ABSTRACT  To explore the importance of equol on health outcomes in future studies, it was necessary to develop a method to reduce equol production. Female monkeys (n = 22) fed a soy diet were treated twice daily with vehicle (control; n = 4), doxycycline (2.5 mg/kg; n = 4), metronidazole (125 mg/d; n = 3), kanamycin (1000 mg/d; n = 4), vancomycin (100 mg/d; n = 3) or kanamycin+vancymycin (n = 4). Plasma samples were collected 4 h postfeeding at baseline, after 4 wk of treatment and 8 wk after the end of treatment and analyzed for isoflavonoid concentrations. Fecal swabs were collected at baseline and at the end of antibiotic treatment for analysis of Gram(-) and Gram(+) bacterial growth. Equol concentrations were reduced (P < 0.05) compared with baseline by 80, 93, 98 and 99% after treatment with metronidazole (955 ± 164 vs. 193 ± 53 nmol/L), kanamycin (545 ± 211 vs. 37.1 ± 17.6 nmol/L), vancomycin (607 ± 163 vs. 8.9 ± 8.2 nmol/L) and kanamycin+vancymycin (721 ± 169 vs. 17.4 ± 17.3 nmol/L), respectively. Daidzein concentrations were increased (P < 0.05) compared with baseline by treatment with doxycycline (336 ± 87 vs. 576 ± 76 nmol/L), kanamycin (168 ± 67 vs. 374 ± 15 nmol/L), and kanamycin+vancymycin (166 ± 35 vs. 384 ± 78 nmol/L). Similar increases (P < 0.05) in dihydrodaidzein were observed after treatment with kanamycin (31.2 ± 6.2 vs. 479 ± 188 nmol/L) and metronidazole (56.0 ± 27.9 vs. 414 ± 212 nmol/L). Isoflavonoid concentrations returned to baseline values after antibiotic treatment was terminated. Gram(+) bacterial growth was reduced by all treatments, including Control, compared with baseline. In conclusion, treatment with antibiotics resulted in a marked reduction in plasma equol concentrations and altered plasma isoflavonoid patterns in cynomolgus monkeys.


KEY WORDS: • cynomolgus monkeys • soy • isoflavonoids • equol • antibiotics

There is high interest in the potential health benefits of soy consumption, particularly related to decreasing risk for cardiovascular disease and hormone-related cancers. Of these potential health benefits, the major research emphasis has been on the potential cardiovascular benefits of soy and its isoflavones. Anderson and co-workers (1) published a meta-analysis indicating that dietary soy consumption by human subjects was associated with reductions in LDL cholesterol of ~13%, reductions in plasma triglycerides of ~10% and increases in HDL cholesterol of ~2%. Our group has conducted a number of studies to examine the effects of soy protein on plasma lipids, lipoproteins and the extent of atherosclerosis in rhesus and cynomolgus monkeys fed atherogenic diets. Compared with casein/albumin, soy protein resulted in reductions in LDL+VLDL cholesterol of 30–40%, increases in HDL cholesterol of ~15% and reductions in the extent of atherosclerosis (2,3).

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In addition to the potential cardiovascular benefits, it has been reported that soy and/or soy isoflavones may reduce cancer risk (4–6). Epidemiologic studies indicate that populations consuming soy habitually tend to have lower rates of breast cancer with increased soy exposure (7–10). Also, studies in rodents suggest that soy prevents the progression of induced carcinogenesis (17,18). Beneficial effects of soy on breast and uterus have also been observed in nonhuman primates. Foth and Cline (19) demonstrated that dietary soy treatment antagonized estradiol (E2)4-induced mammary gland cell proliferation, slightly reduced E2-stimulated cell proliferation in the endometrium and reduced the incidence of endometrial hyperplasia in monkeys treated with E2.

In contrast to the robust reductions (~30%) in plasma LDL+VLDL cholesterol and increases (~15%) in HDL cholesterol in nonhuman primates by dietary soy protein administration, results of recent studies in human patients show only modest changes in plasma lipoprotein concentrations. Several studies have reported reductions in plasma LDL cholesterol concentrations in human subjects of 2.6–6.5% (20–22), whereas most found no effect on HDL cholesterol concentrations. Recently, Lichtenstein et al. (23) found very small

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reductions in LDL cholesterol (2%) and increases in HDL cholesterol (3%) when soy/soy isolates were administered to postmenopausal women in amounts comparable to those used in the monkey studies.

