Serum isoflavones and soya food intake in Japanese, Thai and American end-stage renal disease patients on chronic haemodialysis

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Abstract

Background. Soya foods, a staple in several Asian countries, have received increasing attention because of their nutritional properties and their high isoflavone content. We have shown recently abnormal pharmacokinetics of soya isoflavones following acute oral intake, in soya-naive end-stage renal disease (ESRD) patients. No information is available, however, about blood levels of soya isoflavones in ESRD patients with habitual soya intake. Additionally, no information is available about the conjugation profile of these compounds in ESRD patients.

Methods. To assess the relationship between habitual soya intake on blood isoflavone levels in ESRD patients, we recorded dietary soya food intake and analysed circulating levels of soya isoflavones in randomly selected, clinically stable haemodialysis patients from the United States (n = 20), Thailand (n = 17) and Japan (n = 20). Dietary records and three weekly blood samples were collected from each participant. Combined isoflavones and individual genistein, daidzein, glycitein and O-desmethylangolensin (DMA) were analysed in serum by liquid chromatography/mass spectrometry. Lipid phase micronutrients, including tocopherols, carotenoids and retinol were also measured to compare ethnic differences in isoflavones with those of more common lipid-soluble antioxidant micronutrients.

Results. Soya intake was higher in Japanese than in Thai patients and it was negligible in the US patients. Blood levels of genistein were very elevated and significantly higher in the Japanese patients (1128 ± 205 nM), as compared with the Thai and US patients (258 ± 64 and 168 ± 49 nM, respectively; P < 0.001). The other isoflavones followed the same trend. Daidzein was more concentrated than genistein in the dialysis patients. Robust correlation was present between weekly soya intake and blood isoflavone levels (r = 0.56, P < 0.001). Despite very high total isoflavone concentrations, the levels of unconjugated and sulphated isoflavones in the Japanese patients were comparable to those described in healthy subjects. Compared with the striking difference in isoflavones, more easily accessible dietary antioxidants, including tocopherols, carotenoids and retinol, differed only minimally or not at all in the three groups.

Conclusions. ESRD patients appear to accumulate isoflavones as a function of dietary soya intake, resulting in blood concentrations that are higher than those reported in subjects with preserved kidney function. Even in the presence of very elevated total isoflavone levels, the concentrations of the unconjugated and sulphated fractions are comparable to those of healthy subjects. A discrepancy is noted between accumulation of soya isoflavones and other more common lipid-soluble antioxidant micronutrients.

Keywords: diet; genistein; phytochemicals; soya; uraemia

Introduction

Soya food consumption is habitual and therefore high in South-East Asia, while it is only minimal in North America. This constitutes a major discrepancy in human nutrition between the two continents and it has been proposed as a contributor to the lower incidence of cardiovascular disease and cancer and to the longer life expectancy in Asia than in North America. This has been an object of vigorous study and debate for several decades [1].
Dietary soya isoflavones in ESRD may be of particular significance to end-stage renal disease (ESRD) populations. Indeed, studies in subjects with normal renal function have shown that these micronutrients affect favourably several pathologic events that are also part of the renal failure syndrome, including activation of the acute-phase response, high oxidative stress burden, accelerated atherosclerosis, decreased vascular compliance, metabolic bone disease and reproductive dysfunction [4–7]. In this context, if the biological effects of soya isoflavones in renal failure are comparable to those present, they may impact the clinical presentation of chronic renal disease.

The isoflavones are excreted primarily by the kidney and it can be expected that their pharmacokinetics are disrupted in renal failure. This was confirmed by our recent observation that ESRD patients had sustained high blood levels of the isoflavones following ingestion of one serving of soya, without a corrective effect of haemodialysis therapy [8]. This acute study, however, did not address the blood levels of isoflavones during habitual soya food intake. It is possible that chronic intake of soya foods may activate alternative metabolic and excretory pathways, resulting in blood levels of isoflavones lower than predictable from the above acute study. In addition, current knowledge about the isoflavones in renal failure is limited to total blood levels, while no information is available about the relative amounts of the unconjugated and conjugated compounds. This leaves open the possibility that the isoflavone conjugation profiles may differ between ESRD patients and healthy subjects. Such difference, if present, could have biological implications because of the relation between conjugation and activity. Finally, it was suggested recently that the antioxidant effect of soya isoflavones may depend on intimate interaction with tocopherols and endogenous antioxidants [9]. Investigation of the effect of soya isoflavones in renal failure may, therefore, benefit from concomitant analysis of endogenous and exogenous antioxidants.

