Oestrogenic activities of food supplements and beers as assessed by a yeast bio-reporter assay

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Abstract

Mounting evidence of the effects of endocrine-disrupting chemicals (EDCs) in humans has led to assaying a vast array of food items (processed or packaged) as possible sources of human exposure to oestrogens. In this study, we investigated the current situation in this respect of different food supplements and beer brands. Eleven different food supplements and twenty-four beer brands were obtained from Helsinki, Finland. Sample preparation was carried out by established methods while oestrogenic activities were assessed by a yeast bioluminiscent assay, using two recombinant yeast strains (*Saccharomyces cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMA64/luc). All the food supplements as well as 81% of the beer samples tested were found to be oestrogenic, with oestradiol equivalent concentrations of food supplements and beer brands ranging from 7.5 to 11.5 µg/ml and from below detection limits to 43.6 ng/ml, respectively. The oestrogenic activities detected in beer samples were not dependent on the beer's alcoholic content, country of production, or the size of the production brewery. The results of our study imply that both food supplements and beers can be a significant source of human exposure to oestrogens. Therefore, further studies and regular surveillance are warranted.

Keywords: Endocrine-disrupting chemicals; oestrogenic activity; bioassay; beer; phytoestrogens; isoflavones; food supplements; isoxanthohumol

Introduction

Food supplements are concentrated sources of nutrients or other substances with nutritional or physiological effect, whose purpose is to supplement the normal diet. They are originally used to correct nutritional deficiencies and to maintain adequate intakes of certain nutrients. In the European Union (EU), food supplements are regulated as foods, and the legislation focuses on vitamins and minerals used as ingredients in food supplements, thus making the toxicological safety of food supplements an inconclusive and somewhat controversial issue. However, food supplements have been reported to be of potential public health concern, due to, for example, the presence of and/or contamination by endocrine-disrupting chemicals (EDCs), which may be natural (such as phytoestrogens) or synthetic. Recent evidence has shown that food supplements can be contaminated with low quantities of steroids or stimulants (such as ephedrine) not specified on the label. A broad-based investigation of the international nutritional supplement market revealed that between 3 to 25% of dietary food supplements were inadvertently or deliberately contaminated with steroid hormones [1]. In several studies, the presence of pro-hormones (supplements designed to enhance muscle size and strength rapidly) in non-hormonal nutritional supplements has been examined. Results from an international study, involving the European Union and the United States, showed that 14.8% (94 out of 634 samples) of the investigated non-hormonal supplements contained one or more anabolic steroids not declared on the label [2].

Another source of compounds with hormonal activity in nutritional supplements is plant-derived phytoestrogens. At present, controversy remains as to the health impacts of phytoestrogens. While epidemiological studies have shown that phytoestrogens (such as genistein) may protect against breast cancer and cardiovascular diseases [3] (although genetic composition

seems to play a notable role for breast cancer [4]), experimental studies have suggested they could enhance the proliferation or metastasis of some types of cancer [5].

On the other hand, beer is consumed in increasing volumes globally, and has recently become more controversial as a source of dietary exposure to oestrogens. The oestrogenic activity of beer is due to the presence/use of hops (*Humulus lupulus* L.) both as a preservative and as a flavouring agent in it [6]. The oestrogenic activity of hops was first attributed to xanthohumol without convincing evidence [7]. Today, it is known that in addition to xanthohumol, hops also contain isoxanthohumol (IX), 6-prenylnaringerin (6-PN) and 8-prenylnaringerin (8-PN) as some of its major constituents. The most potent phytoestrogen of them is 8-PN, with its oestrogenic activity being equal to, or greater than that of other established plant oestrogens [6].

Although xanthohumol is the predominant prenylchalcone (prenylated flavonoids found in hops and beer) present in beer, most of it is transformed into IX by thermal isomerization during wort boiling [8]. IX is further converted to 8-PN in the gastrointestinal tract by the intestinal microbe, *Eubacterium limosum*. Thereby, the human exposure to 8-PN may grow more than 10-fold [9]. These observations have led to intense research aiming at deciphering the bioactivities of the ultimate metabolite, 8-PN. *In vivo*, 8-PN showed estrogenic activity [10], inhibited angiogenesis [11] and metastasis [12], exhibited antiandrogenic activity [13], as well as prevented bone loss in rats [14].