These differences between monkeys and human subjects in plasma lipoprotein responses to soy may be due in part to species differences in isoflavone metabolism. In general, ~33% (22–47%) of human subjects consuming soy products produce measurable quantities of equol (24–32). Unlike humans, nonhuman primates produce higher levels of equol (33–35). Furthermore, plasma concentrations of isoflavones in postmenopausal cynomolgus monkeys fed a soy-based diet are comprised predominantly (~60% of the total plasma isoflavones) of equol (33).

The precise role of equol in health outcomes has been difficult to evaluate due to the variable incidence of equol production in human subjects consuming soy. It has been suggested that the variability in equol excretion is due to differences in the absorbance and degradation of the isoflavones present in soy. Xu et al. (36) reported in women that the bioavailability of soy isoflavones depends upon the intestinal microflora, and it has been demonstrated that daidzein and its metabolites appear at higher levels in urine than genistein and its metabolites (12,37). One of the primary metabolites of the bacterial degradation of daidzein is the isoflavone equol (30,37,38).

Because equol production is a function of the bacterial metabolism of daidzein, the use of antibiotics to alter bacterial populations in the intestine provides a potential means to modulate plasma equol concentrations. The objective of the present study was to determine whether oral treatment of cymomolgus monkeys with antibiotics could reduce plasma concentrations of equol.

### MATERIALS AND METHODS

#### Animals.
Adult, long-term, ovariectomized (> 1 y old), female cynomolgus macaques (Macaca fascicularis, n = 22) were used for this study. Monkeys were group-housed (2–5/pen) and fed a soy-based diet (Table 1). The soy-based diet contained 23.88% protein (15.0% soy protein isolate, 4.5% casein, and 4.4% lactalbumin). The soy protein isolate was generously provided by DuPont Protein Technologies (St Louis, MO). All procedures involving monkeys were conducted in compliance with state and federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University School of Medicine Animal Care and Use Committee.

#### Experimental design.
Monkeys were randomly assigned to be treated orally twice daily for 4 wk with 1 vehicle (Control; n = 4; 1.0 mL fruit punch); 2 doxycycline (DOX; 2.5 mg/kg; n = 4; Major Pharmaceuticals, Livonia, MI); 3 metronidazole (MET; 125 mg/d; n = 3; Major Pharmaceuticals); 4) kanamycin (KAN; 1000 mg/d; n = 4; Kantrex; Bristol-Myers Squibb, Princeton, NJ); 5) vancomycin (VAN; 100 mg/d; n = 3; American Pharmaceutical Partners, Los Angeles, CA) or 6) kanamycin + vancomycin (KAN+VAN; n = 4).

Blood samples were collected 4 h postfeeding at three time points, i.e., before initiation of antibiotic treatment (baseline), at the end of antibiotic treatment (postantibiotic) and 8 wk after the end of antibiotic treatment (washout period). For blood collection, monkeys were sedated (ketamine HCl, 100 g/L; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and a blood sample was collected from the femoral vein into an EDTA Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Blood samples were centrifuged at 4°C for 25 min at 1000 × g and plasma stored at −70°C until analyzed for isoflavonoid concentrations by liquid chromatography-photodiode array-electrospray ionization-MS (LC/PDA/ESI-MS).

#### Plasma isoflavonoid determinations.
Analysis of isoflavonoids was carried out using LC/PDA/ESI-MS as established recently for isoflavonoids (genistein, dihydrogenistein, daidzein, dihydrodaidzein, glycitein, equol, O-desmethylangolensin) and other phenolic phytochemicals from blood, urine and breast milk (39). In brief, 0.1 mL triethylamine buffer (pH 7; 0.2 mol/L) was added to 0.45 mL plasma or serum followed by the addition of formononetin as an internal standard. This mixture was incubated with 0.04 mL β-glucuronidase and 0.04 mL arylsulfatase for 12–17 h at 37°C. Hydrolyzed analytes were isolated by partitioning into diethyl ether after precipitating proteins with 0.45 mL acetonitrile. The combined organic phases were evaporated and the residue was redissolved in 0.1 mL methanol and 0.1 mL of 0.2 mol/L acetate buffer (pH 4). This redissolved solution (10–20 μL) was injected onto a HydroBond PS 18 (100 × 3.0 mm; 5 μm) column (MAC-MOD Analytical, Chadds Ford, PA) with a flow rate of 0.25 mL/min using a methanol/acetonitrile/water gradient and detected by MS using a quadrupole ion trap MS model Surveyor-Advantage (Thermo Finnigan, San Jose, CA). All analytes were monitored by screening the acquired set of MS data at the mass range M-0.5 to M+1.5 with M being the nuclide mass of the respective analyte and by selected reaction monitoring using product masses diagnostic for the respective analyte. Interassay variability for isoflavonoids and flavonoids was found to vary between 5 and 17% depending on the analyte type and analyte concentration (39,40).

