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The *E-screen* test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues

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ABSTRACT

Endocrine-disrupting chemicals (EDCs) may lead to adverse systemic effects by interfering with normal hormone homeostasis, and diet is considered to be among the main routes of EDC exposure. The present study investigated the total estrogenic activity of fruits and vegetables by calculating the 17-β-estradiol equivalent quantity (EEQ) using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation assay (*E-screen* test) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay. Of the 24 analyzed fruits and vegetables, 14 contained from 1 to 4 pesticide residues in concentrations ranging from 0.02 to 1.19 ppm, whereas the other 10 did not contain any pesticide residues. The EEQ values for all positive samples ranged from 0.010 to 0.616 μg/100 g for the above *in vitro* tests. Our study demonstrates that estrogenic activity was present in fruits and vegetables and that the concentration of allowable pesticide residues and EEQ values were positively correlated; however, no correlation was found by comparing the estrogenic activity and the intrinsic content of phytoestrogens obtained from the available literature. A theoretical adult dietary intake of 0.7–0.9 ng EEQ/L/day from fruits and vegetables was calculated.

1. Introduction

The study of the chemical disruption of the endocrine system has been an active area of research during the last decade and has captured the attention of governments, policy makers, and media (De Rosa et al., 1998; Choi et al., 2005). An endocrine-disrupting compound (EDC) is defined as "exogenous substance or mixture that alters the function of the endocrine system and generate noxious effects on the health of a safe body, its descendants, or its sub-population" (WHO, 2002); at the European Union level, EDCs are included in the list of so-called emerging contaminants (EC, 2001). In terms of adverse health effects, there is concern that substances with endocrine-disrupting properties may be causally involved in a number of diseases or conditions, such as hormone-dependent cancer, reproductive disorders, a decline in fertility, or obesity (Diamanti-Kandarakis et al., 2009; Shaw, 2009; Hotchkiss et al., 2008). However, causality between EDCs and effects on human health remains controversially debated in both science and the public (Wagner and Oehlmann, 2009).

EDCs are ubiquitous in the environment because of their very frequent use in residential, industrial, and agricultural applications; in particular, the origin and fate of these contaminants can lead to their transmission in the food chain (Schwartz, 2001). It is widely accepted that food and diet are among the most important exposure routes for EDCs. Therefore, a normal human diet results in exposure to a complex mixture of xenoestrogens that enter systemic circulation in the body. There are many types of EDCs in food, ranging from natural compounds (e.g., hormones, phytoestrogens, and mycotoxins) to synthetic compounds (e.g., pesticides, pharmaceuticals, and industrial or process chemicals). The natural contribution of phytoestrogens

Abbreviations: COU, coumestrol; DMEM, Dulbecco's Modified Eagle Medium; E2, 17b-estradiol; EC50, effective concentration 50; EDCs, endocrine disruptor compounds; EEF, estradiol equivalency factor; EEQ, estradiol equivalency quantity; EFSA, European food safety authority; EU, European; FCS, fetal calf serum; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography ISO, isoflavones; LIG, lignans; MRLs, maximum residue levels; PE, proliferative effect; RPE, relative proliferative effect; rS, spearman rank correlation; SPE, solid phase extraction; Tam, tamoxifen; TRANS, increased rate of luciferase gene expression; WHO, World Health Organization.

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includes some isoflavonoids, flavonoids, stilbenes, and lignans; however, their role in endocrine disruption remains highly controversial (Patisaul and Jefferson, 2010). Indeed, the lack of consistency in epidemiological and experimental results places these chemicals in EDC category III, a category that includes compounds for which *in vitro* data exist but for which data from experimental animals concerning adverse effects on endocrine homeostasis are weak or lacking (Foster and Agzarian, 2008). Among synthetic endocrine compounds some pesticides regularly used in agriculture have shown weak estrogenic responses *in vitro*, for example, tolclofos-methyl (Andersen et al., 2002) and triadimenol (Vinggaard et al., 1999). Imazalil showed weak anti-estrogenic activity in an *in vitro* gene-reporter assay (Kojima et al., 2005) and a negligible proliferation response in an MCF7 cell proliferation assay (Soto et al., 1994). Endosulfan also showed an estrogenic response in several *in vitro* tests (Soto et al., 1994; Andersen et al., 2002). New pesticide regulations were recently introduced by the European Parliament and contain, for the first time, specific reference to endocrine-disrupting properties (on 21 October 2009, regulation (EC) No 1107/2009 replaced Council Directive 91/414/EEC). Although it is clear that substances with endocrine-disrupting properties should be avoided, there is no clear consensus of how to identify and evaluate endocrine-disrupting properties, and no guidance is yet provided in the new European Regulation (Flynn, 2011).

The assessment of EDC food contaminants is a continual challenge and has traditionally been performed mainly through analytical chemistry with respect to the detection of a few, specific chemicals (De Brabander et al., 2009). However, despite rapid improvements in analytical chemistry, merely evaluating single chemicals ignores the potential mixture effects between different compounds and the effects caused by as-yet-unidentified compounds (Connolly et al., 2011; US EPA, 2013). Another concern is that, although some of these EDCs have been deemed to be relatively safe at low individual levels of consumption, they may combine with other low-level EDCs to create low-level cocktail or mixture effects (Kjaerstad et al., 2010; Kortenkamp, 2007; Payne et al., 2000). Considering these points, the most appropriate way of detecting and studying the effects of EDCs and their mixtures may be through the use of bioassay systems that utilize the natural ligands and pathways. This can be achieved through, preferably, *in vitro* bioassays, which, although they cannot assess behavioral effects, have the benefit of closely related natural systems without the use of animal testing. Such bioassays can detect compounds based on their effects, enable the detection of the effects caused by currently unidentified compounds, and integrate the effect of complex chemical mixtures (Wagner and Oehlmann, 2011). Moreover, quantification of estrogenic activity as the 17-β-estradiol equivalent quantity (EEQ) facilitates the estimation of the total dietary intake of estrogenicity. Although such assessments provide valuable information on the human exposure to estrogen-like compounds, they are rare in the literature (Safe, 2000; Shaw and McCully, 2002; Behr et al., 2011; Schilirò et al., 2011).