A number of yeast bioluminescent assays have been developed in recent times to detect the presence of endocrine disrupting chemicals in different matrices. However, the yeast bioluminescent assay employed in this study uses two recombinant yeast strains (*Saccharomyces cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMA64/luc). The yeast strain *S. cerevisiae* BMA64/luc helps to detect the cytotoxic effect of the test compound because it expresses

luciferase constitutively, while the human oestrogen receptor–agonist complex is activated in *S. cerevisiae* BMAEREluc/ERα upon binding of an oestrogenic compound, thus resulting in light emission. These assays are faster than corresponding colorimetric assays, taking only a few hours to be performed.

To shed more light on these issues, the current study sought to determine the oestrogenic activities of a representative selection of food supplements and beer brands marketed in Finland, with a view to furthering our understanding of their potential as a source of human exposure to oestrogens.

Materials and methods

Sample collection and preparation

Food supplements

Fifteen different food supplements were randomly purchased from a local shop (Prisma, Viikki) in Helsinki, Finland. All food supplements investigated were packaged as tablets. Samples were prepared for oestrogenic assay by directly dissolving the tablets and capsule in 10 % ethanol. To be sure that the 10 % ethanol used as vehicle was not cytotoxic to the yeast, it was used as a negative control substance in the yeast bioluminescent assay. All food supplement products were readily and completely dissolved in ethanol.

Beer samples:

A total of twenty-one different brands of beer and 3 controls (unhoped beer samples) were investigated. The selection of beer samples was based on the following grounds: beers produced in the two continents with the highest beer consumption (Europe and the USA), beer production style (e.g. larger, pilsner, ale, etc.), alcoholic content, brewery size, anticipated oestrogenic contents, and hopping style. Our idea was to get an as broad and representative sample of the beer brands currently on the market in Finland as possible regarding all these properties.

Possible oestrogenic compounds in beer samples were extracted by the solid-phase extraction method as previously described [15], with the slight modification that 100 ml of the samples were first mixed with 25 ml of ethanol (extraction solvent) to increase the extraction efficiency of the test system. All beer products mixed readily and completely with ethanol.

In addition to the different beer brands, Menohop (a product developed using hops for the treatment of menopause-related problems in women) was also analysed for comparison.

Yeast bioluminescent assay

The yeast bioluminescent assay was performed as previously described [16]. Briefly, yeast strains (*S. cerevisiae* BMAEREluc/ERα and *S. cerevisiae* BMA64/luc) were grown overnight until the optical density at a wave length of 600 (OD_{600}) reached 0.4 cfu/ml. Necessary dilutions were made, and the yeast strains were further grown at 30°C in a shaker for 2.5 hours until OD600 reached 0.6. Different concentrations of the test compounds, the yeast strains, as well as 10 x luciferin solutions were placed in a micro-well-plate and then incubated at 30°C for 2.5 hours before the luciferase activity was measured. Oestradiol, genistein and IX served as positive controls, while progesterone and testosterone served as negative controls. The data are given as oestradiol (EEQ) or genistein equivalents.

Data analysis

The fold induction, fold induction corrected (FIC), and limit of detection (LOD) were calculated as described previously [17]. Sigmoidal dose–response curves for increasing concentrations of oestradiol, genistein and isoxanthohumol were obtained using the software Prisma 5.0 (GraphPad software Inc. San Diego, CA). The oestradiol, genistein and IX equivalents of food samples showing oestrogenic activity were calculated from probit transformation of the curves.

Results and Discussion

Recent evidence of a decline in male sperm count coupled with increased incidence of various types of cancer amongst young men and women [18], as well as of neurobehavioural diseases observed in the populations of different countries [19] have led researchers to search for possible causes of such inauspicious human conditions. These phenomena have epidemiologically been associated with exposure to EDCs, particularly during the intrauterine phase or during critical periods of postnatal development [20]. More recently, some food items (including those of plant origin) have begun to gain increasing attention as possible sources of human exposure to xeno- and phytoestrogens. Thus, our study was aimed at evaluating the current situation of food supplements and beers with respect to their oestrogenic activities.

The oestrogenic activities of food supplements measured are presented in Table 1. All food supplements (15) investigated in this study were positive in the yeast bioluminescent assay, with their oestradiol and genistein equivalent concentrations ranging from 7.9 to 11.5 µg/ml and 74 to 240 µg/mg, respectively. These narrow ranges obtained with all samples suggest the presence of a common or similar substance/compound in slightly varying concentrations.