#### Fecal bacteria determinations.
Analysis of bacterial populations from individual fecal samples was performed at baseline and at the end of the antibiotic treatment period. Fecal swabs were collected by insertion of a sterile cotton swab into the rectum. Fecal samples were cultured on Blood Agar McConkey Bileplats (Laboratory Supply, Winston-Salem, NC) to examine differences in Gram(-) and Gram(+) bacterial growth. Agar plates were cultured at 37°C for 48 h and scored for bacterial growth. Growth scores were assigned to each plate using the following criteria: 0 = no growth; 1 = light growth (0–30% plate coverage); 2 = moderate growth (30–60% plate coverage); or 3 = heavy growth (60–100% plate coverage).

#### Statistical analysis.
Data were analyzed by ANOVA using the General Linear Models (Proc GLM) of SAS (Version 6.12 for Windows; SAS Institute, Cary, NC). Plasma isoflavonoid concentrations and bacterial growth scores were analyzed for differences between antibiotic treatment, sample collection time point and treatment.
× time point interactions. For post-hoc comparisons between groups, 
\( t \) tests using pooled variance (comparable to Fisher's least significant difference test) were used. A \( P \)-value < 0.05 was considered significant. Values are means ± SEM.

RESULTS

Plasma isoflavones. Plasma levels of equol were dramatically reduced in monkeys treated with MET \((P < 0.01)\), KAN \((P < 0.05)\), VAN \((P < 0.05)\) and the KAN+VAN \((P < 0.01)\) combination compared with baseline (Fig. 1). Plasma equol concentrations returned to baseline values 8 wk after the end of the antibiotic treatment.

In contrast to the decrease in equol concentrations, plasma concentrations of daidzein were increased by antibiotic-specific treatment. Compared with baseline values, treatment with DOX \((P < 0.01)\), KAN \((P < 0.05)\) and KAN+VAN \((P < 0.05)\) resulted in increased plasma daidzein concentrations, which returned to baseline values after the washout period (Fig. 2). Plasma concentrations of dihydroidaizidein were increased in monkeys treated with KAN \((P < 0.01)\) and MET \((P < 0.01)\), and the concentrations of this daidzein metabolite also returned to baseline after 8 wk without antibiotic treatment (Fig. 3).

In addition to daidzein and its metabolites (equol and dihydrodaidzein), antibiotic treatment also affected plasma concentrations of genistein, dihydrogenistein and glycitein. Genistein concentrations were increased compared with baseline in the plasma of monkeys treated for 4 wk with DOX \((172 ± 55 vs. 591 ± 181 \text{ nmol/L}; \ P < 0.01)\) and KAN+VAN \((76.3 ± 27.3 vs. 432 ± 165 \text{ nmol/L}; \ P < 0.01)\), whereas concentrations of dihydrogenistein were increased by treatment with DOX \((35.1 ± 7.5 vs. 360 ± 254 \text{ nmol/L}; \ P < 0.01)\) and MET \((65.7 ± 37.1 vs. 376 ± 84 \text{ nmol/L}; \ P < 0.05)\). Plasma glycitein concentrations were elevated in monkeys administered DOX \((57.1 ± 18.2 \text{ vs. 97.6 ± 6.7 \text{ nmol/L}; } P < 0.01)\) and KAN \((26.6 ± 11.8 \text{ vs. 57.2 ± 8.4 \text{ nmol/L}; } P < 0.05)\) for 4 wk compared with baseline. In each instance, isoflavonoid concentrations returned to baseline levels within 8 wk of cessation of antibiotic treatment. These data indicate that different antibiotics uniquely alter the plasma isoflavone profile.

The concentrations of individual isoflavonoids in relation to each other and to total plasma isoflavonoid concentrations are depicted in Figure 4. Although concentrations of total plasma isoflavonoids generally remained unchanged over treatment periods, DOX-treated monkeys exhibited higher \((P < 0.01)\) total isoflavonoids compared with baseline. At baseline, mean total isoflavonoids for all groups was 1194 ± 71 \text{ nmol/L} with ~57% of total isoflavonoids comprised of equol \((679 ± 75 \text{ nmol/L})\).

