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Equol production changes over time in premenopausal women

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Abstract

Equol (EQ) is a metabolite produced by gut bacteria through the chemical reduction of the soy isoflavone (IFL) daidzein (DE), but only by 30–60% of the population. EQ is believed to provide benefits derived from soy intake and its production is widely viewed as a relatively stable phenomenon. In a randomized, cross-over intervention with soy foods, 79 premenopausal women were challenged with 6-months each of a high-soy and low-soy diet separated by a 1-month washout. Overnight urine (OU) was collected at three time points during each diet period and analyzed for DE and EQ by liquid chromatography tandem mass spectrometry (LC-MS/MS). Remaining an EQ producer (EP) or nonproducer (NP) or changing towards an EP or NP was assessed using a ratio of EQ/DE ≥ 0.018 combined with a DE threshold ≥ 2 nmol/mg creatinine as cutoff. We observed 19% and 24% EP during the low-soy and high-soy diet period, respectively, and found that 6–11% of our subjects changed EQ status *within* each study period (on average of 1.2 times) while 16% changed *between* the two diet periods. This finding challenges the widely held conviction that EQ production within an individual remains stable over time. The precise factors contributing to changes in EQ status, however, remain elusive and warrant further investigation.

Keywords

equol; soy; health benefits

Introduction

Equol (EQ) is a metabolite produced by intestinal bacteria through the chemical reduction of the soy isoflavone (IFL) daidzein (DE) [1, 2] and has been hypothesized to play an important role regarding the health benefits observed from soy or IFL consumption [3]. These benefits include protection against breast, prostate, lung, and colorectal cancer, osteoporosis and cardiovascular disorders, as well as alleviation from menopausal symptoms [4–11]. Recent epidemiological studies have provided strong evidence about the protective effect of soy or IFL exposure against breast cancer in adulthood [10, 12, 13], particularly when exposure occurs at an early age [14–18], however, the role of EQ in this respect remains uncertain.

The prevalence of EQ producers (EP) has been estimated to be 30–35% in Western populations [19–21] and up to 60% in vegetarians [22] or Asians [23]. However, it is widely debated as to whether the ability to produce EQ is stable over time and results necessarily in favorable health outcomes [reviewed in24, 25, 26]. Previous studies have indicated that EQ

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production is stable not in all, but only "in most individuals over 1–3 years" [27], or only "over 1 year" [28, 29], or "in 85% of individuals over 1.5 years" [23].

Diet has been suggested to influence an individual's ability to produce EQ [19, 20, 30, 31], however, this could not be confirmed [22, 32–37]. Recently, we observed a relatively high prevalence (up to 35%) of postmenopausal women who became EP (CR+) in a well controlled intervention study with isolated soy protein containing a daily IFL dose of approximately 90 mg [38]. Unexpectedly, more CR+ occurred in the placebo group exposed to soy foods from the habitual diet *versus* the treatment group exposed to soy protein.

In the current study, we aimed to evaluate the role of soy food consumption and a reduced IFL dose on EQ producer status by determining the consistency of urinary EQ excretion over time in a cohort of premenopausal women challenged with 6-months each of a high-soy and low-soy diet of soy foods in a cross-over designed intervention separated by a 1-month washout. To maintain consistency between, and facilitate comparison with our previous study, determination of EQ producer status was performed using a urinary EQ/DE ratio of 0.018 in combination with a DE excretion threshold (2 or 5 nmol per mg creatinine) or an absolute urinary EQ excretion (0.5 or 1.0 nmol per mg creatinine).