To further characterize the metabolism of soya isoflavones in chronic renal disease, and as a preliminary step in the investigation of their biologic activity in renal failure, we have surveyed small samples of haemodialysis patients from countries with different habitual soya intake. Specifically, we have (i) assessed total circulating levels and conjugation patterns of soya isoflavones in ESRD patients from Japan, Thailand and the US, (ii) correlated the circulating isoflavone levels with dietary intake and (iii) compared ethnic differences in isoflavone levels with those of other antioxidant micronutrients.

**Subjects and methods**

**Study subjects and protocol**

Chronic ambulatory haemodialysis patients were recruited from the renal replacement therapy (RRT) programmes of Lexington, KY (USA), Kanagawa (Japan) and Bangkok (Thailand). Only 50- to 70-year-old subjects were included in the study, because between-centre differences in prevalence of young patients on chronic dialysis might have caused age unbalance between groups. Other inclusion criteria were residual renal function <10% and ability to accurately report food intake. Exclusion criteria were intake of antibiotics within the last 2 months, acute or chronic gastrointestinal illness that would interfere with food absorption and liver failure. The nephrologist in each centre identified all patients that met inclusion and exclusion criteria and proceeded systematically to recruit participants from the first through the last treatment shift of the week, until the target sample size of 20 subjects was reached. Six people dropped out of the US group and three from the Thai group, due to acute illness or access problem. To maintain comparable sample size between centres, six extra US subjects were recruited later during the investigation. General clinical information, including demographics, cause of chronic renal disease, anthropometrical parameters, length of enrolment on chronic dialysis, residual urine output, type of dialyser and measure of urea removal were also collected after enrolment. The Japanese and US patients were routinely dialysed three times per week, while the Thai patients were dialysed twice per week, reflecting different dialysis practices between countries. All subjects were anuric, with the exception of two US patients (250 and 400 ml/day, respectively).

Three blood samples were collected in each patient prior to the first haemodialysis treatment of the week, during consecutive weeks. Serum was stored below –20°C until the time of analysis. Sample transportation from the collection site to the analytical laboratories in Lexington, KY and Honolulu, HI, USA was done in dry ice. Dietary questionnaires were designed by the clinical dieticians from the respective RRT programmes. These focused on the most popular soya foods in each country and were, therefore, different between patient groups. The Japanese questionnaire included tofu, miso, natto, bean snack, toasted soya nuts, cooked beans, bean stalks, vinegar miso, tofu burger and cooked rice and soyabeans. The Thai questionnaire included soya milk, tofu, bean sprouts, bean curd dessert and soya powder for coffee. The US questionnaire included soya milk, vegeburger, soya protein isolate, tofu, tempah, miso, green soyabeans, textured vegetable protein, Ener-G egg replacer, soya cheese, soya flour, soya bacon, soya sausage, bean sprouts, Advera, Ensure, Osmolite and Promote (Ross Products Division, Columbus, OH, USA) and Isocal and Sustacal (Mead Johnson Nutritional, Evanville, IN, USA).
The questionnaires were designed to capture the number of soya foods servings per day, but not to provide precise information about the serving size, due to the tendency of all three cultures to mix soya products with variable amounts of other foods. The food questionnaires were compiled by the patients with dietician assistance on the dialysis days, for 21 consecutive days starting 1 week prior to the first blood collection.

The Ethics Committee guidelines of the respective countries were followed, in compliance with the International Ethics Guidelines for Biomedical Science, as published in 1982 by the Council for the International Organization of Medical Science [10].