Table 2 shows the oestradiol and IX equivalent concentrations of various brands of commercial beer produced in different countries. Seventeen (81%) out of the 21 beer samples examined were positive in the test system. The oestradiol and IX equivalent concentrations of the tested beer samples were not dependent on their alcohol content, brewery size or country of production (Table 2). Beer sample 9 (BS9), with alcoholic content of 4.6% and produced in the Netherlands, had the highest oestrogenic activity (43.6 ng/ml EEQ), followed by BS20 (10.5% ethanol, produced in Denmark), whose oestrogenic activity was 31.8 ng/ml EEQ. Four (BS3, BS12, BS15 and BS19) out of the 21 tested beer samples were negative in the yeast

bioluminescence assay. Although the reason for the low oestrogenicity in these cases is somewhat unclear, the outcome was not associated with their alcoholic content or their country of origin [Finland (3) and Germany (1)]. Interestingly, all unhoped beer samples (3) used as control in this study failed to yield any oestrogenic response, further substantiating the notion that the hop plant is the major source of the oestrogenicity in beer.

IX was previously reported to be non-oestrogenic in a similar yeast bioreporter assay [21]. Although Milligan *et al.* [21] did not report the concentration used in that study, we, however, found IX to be oestrogenic at concentrations of 1 mg/ml or greater. Below this concentration, IX was found to lose its oestrogenic activity. Using phenobarbital-induced rat liver microsomal enzyme mix (S9), we therefore investigated the possibility that IX might gain oestrogenicity at low concentrations following metabolic activation; however, we failed to find any (data not shown). The results of this study thus suggest that IX has relatively weak oestrogenic potency and is non-oestrogenic at low concentrations with or without metabolic activation, but it should be kept in mind that the rat liver S9 mix may not contain the enzyme activity critical for IX conversion to 8-PN; *i.e.* the reaction that takes place in human distal colon [22].

The oestrogenic activities of beer samples were not unexpected but warrant attention. The oestrogenicity of beer is associated with the presence of IX and 8-PN [6], of which 8-PN is an exceptionally potent phytoestrogen, being only about 100 times weaker activator of oestrogen receptor-α in a yeast reporter-gene assay than oestradiol [23]. Exposure to 8-PN has previously been linked to menstrual disturbances in female hop workers [7], and it also reduces hot flushes in post-menopausal women. Apart from drugs produced from hops (such as the Menohop used here as a positive control), beer consumption is today the only appreciable source of human exposure to IX and 8-PN, since hop-picking is now performed mechanically [6]. Although exposure to

especially 8-PN has aroused concern due to the high oestrogenic potency of this compound, Milligan *et al*. [6] have argued that the concentrations of IX and 8-PN in beer are not sufficient to cause any detrimental health effects. However, Possemiers et al. [22] have contended that intestinal conversion of IX upon moderate beer consumption can lead to 8-PN exposure values that might fall within the range of human biological activity. It is noteworthy that the highest oestrogenic activities of beer brands measured in this study (per l beer) fall only slightly lower than that of Menohop (per g), a widely-used hop-based product for the treatment of menopause-related problems in women.

Earlier, Plotan et al. [24] reported that 71–89% of sport supplements exhibited oestrogenic activity in an *in vitro* reporter-gene assay. Some other previous studies have examined isoflavone contents and oestrogenic activities in food supplements intended for alleviating menopausal complaints in women. One of such products was also included in our study (Menohop in Table 1). Reiter et al. [25] reported that only 26.3% (five out of 19) of the high-dose isoflavone preparations they analysed contained the isoflavone content or more specified in the package label. Using a similar oestrogen receptor-α and reporter gene-based yeast assay to that of ours, they recorded oestrogenic activities of up to 200 nmol/g (54 µg/g) EEQ, which is practically equal to the level measured by us in the Menohop preparation (Table 1). On the other hand, Andres *et al.* [26] found that the isoflavone supplements they examined contained approximately the amounts of isoflavones claimed by the manufacturers in their product information, and the highest oestrogen receptor-α–mediated EEQ value (obtained by a human embryonic kidney cell-based reporter-gene assay) was 11.6 µg/capsule. Judkins *et al*. [1] and Geyer *et al*. [2] have also reported hormonal steroid contaminants in various types of food and sport supplements. Interestingly, the contaminants were not listed in the packaging material of the supplements, further calling for

serious concern. Controversies exist as to the health impacts of isoflavones. The purported health benefits are quite variable in different studies. While isoflavones are reported to lower total cholesterol [27], reduce the incidence of breast cancer [28], and diminish the risk of prostate cancer [29], they are also known to increase the incidences of goitre and thyroid enlargement in certain nutrient-deficient individuals [30].