Methods

Study Design

The Breast, Estrogens, and Nutrition study (BEAN) was a randomized, 2 x 6-month crossover soy trial with a 1-month washout period; all details were reported previously [39]. Briefly, 96 premenopausal women (ages 18–50 years) who consumed 5 or less servings of soy per week completed diet and anthropometric questionnaires followed by randomization into 2 groups: Group A (n=48) began the study with high-soy consumption, and, following the 1-month washout, switched to low-soy consumption, whereas the order of diets was reversed in Group B (n=48). During the high-soy period, participants added 2 servings of selected soy foods per day to their regular diet, which approximated 50 mg isoflavones [40], an amount comparable to daily intakes reported for some Asian countries [41]. All soy foods were provided to the participants at the beginning and middle of the high-soy period. There was no restriction on further soy food consumption during the high-soy period and no time regime on daily soy food consumption was imposed. During the low-soy period, participants maintained their usual diet but were asked to limit their soy intake to less than 3 servings per week and consume no soy-containing supplements. A total of 8 overnight urine (OU) specimens (at months 1, 3, 6, 8, 10, and 13 plus at baseline and washout) were collected during the study and used for the presented analyses. For these OU specimens, subjects voided their bladders before retiring, then collected urine during the entire night including the first morning void. All except 2 urine specimens were delivered to the laboratory on the morning of the last collection and were immediately processed followed by storage at −80°C until analysis. The 2 urine specimens not delivered were sent by mail; these samples consisted of only morning urine.

Adherence to the study regime was assessed by urinary IFL analyses and several unannounced telephone 24-hour dietary recalls during each diet period. Compliance was defined as consuming >40 mg and <10 mg IFL during the high-soy and low-soy diets, respectively. Prior to cessation of the study, 14 women (15%) dropped out. Of the remaining 82 individuals, 79 provided a complete set of the 6 OU specimens relevant to the study periods (those collections without baseline and washout samples) that were subsequently used for IFL analyses. For comparisons between high-soy and low-soy diets, only 6 OU specimens were considered, as baseline and washout time periods were deemed invalid for evaluating differences in EQ status. This study was conducted according to the guidelines

laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee on Human Subjects at the University of Hawaii and by participating clinics. All participants signed an informed consent form. A Data Safety Monitoring Committee annually reviewed study progress, reasons for dropouts and any reported symptoms.

Biochemical analyses

Urinary creatinine concentrations were measured using a Roche-Cobas MiraPlus Chemistry autoanalyzer. Daidzein (DE), EQ, genistein and O-desmethylangolensin levels were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously detailed [42]. IFL excretion was expressed as nmol/mg creatinine to convert OU concentrations to an excretion value and to adequately adjust for variable urine volume.

Statistical Analysis

As proposed in our previous study [38], determination of EQ status in OU was performed using four different methods based on previous suggestions in the literature which included using a relative EQ:DE ratio cutoff of 0.018 [22] but -as detailed previously- with a DE threshold exceeding either 2 or 5 nmol per mg creatinine (hereafter referred to as EQ/DE 2 or EQ/DE 5, respectively). Thus, the EQ/DE cutoff to define EP versus NP was only considered among subjects who reached the given DE threshold. Alternatively, EQ status was also determined using absolute EQ concentration cutoffs of either 0.5 or 1.0 nmol per mg creatinine (hereafter referred to as EQ 0.5 or EQ 1.0, respectively) which are cutoffs used variably in the literature [28, 32]. Individuals whose OU concentrations remained above or below the cutoffs for all 6 OU measurements during the BEAN study were defined as either EP or NP, respectively. Individuals who changed between EP and NP status were defined as crossers (CR) and, within this CR group, those switching ever from NP to EQ producer status were defined as CR+ while those switching in the opposite direction were defined as negative crossers (CR−). The SAS statistical package was used for analysis.

Results

Of the 96 women who entered the study, we included data for 79 after excluding those who dropped out (n=14) or had incomplete OU collections (n=3). Table 1 shows the percent EP, NP, and CR for these 79 individuals during the high-soy and low-soy diet periods after each of the relative (EQ/DE 2, EQ/DE 5) and absolute (EQ 0.5, EQ 1.0) cutoffs were applied. Using relative cutoffs (which included a DE threshold), we observed 23–24% EP during the high-soy diet and 9–19% EP during the low-soy diet. When absolute cutoffs were applied (no DE threshold), we observed a similar proportion of EP during the high-soy diet (16%–22%) but much fewer EP (3–4%) during the low-soy diet. The %CR was higher (10–18%) and %NP was lower (65–67%) during the high-soy diet compared with the low-soy diet (6–11% and 75–86% for CR and NP, respectively) among all EQ status cutoffs.

