc administered with the same volume of vehicle solution according to the same schedule.

All animals were decapitated 24 hrs after the last treatment. Blood was collected from the trunk. The sera were stored at −70°C.

Serum Analysis. Total serum cholesterol and triglycerides were determined by the enzymatic method using colorimetric CHOD/PAP and GPO-PAP assays, respectively, according to the manufacturer's instructions (Serbolab, Kragujevac, Serbia) on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). The HDL-C measurement was performed after phosphotungstic acid magnesium chloride precipitation. LDL cholesterol was determined by the direct homogeneous method with the reagent LDL-C Select FS (DiaSys Diagnostic Systems GmbH, Holzheim, Germany), according to the manufacturer's specifications.

Statistical Analysis. Statistical analyses were performed using Statistica 6.0 software (Statsoft, Tulsa, OK). The data for the experimental groups were tested for normality of distribution with the Kolmogorov-Smirnov test and then were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range test was used for post-

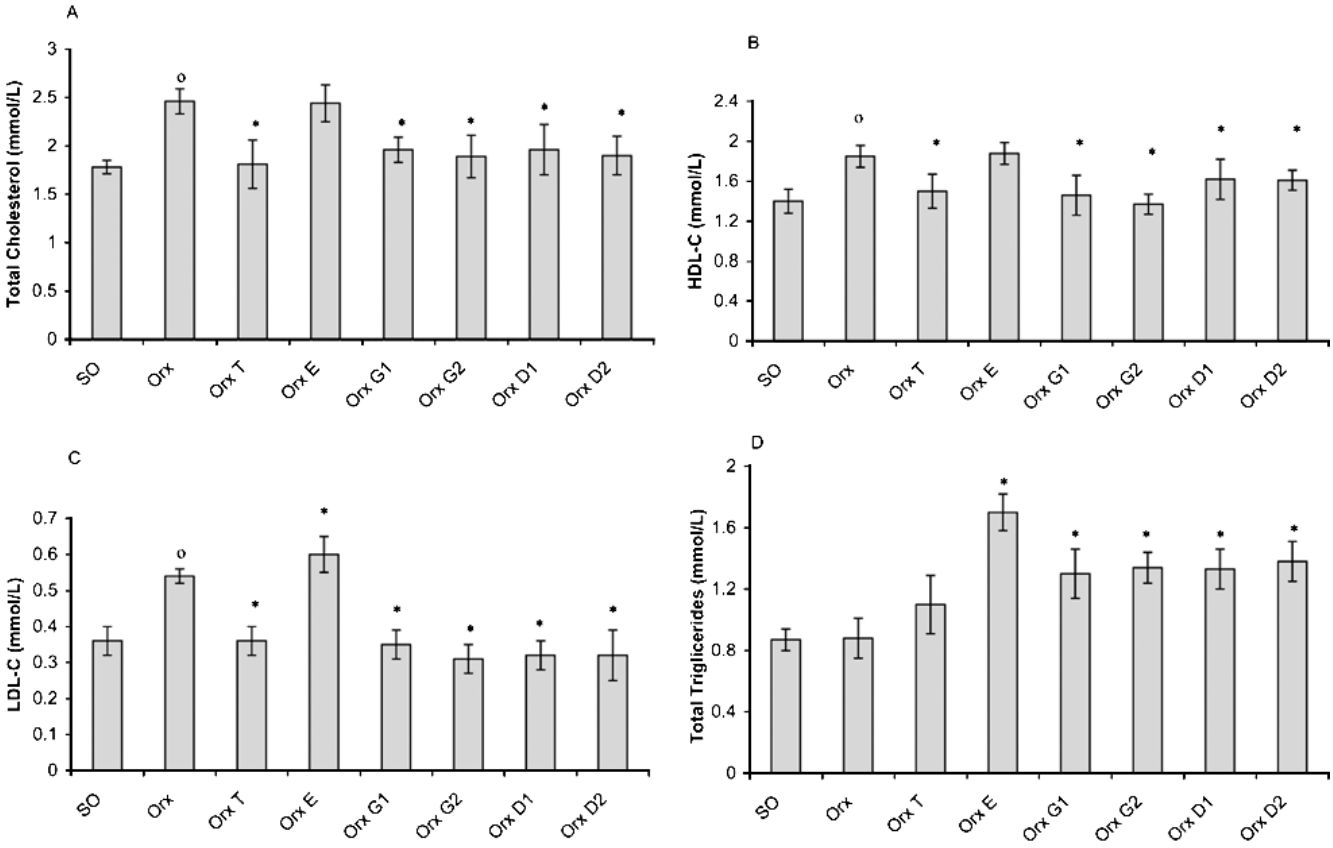

Figure 1. The serum parameters of lipid metabolism after orchidectomy and subsequent sc administration of testosterone (T) and estradiol (E) as controls, and lower and higher doses of genistein (G1 and G2, respectively) and daidzein (D1 and D2, respectively) in male orchidectomized middle-aged rats. The results are means ± SD ($n = 8$); *$P < 0.05$ versus orchidectomized control (Orx) group; °$P < 0.05$ versus sham-operated control (SO) group.

hoc comparison of means between the groups. A confidence level of $P < 0.05$ was considered statistically significant. The data are presented as means ± standard deviation (SD).

