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ORIGINAL CONTRIBUTION

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Effects of a soy milk supplement on plasma cholesterol levels and oxidative DNA damage in men – a pilot study

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Abstract *Background:* Phytoestrogens are a major component of Asian diets and may be protective against certain hormone-dependent cancers (breast and prostate) and coronary heart disease. They may also have antioxidant function in scavenging potentially harmful free radicals and thus decreasing oxidative attack on DNA.
Aims of the study: A pilot study to determine the effects of a phytoestrogen supplement, in the form of soy milk, on plasma LDL and HDL cholesterol levels and DNA damage in men.
Methods: Ten healthy men participated in the study and were assigned to one of three groups consuming 1 litre of either soy milk, rice dream (vegetable protein control) or semi-skimmed cow's milk (animal protein control) each day for 4 weeks.
Results: The soy supplement caused significant increases in plasma genistein and daidzein concentrations despite considerable inter-individual variation ($P<0.001$). Supplementation with soy resulted in a decrease in oxidative damage to DNA bases detected using the comet assay compared with controls ($P<0.05$). However, there was no significant effect of the soy supplement on plasma cholesterol or triglyceride levels in comparison with control groups.
Conclusions: A 4 week soy milk supplementation in healthy volunteers does not alter serum cholesterol levels but can have a protective effect against oxidative DNA damage in lymphocytes.

Key words Phytoestrogen – cholesterol – DNA damage – comet assay – antioxidant

Introduction

Plant phytoestrogens are diphenolic compounds which have weak oestrogenic and antioestrogenic effects in mammalian tissues. Two main classes are the isoflavones, found mainly in soybeans, and lignans with precursors in almost all plant based foods. Isoflavones are a major component of Asian diets and may confer some degree of protection against many hormone-dependent diseases that commonly affect Western populations. These include breast and prostate cancer as well as heart disease. The proposed mechanisms of action of phytoestrogens involve binding to oestrogen receptors (ER) as well as induction of cancer cell differentiation (1), inhibition of protein tyrosine kinase and DNA topoisomerase activities (2, 3), suppression of angiogenesis (4) and antioxidant effects (5, 6).

Epidemiological evidence suggests that diets rich in plant based foods are protective against many cancers and heart disease (7); however, correlations with specific nutrients or antioxidants are unclear. At the same time, it is generally accepted that antioxidants can protect against cancer by removing reactive oxygen species before they have a chance to induce damage to DNA (8). In support of this, the "comet assay" (single-cell gel electrophoresis) was used to demonstrate the protective effect provided by antioxidant supplementation on H_2O_2-induced strand

breakage in DNA and endogenous oxidative damage to bases in DNA (9).

The aim of the present study was to determine whether a soy supplement, possibly as a result of oestrogenic and antioxidant properties, could induce a protective effect on DNA damage and/or lower plasma cholesterol levels in men.

Materials and methods

Materials

Provamel soy milk (Vandemoortele UK Ltd.) and Rice Dream (Clearspring Ltd., Surrey, UK) were purchased from Holland and Barrett Ltd. (Aberdeen, UK). Semi-skimmed cow's milk was purchased from Asda Superstores (UK). Histopaque 1077 and RPMI medium without phenol red and bovine serum were obtained from Sigma Chemical Company (Dorset, UK). Foetal calf serum (FCS) was purchased from Globepharm Ltd., (Surrey, UK). Dr R. Cunningham (Department of Biological Sciences, State University of New York, Albany, NY) generously supplied the *E. coli* strain overproducing the endonuclease III.

Subjects and experiment design

Ten healthy adult men were recruited from the Grampian Fire Brigade. They were all between the ages of 20-50 years, non-vegetarians and were not taking any prescribed medication. Smokers and individuals taking health supplements or with a history of heart disease were excluded. The men were assigned randomly to one of three groups and asked to consume 1 litre/day of either soy milk (n=4), rice milk (vegetable protein control, n=3) or semi-skimmed cow's milk (animal protein control, n=3) for four weeks. Fasting blood samples were obtained by venepuncture on two occasions prior to and weekly throughout the supplementation period. A washout blood sample was collected two weeks after the supplementation period.

Preparation of plasma and lymphocytes

Blood was centrifuged at 1500g for 15 min at 4 °C within 1 hour of collection; plasma was harvested and stored in aliquots at -80 °C until use. Lymphocytes were isolated after mixing the blood with RPMI medium and centrifuging over Lymphoprep (Histopaque 1077) at 700g for 30 min at 20 °C. Lymphocytes were washed twice in RPMI medium with 10% foetal calf serum (FCS) and resuspended in freezing medium (90% FCS, 10% dimethyl sulphoxide [DMSO]) to give a density of 3 x 10^6/ml. Aliquots were frozen slowly, no more than -1 °C per min, to prevent cell damage, and stored at -80 °C.