Analytical methods

Analysis of serum isoflavones was carried out on pooled samples from the three weekly blood drawings, utilizing high pressure liquid chromatography/photodiode array/electrospray/mass spectrometry (HPLC/PDA/ESI-MS; ion-trap system model LCQ-Classic; Thermo Finnigan Corp., San Jose, CA, USA), as reported recently [11], with the following modifications. Analysis of unconjugated and sulphated isoflavones was carried out by pre-incubating the samples in the absence of cleaving enzymes or with exclusively the highly selective sulphatase type VI. The HPLC flow rate was maintained at 0.25 ml/min and, at the time of sample injection, the mobile phase consisted of 10% methanol, 10% acetonitrile and 80% water (hereafter conventionally reported as 10/10/80). After injection, the percentage of solvents was changed linearly to 30/30/40 in 10 min, then held at 30/30/40 for 7 min, then changed linearly to 45/45/10 in 1 min and, finally, to 10/10/80 in 2 min with equilibration at this ratio for 15 min before the next injection. Mass spectrometric measurements were calibrated with authentic isoflavone standards and the mass screening covered the range 100–400 atomic mass unit. The divert valve was engaged during the elution period of analytes (9.9–21.6 min of the run time). Mean quantitation limits in dialysis patient samples were 1.08 pmol for daidzein, 4.2 pmol for equol, 0.015 pmol for glycitein, 0.86 pmol for genistein and 0.081 pmol for O-desmethylangolensin (DMA). Detection limits of pure standards were lower by a factor of 5–20. The intra- and inter-assay variability were, respectively, 0% and 11% for daidzein, 13% and 15% for glycitein, 12% and 18% for genistein and 14% and 19% for DMA. Recovery of analytes through the whole procedure varied from 79% to 100% for daidzein, from 96% to 100% for glycitein, from 84% to 100% for genistein and from 77% to 100% for DMA.

Analysis of lipid phase micronutrients was carried out with a binary solvent HPLC system, equipped with a C18 column and a photodiode array detector (Beckman, Fullerton, CA, USA), using 0.5 ml serum aliquots, as reported by us previously [12].

Routine chemistry, including albumin, was analysed in serum using the Vitros DT60 II Chemistry System (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA).

Reagents

All reagents for serum isoflavones analysis were as reported recently [11]. In addition, sulphatase type VI was from Sigma Chemicals Co., St Louis, MO, USA. Standards for all trans-retinol, tocopherols and all trans-carotenes were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Tocot and β-cryptoxanthin were a gift from Hoffmann-LaRoche (Basel, Switzerland). All trans-ethyl-β-apo-8’-carotenoaetc was obtained from Fluka (Ronkonkoma, NY, USA).

Statistical analysis

The number of patients per group was chosen arbitrarily, since no information was available prior to this investigation about isoflavone blood levels in ESRD patients with different levels of habitual soya food intake. All variables are expressed as means ± SEM. The Kolgomorov–Smirnov test was used to assess normality of distribution and the Levene test to assess homogeneity of variance. One-way analysis of variance with post-hoc Bonferroni test was used for comparisons. For this test, the isoflavone, tocopherol, carotene and retinol levels were transformed to their natural log to correct for variation from normality. Pearson correlation coefficient with two-tailed test of significance was utilized to analyse associations between diet and isoflavone blood levels. SPSS 10.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Patient characteristics, intake of soya foods and general chemistry

As shown in Table 1, the three groups were comparable for age, gender and presence of diabetes mellitus as the primary cause of ESRD. There were 14 African Americans and six Caucasians in the US group. The Japanese patients had been on RRT for longer than the Thai and US patients. The North American patients had markedly larger body mass than the Asian patients, while the urea removal (single pool Kt/V) was highest in the Thai group, reflecting twice weekly treatment, and it was comparable between Japanese and North American patients. Creatinine and albumin were modestly, albeit significantly, higher in the Japanese than in the Thai and US patients, while the urea removal was higher in the US group. The Japanese patients had been on RRT for longer than the Thai and US patients. The North American patients had markedly larger body mass than the Asian patients, while the urea removal (single pool Kt/V) was highest in the Thai group, reflecting twice weekly treatment, and it was comparable between Japanese and North American patients. Creatinine and albumin were modestly, albeit significantly, higher in the Japanese than in the Thai and US patients, while the urea removal was higher in the US group. The Japanese patients had been on RRT for longer than the Thai and US patients. The North American patients had markedly larger body mass than the Asian patients, while the urea removal (single pool Kt/V) was highest in the Thai group, reflecting twice weekly treatment, and it was comparable between Japanese and North American patients. Creatinine and albumin were modestly, albeit significantly, higher in the Japanese than in the Thai and US patients, while the urea removal was higher in the US group.