Results

Orchidectomy of middle-aged male rats induced a significant increase of TC, LDL-C, and HDL-C cholesterols in comparison with SO animals (approximately 28%, 24%, and 23%, respectively; $P < 0.05$; Fig. 1A–C). Subsequent subcutaneous administration of higher doses of G and D significantly decreased these parameters, with the most substantial effects on LDL-C reduction both in Orx (approximately 40% and 42%, respectively; $P < 0.05$; Fig. 1C) and IA (approximately 27% and 33%, respectively; $P < 0.05$; Fig. 2C) rats. No significant differences between the treatments with either G or D on the serum lipid profile were observed (Figs. 1A–D and 2A–D). The detected effects were not dose dependent in Orx rats, whereas only the higher doses of G and D altered the serum lipid levels in the IA model (Figs. 1A–D and 2A–D).

Lower serum cholesterol levels were also observed after testosterone administration in both Orx and IA (Figs. 1A–C and 2A–C) rats. The most pronounced effect was observed on LDL-C in the Orx group (approximately 35%; $P < 0.05$; Fig. 1C). Testosterone did not affect serum triglyceride levels (Figs. 1D and 2D).

Estradiol treatment brought about an increase in LDL-C in IA animals (approximately 27%; $P < 0.05$; Fig. 2D), and it increased serum triglycerides (approximately 90%; $P < 0.05$; Figs. 1D and 2D), as did the higher doses of G and D in both Orx (approximately 53% and 57%, respectively; $P < 0.05$; Fig. 1D) and IA (approximately 60% and 64%, respectively; $P < 0.05$; Fig. 2D) rats.

Discussion

In this work we showed that the sc administration of higher doses of purified soy isoflavones G and D significantly decreased the total cholesterol and lipoprotein HDL, with the most substantial effect exerted on LDL cholesterol concentrations in both Orx and IA models of andropause.

Our findings are consistent with the results of other researchers who have demonstrated that purified isoflavones exert a beneficial, cholesterol-lowering effect (18–21). However, we do not exclude the possibility that soy protein and other soy components exert the same or similar effects,