Fecal bacterial growth. No differences in Gram(−) bacteria growth were observed after treatment with antibiotics; however, a reduction in growth was observed in the Control

FIGURE 1 Plasma equol concentrations in cynomolgus monkeys fed a soy-based diet and orally dosed with vehicle (control; \(n = 4\)), doxycycline (DOX; \(n = 4\)); samples from only 3 monkeys were available at the washout collection), metronidazole (MET; \(n = 3\)), kanamycin (KAN; \(n = 4\)), vancomycin (VAN; \(n = 3\)) or kanamycin + vancomycin (KAN+VAN; \(n = 4\)) for 4 wk. Values are means ± SEM. *Different from baseline, \(P < 0.05\).

FIGURE 2 Plasma daidzein concentrations in cynomolgus monkeys fed a soy-based diet and orally dosed with vehicle (control; \(n = 4\)), doxycycline (DOX; \(n = 4\)); samples from only 3 monkeys were available at the washout collection), metronidazole (MET; \(n = 3\)), kanamycin (KAN; \(n = 4\)), vancomycin (VAN; \(n = 3\)) or kanamycin + vancomycin (KAN+VAN; \(n = 4\)) for 4 wk. Values are means ± SEM. *Different from baseline, \(P < 0.05\). See Figure 1 for abbreviations.

FIGURE 3 Plasma dihydrodaidzein concentrations in cynomolgus monkeys fed a soy-based diet and orally dosed with vehicle (control; \(n = 4\)), doxycycline (DOX; \(n = 4\)); samples from only 3 monkeys were available at the washout collection), metronidazole (MET; \(n = 3\)), kanamycin (KAN; \(n = 4\)), vancomycin (VAN; \(n = 3\)) or kanamycin + vancomycin (KAN+VAN; \(n = 4\)) for 4 wk. Values are means ± SEM. *Different from baseline, \(P < 0.05\). See Figure 1 for abbreviations.
FIGURE 4  Total plasma isoflavonoid concentrations in cynomolgus monkeys fed a soy-based diet and orally treated twice daily with vehicle (control; n = 4), doxycycline (n = 4; samples from only 3 monkeys were available at the washout collection), metronidazole (n = 3), kanamycin (n = 4), vancomycin (n = 3) or kanamycin + vancomycin (n = 4) for 4 wk. Values are means ± SEM. See Figure 1 for abbreviations. O-DMA, O-desmethylenolensin.

group. Gram(+) bacteria growth was reduced by all antibiotic treatments; however, a reduction in Gram(+) bacteria was also observed in the Control group.

DISCUSSION

It has been suggested that the isoflavone equol, which was first identified in the urine of humans by Axelsson et al. (41), may have some chemoprotective properties. Although little research into the chemoprotective effects of equol was conducted until the last decade, Adlercreutz et al. (42) reported that the combined urinary equol and enterolactone concentrations were positively associated with plasma levels of sex hormone binding globulin (SHBG). This increase in SHBG may be cardioprotective by reducing the amounts of free steroids in the plasma. More recently, it was demonstrated that equol treatment of MCF-7 cells suppressed estrogen-induced expression of the estrogen-responsive gene pS2 (43). In a case-control study designed to examine the relationship between urinary phytoestrogen excretion and breast cancer risk, Ingram et al. (12) demonstrated that high excretion of equol was significantly associated with a reduction in breast cancer risk. Furthermore, premenopausal equol excretors have lower plasma concentrations of multiple estrogens and androgens, but higher concentrations of SHBG and progesterone, indicative of reduced breast cancer risk (44).

The precise role of equol in health outcomes has been difficult to evaluate due to the variable incidence of equol production in human subjects consuming soy. In a dietary crossover study of lignan and isoflavone excretion, Kirkman and co-workers (24) reported that 4 of 18 individuals (22.2%) excreted high amounts of equol while consuming the soy diet. Kelly et al. (25) observed a similar percentage of high equol producers (33.3%) in a study in which 12 subjects were challenged with 40 g of whole soy flour for 2 d. Similar results also have been reported by other investigators (26–32). Results from larger studies suggest potential gender differences in equol production. In a study of 30 men and 30 women consuming a powdered soy protein beverage, 43% of the men and 27% of the women had high concentrations of urinary equol (26,27), whereas Morton et al. (32) reported that 38% of male and 38% of female Japanese subjects had plasma concentrations of equol > 20 nmol/L.

The physiologic differences between equol producers and equol nonproducers have not been fully elucidated. Lu and Anderson (29) demonstrated that some women (3 of 5 initially categorized as nonequol excretors) developed the ability to produce equol after 2 wk of soy milk ingestion. Rowland and co-workers (30) reported that equol excretors consumed less fat and more carbohydrate as a percentage of energy than nonequcretors of equol. However, Lampe et al. (31) demonstrated that equol excretor status did not change during chronic soy ingestion and was not associated with dietary carbohydrate intake.