Isoflavone levels

Initially, we analysed the total (conjugated + unconjugated) isoflavones in the three groups. The concentration of genistein was different among groups (Japanese 1128 ± 205 nM vs Thai 258 ± 64 nM vs US 168 ± 49 nM; F = 30.7, P < 0.001; Figure 1). Post-hoc analysis showed that genistein was higher in the Japanese group than in the Thai and US groups (P < 0.001), although Thai and US did not differ significantly from each other. The same pattern and level of significance was also observed for the other
isoflavones, with the exception of DMA that was higher in the Japanese than in the US patients ($P = 0.001$), but displayed intermediate concentrations in the Thai patients (Figure 1).

The relative concentration of unconjugated, sulphated and total isoflavones were analysed in the Japanese patients only. This group was selected because of its high total isoflavone levels (see above). Table 2 shows that the concentrations of unconjugated genistein and sulphated genistein were approximately $10^4$- and $10^2$-folds lower than total genistein. The other isoflavones followed the same pattern with the mean values of the unconjugated isoflavones ranging from 0.16 to 0.86 nM, the sulphated isoflavones from 4 to 50 nM and the total isoflavones from 112 to 4163 nM. Unconjugated daidzein and DMA were below the quantitation limit in several patients, while genistein, glycitein and combined isoflavones were undetectable in only a few. Sulphated genistein and DMA were undetectable in few cases.

**Correlation between soya food intake and isoflavone levels**

The dietary survey showed that soya consumption by the three groups was consistent with the dietary habits of the general population in the respective countries, being highest in the Japanese, intermediate in Thai and negligible in the US patients [$3.4 \pm 1.7$ vs $1.9 \pm 1.4$ vs 0 servings per week (s/week); $P < 0.001$]. The soya foods consumed most frequently by the Japanese patients were miso soup (1.2 s/week), nattou (0.65 s/week), bean stalks (0.45 s/week) and tofu (0.36 s/week), while the Thai patients consumed more frequently bean sprouts.
A robust correlation was present between soya intake and genistein levels \( (r = 0.56, P < 0.0001; \text{Figure 2}) \), as well as for daidzein \( (r = 0.658, P < 0.0001) \), glycine \( (r = 0.614, P < 0.001) \) and DMA \( (r = 0.381, P < 0.001) \). Two US patients had genistein levels 991 and 475 nM, despite no known intake of soya foods. This finding is unusual but not unprecedented in dialysis patients from the Lexington area [8].

**Antioxidant lipid-soluble micronutrient levels**

The Japanese patients had a carotenoid concentration \( \approx 70\% \) higher than the other two groups \( (F = 15.5, P < 0.001; \text{Figure 3}) \). Tocopherol and retinol were not different among groups. In contrast, the difference in total isoflavones concentration was much more striking, with \( \approx 8\)-fold higher levels in the Japanese than the US patients and 4-fold higher in Japanese than Thai \( (F = 29.9, P < 0.001) \).

**Discussion**

The last decade has been marked by increasing interest in the biological activity of soya isoflavones. Recent technical advances in the analysis of isoflavones in biological fluids led to the observation that these compounds have a relatively short half-life and considerable circadian fluctuation in human blood [8,13–15]. It was shown that the plasma concentrations of genistein and daidzein range between 20 and 3000 nM, with fasting trough levels between 20 and 200 nM in Japanese and Finnish adults regularly consuming moderate quantities of soya foods [3,15].

We recently reported that renal failure is associated with marked derangement in soya isoflavone pharmacokinetics and sustained high blood concentrations following a standardized soya meal [8]. This acute study, however, was conducted in Northern American ESRD patients who are not customarily exposed to soya. As well, it did not test the possibility that prolonged exposure of these patients to soya may lead to adaptation of intestinal absorption and/or metabolic clearance of the isoflavones.