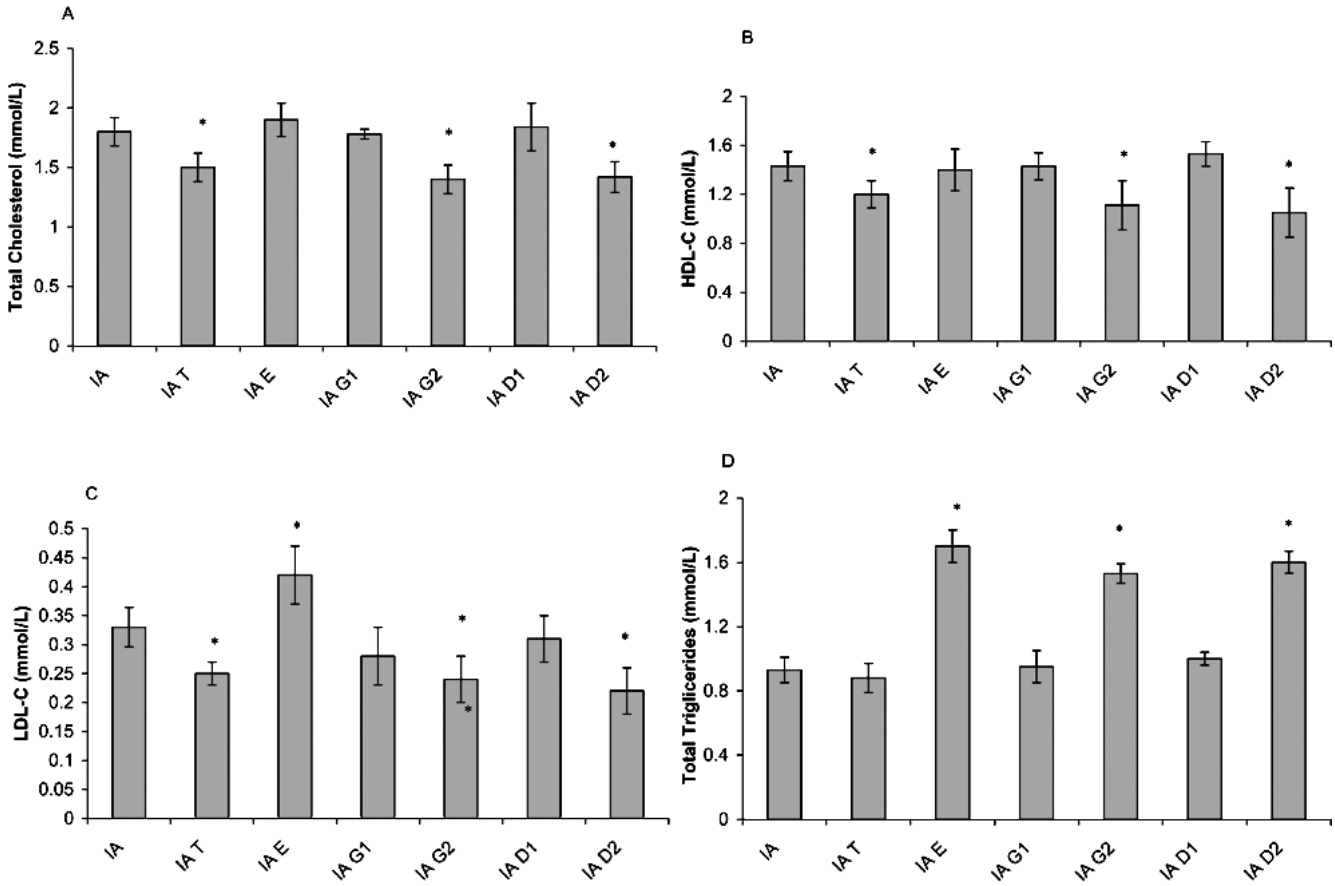

Figure 2. The serum parameters of lipid metabolism after the sc administration of testosterone (T), estradiol (E), and lower and higher doses of genistein (G1 and G2, respectively) and daidzein (D1 and D2, respectively) to intact male middle-aged rats. The results are means ± SD ($n = 8$); *$P < 0.05$ versus the vehicle-treated control (IA) group.

as reported by some researchers (14, 15, 22). Song *et al.* (24) compared the cholesterol-lessening effect of dietary daidzein, isolated soy protein with or without isoflavones, soy germ, and soy germ extract (a rich source of isoflavones) in hamsters. Since all treatments were almost equally effective, the authors concluded that both soy components as well as other extractable elements of soy are responsible for cholesterol-lowering effect of soy foods.

A decrease of HDL lipoprotein cholesterol was detected after either isoflavone or testosterone treatments. Several authors using different experimental approaches reported an increase (20, 21), no change (9, 24) or, as in the present study, a decrease (25, 26) in HDL cholesterol after exposure to isoflavones. A negative effect of higher isoflavone exposure on the serum HDL level is an intriguing possibility. However, in our study it could be due to differences in lipid metabolism between rats and humans. Further studies are needed to clarify this effect.

Treatments with G and D were chosen in order to mimic human exposure to elevated concentrations of isoflavones when nutritional supplements are consumed for therapeutic purposes (27). Both lower and higher doses altered the serum lipid level in Orx rats to a similar extent, whereas only higher doses were effective in the IA model. The difference in response could be due to the increased sensitivity to phytoestrogens of Orx animals that have a mild hypercholesterolemic profile compared with intact rats. Hasler observed that the lipid-lowering potential of soy was more pronounced in individuals with elevated cholesterol levels (28).

In this study, testosterone administration simulated hormone replacement therapy. Consistent with our findings that testosterone treatment improves serum cholesterols, Alexandersen *et al.* (29) reported that replacement of natural androgens inhibited atheroma formation in castrated male rabbits fed with cholesterol.

Treatment with estradiol mimicked hormone replacement therapy that exerts a well-described beneficial effect on the female blood cholesterol profile (30). However, its role in regulating serum lipoprotein concentration in males remains questionable. Researchers have reported a beneficial effect (31), an unfavorable effect (32), and no correlation between estradiol and lipoprotein concentration (33). Under our experimental conditions, estradiol treatment had an unfavorable effect on increasing LDL cholesterol. It also adversely increased serum triglycerides to a higher

extent than did the higher dose of G and D in both Orx and IA rats. The hypertriglyceridemic effect of estradiol has been well described (34, 35).

The reported effects of isoflavones on triglyceride levels are still poorly understood and contradictory. Whereas some authors reported beneficial or no effects (19, 20, 22, 24), others and the present study included, detected an increase in triglyceride levels after a genistein and soy–enriched isoflavone treatment (9, 26, 36). Nevertheless, the observed decrease of HDL-C and increase in triglyceride levels question the beneficial effect of the intake of higher doses of isoflavones. Further long-term studies are needed to clarify these effects.

The molecular mechanism(s) by which G and D affect liver lipid metabolism are unclear. Aside from having estrogenic activity, a recent report described the upregulation of expression of the rat hepatic thyroid hormone receptor β1 by isoflavones (37). The hypocholesterolemic actions of soy could be mediated by such a mechanism. Mezei *et al*. (17) reported that the isoflavones exert a beneficial effect on lipid metabolism through activation of peroxisome proliferator–activated receptors (PPARs) that regulate the transcription of genes involved in lipid metabolism.

To conclude, our results revealed that higher doses of subcutaneously injected soy isoflavones G and D decreased the serum cholesterol levels in male middle-aged rats to the same extent as after testosterone administration. At the same time, they brought about an unfavorable increase of serum triglycerides that is similar to that observed after estrogen administration.

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