DNA damage measured by the comet assay (single cell gel electrophoresis, SCGE)

DNA damage was measured as described previously (10, 11). Lymphocyes were rapidly defrosted at 37 °C, centrifuged at 200g for 3 min at 4 °C and resuspended in RPMI medium with 10% FCS. They were then embedded in low melting point agarose and lysed to remove cellular proteins exposing the DNA as nucleoids. DNA damage was introduced *in vitro* by exposing the lymphocytes to 100 μM H_2O_2, in phosphate buffered saline (PBS) on ice for

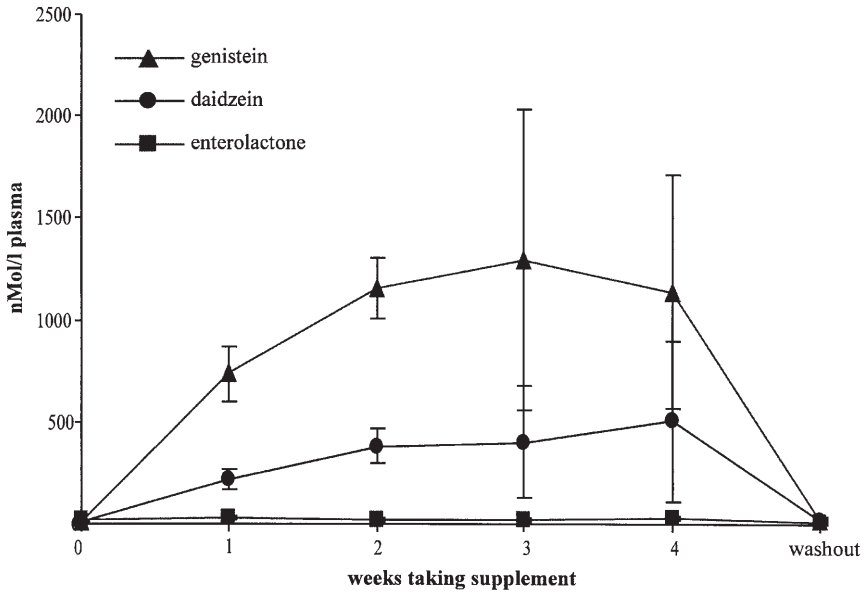

Fig. 1 Plasma phytoestrogen concentrations in volunteers taking soy milk. Data are shown as mean ± SEM (n=4).

5 min prior to embedding in agarose. Nucleoid DNA in agarose was incubated with either buffer (40 mM hepes, (0.1 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA) to detect endogenous strand breakage or with the lesion-specific enzyme, endonuclease III which induces strand breaks at the sites of oxidised pyrimidines (11). The DNA was then allowed to unwind in alkaline solution (0.3 M NaOH, 1 mM EDTA) at 4 °C before electrophoresis for 30 min at 25V. During electrophoresis, breaks in the DNA allow supercoiled loops to extend towards the anode. "Comets" were observed by fluorescence microscopy after staining with 4',6-diamidine-2-phenylindol dihydrochloride (DAPI). Comets fall into five classes, assessed by visual inspection; increasing levels of damage being indicated by an increasing proportion of DNA in the tail of the comet compared with the head. 100 comets were selected at random and classified as zero (undamaged) to four (almost all DNA in the tail); a total score (of between 0 and 400 arbitrary units) could then be calculated for the sample of 100 comets, expressed as arbitrary units (12). Samples were analysed randomly to exclude experimental variation.

Plasma phytoestrogen, cholesterol, triglyceride and antioxidant levels

Plasma genistein, daidzein, equol and enterolactone concentrations were measured, by Dr. Michael Morton, TENOVUS Cancer Research Centre, University of Wales College of Medicine, Cardiff, UK, using isotope dilution GCMS, with a DANI 3800 gas chromatograph coupled to a VG 7070 HS Mass Spectrometer (VG Analytical, Manchester, UK). Plasma total cholesterol and triglycerides were measured by the method of Allain et al., (13) using a Kone Dynamic Discrete Analyser. High density lipoprotein (HDL) cholesterol was determined in the same way as total cholesterol after sample precipitation of low density lipoproteins (LDL) with phophotungstic acid/MgC_{12} (14). Plasma concentrations of α- and γ-tocopherol were measured according to the method of Hess et al. (15). The total antioxidant capacity of plasma was estimated using the ferric reducing ability of plasma (FRAP) assay (16).

Statistical analysis

Results were analysed by ANOVA using the Genstat for Windows Statistics Package.