The aim of the present study is to evaluate the estrogenic properties of fruits and vegetables by calculating the EEQ using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation test or *E-screen* test (Soto et al., 1995) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay (Balaguer et al., 1999). A further aim of this study is to compare the estrogenic activity of food samples to the pesticide residue content and the reported intrinsic content of phytoestrogens, as found in the literature. The results of the two *in vitro* tests were compared to each other. Finally, we combined the results to assess the theoretical blood EEQ levels for adults.

2. Materials and methods

2.1. Fruit and vegetable samples

We analyzed 24 fruits and 7 vegetables (Table 1) supplied by the Regional Environmental Protection Agency (Piedmont A.R.P.A., *Agenzia Regionale per la Protezione dell'Ambiente del Piemonte*) between April 2010 and March 2011. This agency collects commercial plant products destined for human consumption for analyses as part of the regular national monitoring program for pesticide residues in food.

2.2. Detection of pesticide residues

All the fruit and vegetable samples (500 g each) were first homogenized using an Ultra-Turrax according to the provision of Italian Ministerial Decree 27/08/2004 and Regulation No. 396/2005 of the European Parliament. All the procedures for the analysis of pesticide residues in food samples were conducted according to the quality control procedures of the European Commission for pesticide residue analysis in food and feed (EC, 2009) and using the same methods as Schilirò et al. (2011). The determination of N-methylcarbamates was performed using reverse-phase high-pressure liquid chromatography (HPLC) with post-column reaction and fluorescence detection (SCL-10AVP, Shimadzu Corp, Japan) (EC, 2009; Branca and Longo, 2002). Organophosphorus, organochlorine, pyrethroids, triazine herbicides, and other classes of pesticides were determined by Gas Chromatography with Mass Spectrometry (GC–MS) equipped with selective detectors, a quadrupole ion trap, and ITQ Series GC-Ion Trap MS (Thermo Scientific, Ohio, USA) (EC, 2009; Branca and Sacchero, 1997).

2.3. Phytoestrogen database

This study used the database compiled by Thompson (Thompson et al., 2006), which is one of the most frequently updated food phytoestrogen databases in the literature; the database includes most of the foods used in our study (67%) and describes 3 classes of phytoestrogens (isoflavones, lignans, and coumestans). We also used the database compiled by Kuhnle (Kuhnle et al., 2009), which lists the content of isoflavones, lignans, and coumestans for 240 fruits and vegetables.

2.4. Preparation of samples for in vitro tests

After homogenization, the raw fruits and vegetables were subjected to nonspecific extraction to obtain whole-food extracts. The extraction of food samples was performed according to the method proposed by Charles and colleagues (Charles et al., 2002) and modified for this application (Schilirò et al., 2011): 25 g of homogenized sample was added to 25 mL of incomplete cell culture medium, (phenol-red-free Dulbecco's modified Eagle's medium, DMEM) in brown glass beakers protected from direct light. The sample was incubated overnight while being agitated at 4 °C. The sample was then centrifuged at 9000*g* for 15 min, and the supernatant was collected in 50-mL brown glass tubes to obtain a 1 g/mL food extract. Whole-food preparations were processed ahead of time, frozen, and stored at −20 °C. Prior to testing by the *E-screen* assay and the MELN gene-reporter assay, the samples were first thawed at 4 °C overnight, kept at room temperature, filter-sterilized using a 0.22-μm filter, and then diluted in steroid-free experimental DMEM at five dilutions (from 0.001 to 10 mg/mL).

2.5. Cell lines, culture conditions, and chemicals

Estrogen-sensitive human MCF-7 BUS breast cancer cells were kindly provided by Drs. A.M. Soto and Dr. C. Sonnenschein (Tufts University School of Medicine, Boston, Massachusetts, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15 mg/L phenol red, 10% fetal calf serum (FCS), 2% L-glutamine 200 mM, 2% HEPES buffer 1 M, 1% sodium pyruvate 100 mM and 1% penicillin/streptomycin 10 mg/mL at 37 °C in an atmosphere of 5% carbon dioxide and 95% air under saturating humidity.

MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier - France), are MCF-7 cells stably transfected with an estrogen-responsive gene (ERE-βGlob-Luc-SVNeo) carried by integrated plasmids. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thereby inducing the transcription of the luciferase reporter gene (Berckmans et al., 2007). Therefore, the luciferase activity measured is proportional to the concentration of estrogenic compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red supplemented with 5% fetal calf serum (FCS), 2% L-glutamine 200 mM, 1% penicillin/streptomycin, and 1 mg/ml G418 sulfate. The cell line was maintained in an incubator at 37 °C, a relative humidity of 95%, and a CO_2 concentration of 5%. The cells were subcultured once a week, with medium refreshment between subculturing steps. For the experiments, we used cells from passage number 4 to passage number 15. The cells were regularly examined for mycoplasm infection to guarantee experimental work with mycoplasm-free cells and to comply with good cell culture practice (GCCP) guidelines.