CR status was determined very consistently between the four EQ status classification methods during both the low-soy diet (concordance 0.93–1.00, overall reliability 0.99; table 2 plain numbers) and high-soy diet (concordance 0.96–1.00, overall reliability 1.00; table 2 bold numbers). During low-soy and high-soy diet periods, crossings occured an average of 1.2 times per CR and were similarly distributed between CR+ and CR− (data not shown). EQ status changed 7–17 times during the high-soy diet depending on which cutoff was applied and 10–11 times during the low-soy diet when absolute cutoffs were applied. When relative cutoffs (EQ/DE ratios) were used during the low-soy diet, only one crossing (CR+) was observed (data shown in part in table 1).

When comparing the low-soy to the high-soy diet period using the EQ/DE 2 cutoff, we identified 13 women who retained the same EQ status during *both* diet periods: 11 maintained NP status and 2 maintained EP status. Additionally, we identified 2 women who changed EQ status not only *within* one of the diet periods but also *between* these periods: one woman was a CR+ in the low-soy period and an EP in the high-soy period while the other woman was CR+ in the high-soy period and an EP in the low-soy period (data not shown). When considering both diet periods we found 15 EP (20%), 49 NP (65%) and 12 CR (16%; 3CR+, 5CR−, and 4 women crossing more than once) (data not shown).

No significant changes in mean EQ production over time ($p>0.30$) were observed during the low-soy diet period, however, we observed significant decreases ($p<0.01$) in mean EQ production during the high-soy period, irrespective of which EQ status cutoff was applied (data not shown).

Discussion

Over recent years, considerable attention has centered on EQ production strictly on the basis of whether individuals produce or not produce EQ. However, little interest has focused on individuals capable of changing EQ producer status over time. Consequently, EQ production has been viewed as a relatively stable phenomenon [17, 23, 27, 43] with four reports even proposing that individuals are *unable* to change EP phenotypes [37, 43–45].

Findings from the current study and a few others [27, 38, 46, anecdotally in[47], undiscussed in[48]], however, seem to challenge this view. In a study by Frankenfeld and colleagues, 11% of subjects were found to change EQ producer status from a NP to a EP, while 8% changed in the opposite direction [27]. Similarly, Lu and colleagues observed NP to EP changes in 3 of their 6 volunteers after chronic soymilk ingestion [48] and Ko and colleagues reported 8 of 20 NP converted to EP after twice weekly consumption of soymilk for 16 weeks [46]. Equally, we reported changes in EQ producer status over time from anecdotal evidence in a previous report [47] and also very recently from a well-controlled 2.5 year soy intervention study with 350 postmenopausal women [38]. In the latter study, we observed a relatively high proportion of individuals (up to 35%) who were CR (either CR+ or CR−). These EQ producer status changes were unexpectedly not associated with antibiotics use, except for selected groups when antibiotics use correlated with CR+. These findings [38] are comparable to those in our current study where 6% and 11% CR were found during the low-soy and high-soy diet periods, respectively, and where EQ producer status was evaluated using the same matrix (OU) and the same EQ producer status cutoff definitions.

Previous studies suggest an uncertain role of diet on EQ production; some reports claim diet to influence an individual's ability to produce EQ while conflicting findings exist from observational and feeding studies. Two studies reported that long-term and repeated soy ingestion could convert NP to EP [46, 48], while four others stated otherwise [37, 43–45]. Another study even proposed that "once an EQ producer, always an EQ producer" [49]. Furthermore, Frankenfeld and colleagues [27] found no apparent association between diet and changes in EQ production when comparing EQ producer status among the same individuals 1–3 year apart. Discrepancies among these findings may, in part, be related to factors such as small sample size [1, 48], matrix differences (feces vs. serum/plasma vs. urine), study design and duration [27, 30, 31, 35, 37], differences in defining EP [as seen in[22, 23, 28, 29, 31–33, 37, 44, 50]], and/or type and form of soy foods consumed [32, 44, 51, 52].

Our most recent findings from a well controlled soy protein intervention study [38] led to the hypothesis that changes in EQ producer status may have been attributable to differences in absolute IFL exposure and/or dietary intake. While the placebo group in that study was

exposed to relatively low levels of IFLs owing to their habitual diet (e.g., native and/or natural soy foods), the treatment group consumed approximately 90 mg IFL during the study period; an amount almost twice the daily intake reported by Asians [41], about 7.5 times the median intake by Asian-Americans [17] and about 30–90 times the average intake of Americans [53]. It was, therefore, of interest to evaluate the role of native soy foods presented at physiologically relevant levels of intake on EQ producer status. To maintain consistency with our previous study, we utilized the same EQ producer status definitions (see Methods) to facilitate accurate comparisons between studies.