In conclusion, the findings imply that food supplements and commercially produced beer can be significant sources of human exposure to oestrogens. The oestrogenicity of beer was not dependent on the alcoholic content, country of production, or the brewery size. Because of the controversy concerning the effects of phytoestrogens on humans, further studies are warranted.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1: Oestrogenic activities in different food supplements expressed as oestradiol (EEQ) and genistein (GEQ) equivalent concentrations

Supplement (code)	EEQ (μg/ml)	GEQ (μg/g)
FS1	7.9	76
FS2	11.5	240
FS3	9.3	110
FS4	10.7	190
FS5	7.5	74
FS6	10.2	170
FS7	8.4	80
FS8	7.9	76
FS9	9.7	140
FS10	10.9	200
FS11	10.1	170
FS12	8.1	79
FS13	10.7	190
FS14	10.1	170
FS15	9.8	140
Menohop	52.4 (μg/g)	NA

Key: EEQ: Oestradiol equivalent concentration; GEQ: Genistein equivalent concentration; NA: Not applicable.

Table 2: Oestrogenic activities in different beer samples expressed as oestradiol (EEQ) and IX (IXEQ) equivalent concentrations

Beer (code)	Country of production	% OH	EEQ (ng/ml)	IXEQ (mg/ml)
BS1	Finland	4.5	8.4	1.8
BS2	Finland	4.6	2.3	0.1
BS3	Finland	4.5	Nil	NA
BS4	USA		5.9	1.0
BS5	Germany		7.0	1.3
BS6	Czech Republic	4.4	21.4	4.1
BS7	Britain	3.5	29.6	4.9
BS8	Belgium	0.0	2.8	0.2
BS9	The Netherlands	4.6	43.6	7.4
BS10	Scotland	5.0	19.8	3.6
BS11	Mexico	4.5	14.8	2.7
BS12	Germany	4.7	Nil	NA
BS13	Ireland	4.2	2.8	0.2
BS14	Finland	5.2	17.4	3.1
BS15	Finland	7.2	Nil	NA
BS16	Denmark	7.2	10.8	1.1
BS17	Belgium	9.0	18.4	3.3
B18	Sweden	5.3	18.2	3.3
BS19	Finland	7.0	Nil	NA
BS20	Denmark	10.5	31.8	6.4
BS21	USA	7.3	14.5	2.0
Control beer 1*		Nil	Nil	Nil
Control beer 2		Nil	Nil	Nil
Control beer 3		Nil	Nil	Nil

Key: OH: Alcohol; EEQ: Oestradiol equivalent concentration; IXEQ: IX equivalent concentration; NA; Not applicable; * The control beers were made without hops

Supplementary Table 1: Summary of the characteristics of the beer brands analyzed

BEER CODE	NAME OF BEER	COUNTRY OF PRODUCTION	PERCENTAGE ALCOHOL	TYPE OF BEER
BS1	Laitilan kukko	Finland	4.5	Pils
BS2	Karhu II	Finland	3.5	Lager
BS3	Koff III	Finland	4.5	Lager
BS4	Budweiser III	USA	5.0	Lager
BS5	Warsteiner P.	Germany	0.0	Lager
BS6	Pilsner Urquell	Czech Republic	4.4	Lager
BS7	Chiswick	Britain	3.5	English Bitter
BS8	Rainbow al.	Belgium	0.0	Lager
BS9	Heineken	The Netherlands	4.6	Lager
BS10	5.A.M. Saint	Scotland	5.0	Ale
BS11	SOL lager	Mexico	4.5	Lager
BS12	Stortebeker	Germany	4.7	Pils
BS13	Guinness-d	Ireland	4.2	Stout
BS14	Prykmestar	Finland	5.2	Pils
BS15	Koff porter	Finland	7.2	Porter
BS16	Carlsberg E	Denmark	7.2	Strong Lager
BS17	Houblon Chouffe	Belgium	9.0	Ale
B18	St. Eriks	Sweden	5.3	Lager
BS19	Beer Hunter’s	Finland	7.0	Ale
BS20	Hr Fredriksen	Denmark	10.5	Stout
BS21	West coast	USA	7.3	Ale