Results

Plasma phytoestrogen, cholesterol and antioxidant levels

Mean plasma concentrations of genistein and daidzein in subjects consuming the soy milk were increased to approximately l.3 μmol/1 and 0.4, μmol/l, respectively (Fig. 1). There was considerable inter-individual variation with one volunteer achieving maximum plasma concentrations of over 2.7 μmol/l genistein and 1.7 μmol/l daidzein. Only one of the four subjects consuming soy milk metabolised daidzein to the metabolite, equol which reached a maximum plasma concentration of 150 nmol/1

Table 1 Biochemical parameters in human plasma

	Week 0	Week 1	Week 2	Week 3	Week 4
Total cholesterol (mmol/l)					
Soya milk	4.63 ± 0.38	4.49 ± 0.12	5.24 ± 0.57	4.48 ± 0.47	4.62 ± 0.41
Rice milk	5.2 ± 0.25	4.50 ± 0.37	4.62 ± 0.13	5.0 ± 0.36	5.28 ± 0.44
Cow's milk	4.61 ± 0.58	3.85 ± 0.25	4.89 ± 0.35	4.19 ± 0.01	5.13 ± 0.36
Triglycerides (mmol/l)					
Soya milk	0.70 ± 0.11	0.71 ± 0.05	0.80 ± 0.08	0.88 ± 0.15	0.87 ± 0.22
Rice milk	1.2 ± 0.19	1.6 ± 0.13	1.13 ± 0.30	1.23 ± 0.16	1.22 ± 0.28
Cow's milk	0.96 ± 0.21	0.76 ± 0.11	0.88 ± 0.13	1.02 ± 0.54	0.78 ± 0.14
HDL:LDL ratio					
Soya milk	0.39 ± 0.04	0.35 ± 0.04	0.33 ± 0.03	0.41 ± 0.06	0.41 ± 0.04
Rice milk	0.40 ± 0.03	0.36 ± 0.0	0.34 ± 0.05	0.42 ± 0.03	0.42 ± 0.01
Cow's milk	0.38 ± 0.07	0.45 ± 0.06	0.37 ± 0.04	0.39 ± 0.04	0.34 ± 0.09
FRAP (mM ferrous produced)					
Soya milk	0.74 ± 0.1	0.81 ± 0.05	0.94 ± 0.09	0.96 ± 0.13	0.88 ± 0.11
Rice milk	0.75 ± 0.02	0.82 ± 0.03	0.95 ± 0.05	0.97 ± 0.1	0.89 ± 0.06
Cow's milk	0.71 ± 0.04	0.77 ± 0.09	0.76 ± 0.05	0.84 ± 0.03	0.84 ± 0.04

Plasma total cholesterol and triglycerides concentrations (mmol/l), the ratio of HDL:LDL cholesterol and the total antioxidant capacity of plasma using the FRAP assay (mM ferrous produced) in subjects consuming either soy milk (n=4), rice milk (n=3) or cow's milk (n=3) for 4 weeks. Data are shown as means + SEM

Table 2 DNA damage in human lymphocytes

DNA damage (arbitrary units)	Week 0	Week 2	Week 4
Endogenous strand breaks			
Soya milk	57.4 ± 8.7	59.8 ± 11.8	44.1 ± 6.9
Rice milk	30.8 ± 8.6	43.5 ± 10.2	39.2 ± 12.0
Cow's	39.8 ± 6.7	28.3 ± 7.3	29.0 ± 10.2
H_2O_2 induced strand breaks			
Soya milk	83 ± 5.8	96.5 ± 16.8	97.8 ± 11.3
Rice milk	108.3 ± 6.5	70.2 ± 15.3	102.2 ± 4.1
Cow's milk	99.7 ± 29.9	85 ± 6.5	119 ± 21.8

Endogenous DNA strand breakage and H_2O_2-induced DNA strand breakage measured by the comet assay (single cell gel electrophoresis, SCGE) in subjects consuming either soy milk (n=4), rice milk (n=3) or cow's milk (n=3) daily for 4 weeks. DNA damage is expressed as arbitrary units. Data are shown as means + SEM

(not shown). Plasma concentrations of genistein and daidzein in the rice dream and cow's milk groups remained at baseline levels throughout the study period, the maximum concentrations being 70 nmol/1 and 50 nmol/l, respectively (results not shown). There was no significant effect of the soy supplement on plasma total cholesterol, triglycerides or HDL:LDL cholesterol ratios over the study period as compared with controls (Table 1). Plasma α- and γ-tocopherol concentrations remained constant throughout the study period (data not shown) and the total antioxidant capacity of plasmas were also unchanged by the soy milk, rice dream or cow's milk supplements (Table 1).

DNA damage measured by SCGE

The soy supplement had no significant protective effect against endogenous DNA strand breakage or H_2O_2-induced DNA damage (Table 2). However, there was a progressive decrease in oxidative damage to DNA pyrimidines over the four week period, as detected with endonuclease III (Fig. 2). DNA damage levels were unchanged in the subjects fed rice dream or cow's milk (Fig. 2 and Table 2).