Previously, the cutoffs to determine EQ producer status and the matrices used for EQ producer analyses have varied markedly across studies. EQ producer cutoffs such as: serum EQ concentrations above the lower limits of the detection system (LOD) [23], the ability to convert DE to EQ in feces after 96-h incubation [54], >10 % conversion of DE into EQ in feces [29], or, in urine, EQ concentrations >0.182nmol/ml [33], >0.68nmol/ml [55], >1 mg/ml [37], > 900 nmol/24 hr [44], DE thresholds >10 nmol/mg creatinine [28] or use of a log10-transformed urinary EQ:DE ratio of −1.75; absolute ratio of 0.018 [22], have made the comparisons of %EP across studies difficult, if not impossible. Inter-study comparisons are further complicated when arbitrary thresholds are used to distinguish a “good-” or “high-” EQ excretor from a “poor/low-” or “non-” EQ excretor for some individuals may inevitably fall into an intermediary position, as suggested previously [22]. Using various EP cutoffs may be especially problematic when defining EQ producer status *changes* (e.g. identifying CR) as EP cutoffs set very low (e.g. using the LOD) would theoretically and erroneously lead to a greater prevalence of CR than when EQ cutoffs are set higher. For consistency it is, therefore, necessary to standardize the EQ producer cutoff method. Moreover, using urine as matrix for that purpose is most attractive as urine integrates changes over time and obtains an accurate measure of overall IFL exposure. Use of blood as matrix for EP classification is less informative than urine and may lead to erroneous results due to the fast elimination of EQ and the differences in elimination half-lives between DE and EQ, as discussed in detail previously [38].

A definition for EQ status in urine was recently proposed based on the product/precursor ratio (i.e. EQ/DE ratio) in order to account for variable IFL intake [22]. Using a ratio in urine is advantageous as concentrations can be used directly without the need to convert to excretion values [38]. In our previous study[38], we refined the product/precursor definition by including a DE threshold with a value of 2 nmol/mg creatinine (e.g. EQ/DE 2). Doing so assured sufficient substrate exposure and produced a reliable product/precursor measurement and calculation, which resulted in a more robust and dependable method of classifying EQ producer status. This refined method of classifying EQ producer status also avoided erroneous findings that may have resulted from external EQ exposure such as cow milk (products) or EQ supplements [38].

Using the EQ/DE 2 cutoff to define EQ status - notwithstanding differences in soy type, soy dose and study duration - we found 6–11% CR in our present study of premenopausal women consuming native soy foods and 14–35% CR in our previous study of postmenopausal women who consumed soy protein isolate [38].The lower prevalence of CR in our present versus our previous study may be due to the much shorter study duration (6 months versus 30 months, respectively) and/or because CR *between* diet periods were not considered in these numbers (as also shown in Table 1).

The cross-over design of our present study allowed comparisons *between* the low- and high-soy diet periods. Using the EQ/DE 2 cutoff, 16 women reached the DE threshold during the low-soy diet period (Table 1) but only 15 of these 16 women reached the DE threshold also during the high-soy period. Of these 15 women, 2 (13%) changed EQ status *between* the two

diet periods from EP → CR+; one woman during low-soy → high-soy diet transition and the other woman during high-soy → low-soy diet transition. Consequently, for these 2 women, being CR+ in one diet period meant that they not only changed EQ producer status *between* but also *within* diet periods. Moreover, one woman was identified as both a CR+ and CR− during the high-soy period. Considering the two diet periods of the present study and including crossings *between* and *within* diet periods, we identified altogether 16% CR. This is higher than the %age of CR shown in table 1 which displays crossings exclusively *within* diet periods. This indicates that IFL dose plays an uncertain role in EQ status. Consequently, other factors need to be considered as causes for EQ status changes. These findings merit consideration when evaluating the health benefits and effects of soy and/or IFL consumption.