Here we report the circulating isoflavone levels in dialysis patients selected randomly from populations of different ethnicity and with different habitual soya intake. The fasting levels of isoflavones in the Japanese patients were \( \approx 10\)-fold higher than those reported in healthy subjects [15]. This remarkable accumulation of isoflavones and higher concentration of daidzein than genistein do not appear to have major undesirable consequences. As expected, a robust correlation was detected between soya intake and isoflavone levels with both parameters being highest in the Japanese, intermediate in the Thai and lowest in the US patients. Despite this, the genistein levels varied widely within each ethnic group and overlapped between groups. Relatively high levels in US patients who had no known soya intake confirms prior observations [8] and it may be explained by the fact that many popular North American processed foods, including baked goods, pasta products, processed meats and dairy-type products, contain soya constituents that are high in isoflavones [16]. The Japanese and Thai populations also showed wide variability and inconsistencies between reported soya intake and isoflavone levels. Possible explanations include individual variability in intestinal absorption of isoflavones, with some patients experiencing very...
low blood levels despite substantial dietary intake [8], and variable isoflavone content of soya foods, depending on type and method of preparation [1]. Individual and group differences in isoflavone levels are unlikely to depend on urea removal rate and/or frequency of dialysis, since we showed before that the isoflavone blood levels are unaffected by dialysis treatment [8]. Contrary to healthy subjects [8,13], ESRD patients displayed consistently higher blood levels of daidzein than genistein. A possible explanation for this finding is that ESRD is characterized by lack of renal tubular function, which normally provides more efficient excretion of daidzein than genistein [17]. This may have significant biological implications since daidzein, in itself a functionally weak compound, is the major substrate for synthesis of equol, which is bioactive [1,17].

The biological activity of drugs and nutriceuticals is influenced by their conjugation profile. We were, therefore, interested in analysing the isoflavone conjugation profile in ESRD patients with regular soya intake. To this end, we studied the Japanese patients only, with the expectation that their high total isoflavone levels would facilitate the accurate quantitation of the relatively small fraction of unconjugated and sulphate compounds. The mean concentrations of unconjugated and sulphated isoflavones (∼0.4 and ∼30 nM, respectively) were comparable to historical data in healthy subjects [13,15,18]. Inferring by exclusion, these findings suggest that the high total isoflavone levels experienced by the Japanese patients is due primarily to the expanded glucuronide-conjugated pool.

The biological significance of this large pool of glucuronide-isoflavones in renal failure is currently unknown, although recent in vitro studies suggest that these compounds, like other popular pharmaceuticals, are active in the glucuronide conjugated form [19,20]. In addition, the large pool of conjugated isoflavones in the blood of ESRD patients who eat soya undergoes entero-hepatic recirculation. It can be expected, therefore, that these patients experience sustained unconjugated isoflavone blood levels, even in the face of intermittent dietary intake. Finally, it is possible that target tissues are able to hydrolyse the conjugated isoflavones after their uptake as suggested by the recent observation that 10–90% of total isoflavones are unconjugated in rat testes, uterus and ovaries [21].

Finally, we report that these patients’ levels of tocopherols, carotenoids and retinol were similar to those previously reported in ESRD patients [22] and the most pronounced discrepancy among the groups was a 70% higher level of carotenoids in the Japanese than in the Thai and US patients. It appears, therefore, that availability of these micronutrients would not limit isoflavone-mediated antioxidant activity [9], in this small sample of ESRD patients with soya food intake. Interestingly, group differences of these common antioxidants were much less pronounced than the 8- and 4-fold higher isoflavone concentration in Japanese than in US and Thai patients. This observation further emphasizes the uniqueness of soya isoflavones as micronutrients with strikingly different concentrations in Asian, as compared with North American, ESRD patients.

In conclusion, we report that isoflavone levels are remarkably different among haemodialysis patients from different ethnic groups, depending on soya food intake. The levels achieved by patients with routine soya consumption, such as the Japanese patients, are very high. Glucuronide-conjugated isoflavones account for these chemicals accumulating in dialysis patients, while the concentrations of sulphated and unconjugated compounds are comparable to those detected in healthy subjects. Marked differences in isoflavone concentrations are not paralleled by differences in other lipid-soluble micronutrients with antioxidant activity. The clinical relevance of dietary isoflavones accumulation in renal failure patients is currently unknown. We submit, however, that the biological effects demonstrated for these compounds in experimental models and in humans with normal renal function are likely to be also present in renal failure patients.

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Conflict of interest statement. None declared.

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