Discussion

The comet assay is a rapid and sensitive method for analysing DNA damage at the level of individual cells and the introduction of lesion-specific enzymes, which induce strand breakage at sites of oxidised bases, allows the measurement of endogenous oxidative DNA base damage (10, 11). In the present study, the soy milk supplement had no protective effect against H_2O_2-induced strand breakage but progressively decreased the levels of oxidised pyrimidine base damage over the four week period of supplementation. This was associated with large increases in plasma genistein and daidzein concentrations which have weak antioxidant activity *in vitro* (6, 17). The

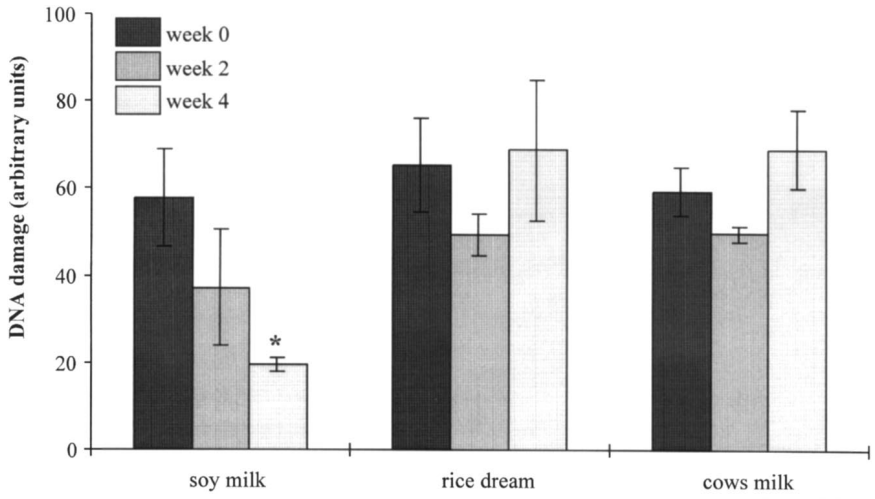

Fig. 2 Effect of soy milk, rice dream and semi-skimmed cow's milk on oxidative base damage to DNA as determined using the comet assay. Data are shown as mean ± SEM (soy milk n=4, rice dream n=3, cow's milk n=3.
* indicates $P < 0.05$

total antioxidant capacity of the plasma was, however, not significantly increased using the FRAP assay possibly owing to the conjugation of these isoflavones in plasma. The circulating forms of isoflavones are predominantly glucuronidated on the 7 position of the A ring, thus removing an hydroxyl group. This is unlikely to enhance, and may impair the antioxidant activity as is seen with the conjugated form of quercetin, rutin (18).

Oxidative DNA base damage in lymphocytes was also decreased during a two week human intervention with carrot juice, however, as in our study, the supplement did not protect against H_2O_2-induced strand breakage (19). These studies highlight the sensitivity of the endonuclease III incubation step in the comet assay allowing the detection of oxidative damage to DNA in short-term supplementation trials.

There was considerable inter-individual variation in the plasma concentrations of genistein and daidzein with one individual reaching maximum concentrations of over 2,7 μmol/1 and 1,7 μmol/1 of genistein and daidzein, respectively. This variation has also been reported in babies fed soy infant formula and is thought to be due to difference in absorption possibly related to gut bacteria (20).

A number of studies have correlated an increased soy:animal protein intake with reduced risk of heart disease (21). This may be due to the oestrogenic effect of isoflavones, lowering plasma concentrations of total and low density lipoprotein (LDL) cholesterol. In agreement with this, the cholesterol-lowering effect of soy in rhesus monkeys was significantly decreased when the isoflavones were removed by alcohol extraction (22). However, in our experiment, the levels of total cholesterol and triglycerides and the ratio of HDL:LDL cholesterol were unchanged despite the several hundred-fold increases in plasma genistein and daidzein levels. The lack of effect may be attributed to a number of reasons: the supplementation period may have been too short, but a similar length of trial caused considerable decreases in plasma total cholesterol levels in normocholesterolaemic premenopausal women fed on a soy based diet (23). The ratio of soy:animal protein was low as the subjects were taking the soy milk along with their normal diets. A similar lack of effect on serum cholesterol levels was observed when subjects were supplemented with tablets of isoflavones along with their normal diets (24). Also, the cholesterol-lowering effect of soy is more significant in hypercholesterolaemic than normo-cholesterolaemic men (25).

In conclusion, a phytoestrogen supplement alone does not lower plasma cholesterol or triglyceride levels in healthy men. However, our results indicate that a soy supplement can have a protective effect against endogenous oxidative DNA damage in lymphocytes possibly due to antioxidant or oestrogenic actions of phytoestrogens.

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