There are a number of strengths of this study which includes the: relatively large number of participants, relatively long study duration, multiple sample collection and measurements, use of a cross-over design (which reduces the effect of confounding covariates as individuals served as their controls), use of a free-living homogenous cohort of premenopausal women, utilization of OU matrix (which helps to ensure both integration of time for IFL accumulation as well as high compliance), measurement of IFLs using state-of-the-art LC-MS/MS methodology, and use of the EQ/DE 2 as a method to determine EQ producer status which confirms the presence of CR that could be directly compared with findings from our previous study. However, our study with premenopausal women precludes generalization of our findings to other populations such as males and non-healthy females. Thus, such populations require further investigations. Nonetheless, this is the first study to examine EQ producer status in a large cross-over designed trial using native soy foods presented at physiologically relevant IFL levels of intake, thus representing a more realistic scenario than previous studies using supra-nutritional or pharmacological IFL doses. Additionally, assignment of EQ producer status was determined using OU and a newly proposed robust method of defining EQ status, two factors which facilitated direct comparison and confirmation with our recently published study using postmenopausal women (38). EQ producer definitions have varied and continue to vary tremendously across studies. Therefore, we suggest using one EQ producer cutoff definition across studies, preferably the one we present here and previously (38) as doing so allows unambigous comparisons regarding EQ production between studies. Lastly, from our findings, we conclude that a single measurement point may not be sufficient to evaluate EQ status.

In summary, we observed 19% and 24% EP in our population of 79 premenopausal women who underwent a 6-month low-soy and a 6-month high-soy diet, respectively, using a EQ to DE ratio of 0.018 with a DE threshold of 2 nmol/mg creatinine (EQ/DE 2), which we conclude to be a most robust method to define EQ producer status. Notably, using this method we found that 6–11% of our participants changed EQ producer status *within* the duration of each study period while 16% changed *between* the two study periods when consuming physiologically relevant IFL levels of intake; a finding which concurs with the our previous study in postmenopausal women (14–35% CR) and challenges the widely held belief that EQ production remains stable over time. However, the precise factors that contribute to changes in EQ status, to date, remain elusive and warrant further investigation.

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Table 1

EQ producers (EP), EQ nonproducers (NP), crossers (CR)[*] and all subjects in the high-soy and low-soy diets using different EQ cutoff methods[†]

EQ cutoff method3	EQ status	Overnight urine	
		High-Soy	Low-Soy
EQ/DE 2 (total n)		(74)	(16)
	EP	24% (18)	19% (3)
	NP	65% (48)	75% (12)
	CR	11% (8)	6% (1)
EQ/DE 5 (total n)		(70)	(11)
	EP	23% (16)	9% (1)
	NP	67% (47)	82% (9)
	CR	10% (7)	9% (1)
EQ 0.5 (total n)		(79)	(79)
	EP	22% (17)	4% (3)
	NP	65% (51)	86% (68)
	CR	14% 11)	10% (8)
EQ 1.0 (total n)		(79)	(79)
	EP	16% (13)	3% (2)
	NP	66% (52)	86% (68)
	CR	18% (14)	11% (9)

EQ, equol; DE, daidzein

[*] Changing from EP to NP or *vice versa*

[ǂ] Cutoff method for equol producer status

EQ/DE 2 = ratio EQ/DE≥0.018 and DE threshold of ≥2 nmol/mg creatinine

EQ/DE 5 = ratio EQ/DE≥0.018 and DE threshold of ≥5 nmol/mg creatinine

EQ 0.5 = EQ excretion of ≥0.5 nmol/mg creatinine

EQ 1.0 = EQ excretion of ≥1.0 nmol/mg creatinine

Absolute number of participants in parentheses

TABLE 2

Concordance between EQ cutoffs for crosser classification in the high soy and low soy groups

	EQ/DE 2	EQ/DE 5	EQ 0.5	EQ 1.0
EQ/DE 2	-	**1.00**	**0.97**	**0.96**
EQ/DE 5	1.00	-	**0.98**	**0.97**
EQ 0.5	0.95	0.94	-	**0.96**
EQ 1.0	0.93	0.94	0.97	-

Bolded numbers for high soy group, plain numbers for low soy group

Overall reliability (intraclass classification) for high soy=1.00 and low soy=0.99

OU, all 6 collections data points