It has been suggested that the variability in equol excretion in human subjects is likely due to differences in the intestinal absorption and degradation of the isoflavones present in soy. Most flavonoids present in plants and plant products are found as aglycones or isoflavone derivatives (45). It is thought that the initial metabolism of glycosidic flavonoids occurs in the small intestine via deglycosylation (46,47). Day and co-workers (48) reported that daidzein-7-glucoside was rapidly deglycosylated by β-glucosidase activity in cell-free extracts of the human small intestine. It also has been reported that lactase phlorizin hydrolase, a specific β-glucosidase present in the small intestine brush border, is one of the enzymes responsible for the initial deglycosylation of daidzein-7-glucoside (49).

In addition to early deglycosylation in the small intestine, flavonoids, such as daidzein, that are not absorbed in the small intestine are transported to the colon where they are metabolized into aglycones and phenolic acids by intestinal bacteria (46,47). Xu et al. (36) reported in women that the bioavailability of soy isoflavones depends upon the intestinal microflora, and it has been demonstrated that daidzein and its metabolites appear at higher levels in urine than genistein and its metabolites (37,50). One of the primary metabolites of the bacterial degradation of daidzein is the isoflavone equol (30,37,38). A recent study reported that three specific human intestinal bacteria, Bacteroides ovatus spp., Ruminococcus productus spp. and Streptococcus intermedius spp., are involved in the conversion of daidzein to equol (51). Because daidzein and its metabolites are more bioavailable than other soy isoflavones, and equol is the primary metabolite of daidzein and has a high affinity for estrogen receptors, the potential effect of equol production on human health is an important issue.

Previous studies have demonstrated wide ranges of plasma equol concentrations in human subjects. Most studies have demonstrated that human equol producers exhibit plasma equol concentrations ranging from ~0 to 130 nmol/L depending upon the type of diet (30,52–55). However, recent studies by Morton and co-workers reported higher plasma equol concentrations in Japanese (32) and Chinese (56) populations.

Unlike human equol producers in which equol appears to be only a small proportion of the total isoflavones in plasma, concentrations of isoflavones in postmenopausal cynomolgus monkeys fed a soy-based diet are comprised predominantly of equol. Clarkson et al. (33) reported that total plasma isoflavone concentrations in cynomolgus monkeys fed a soy-based diet containing isoflavones was ~777 nmol/L with equol accounting for ~459 nmol/L (60% of the total plasma isoflavones). This is comparable to the results from the current study in which mean baseline plasma equol concentrations were ~679 nmol/L (57% of total plasma isoflavones). Rodent species fed soy-based diets have also demonstrated very high plasma equol concentrations (57–59).

Because equol production appears to be a function of the
bacterial metabolism of daidzein, the use of antibiotics to alter bacterial populations in the intestine provides a potential means to modulate plasma equol concentrations. The current study demonstrated that plasma equol concentrations can be reduced in monkeys by the administration of antibiotics. Similar results were observed in humans (60,61). In contrast to Kilkkinen et al. (61), who indicated that it required several months for enteroactone concentrations to return to levels comparable to subjects not taking antibiotics, plasma equol concentrations in the present study returned to baseline values within 8 wk after the end of antibiotic treatment. These differences could be associated with differences between species, antibiotics, or bacterial populations affected by antibiotic treatment.

Interestingly, the results of the current study also demonstrate that individual antibiotics alter plasma isolavone levels in unique patterns. For instance, treatment with kanamycin reduced plasma equol levels while increasing plasma levels of daidzein, dihydrodaidzein and genistein. In contrast, treatment with doxycycline did not affect plasma equol levels, but plasma levels of daidzein, genistein, dihydrogenistein and glycitein were elevated. The biological basis for these differential effects is unclear. However, it is possible that some of the antibiotics examined in the current study may have direct effects on isolavone metabolism, whereas others may alter absorption through the intestinal wall.

Minimal differences in fecal bacterial populations were observed in the current study. Although Gram(+) bacteria were reduced after antibiotic treatment, these results were confounded by the reduction of both Gram(+) and Gram(−) bacteria in the control groups. We currently have no explanation for the reduction in fecal bacteria in the Control monkeys.

In conclusion, the present study demonstrated that oral treatment with antibiotics reduces the plasma concentrations of equol in cynomolgus monkeys, thus establishing a potential model system in which equol production can be modulated. For instance, treatment with antibiotics reduces the plasma concentrations of equol in cynomolgus monkeys, thus establishing a potential animal model with which to examine in the current study may have direct effects on isolavone metabolism, whereas others may alter absorption through the intestinal wall.

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LITERATURE CITED