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Application of the yeast-based reporter gene bioassay for the assessment of estrogenic activity in cow's milk from Poland

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ABSTRACT

Milk contain compounds acting through the estrogen receptor signaling. The still open question whether such estrogens pose a risk for human health, encouraged us to measure the overall estrogenic activity of cow's milk in the *in vitro* yeast reporter bioassay.

First, we assessed the ability of the bioassay to detect estrogens frequently detected in milk. The relative potencies of 16 compounds descended in the order: 17β-estradiol (17β-E2), 17α-ethinylestradiol, diethylstilbestrol, dienestrol, 17α-E2, estrone, zearalenone, estriol, equol, genistein, 17β-E2 glucuronide, bisphenol A, apigenin, daidzein. Flavone, 4-*n*-nonylphenol and 4-*t*-octylphenol shown no activity in the bioassay.The estrogenic activities of milk samples without hydrolysis were below the detection limit, whereas in 50% of the deconjugated samples they varied between 0.29 and 0.49 ng EEQ mL^{-1}. We also compared the estrogenic activity in raw cow's milk collected from rural and industrial locations in Poland. In our pilot study we did not observe statistically significant difference in estrogenic activities in milk collected from the two locations. We found that the daily intake of estrogens with milk may be higher than estrogen levels in human serum. Further studies are warranted to evaluate the significance of milk and dairy as a source of estrogens for humans.

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1. Introduction

The presence of bioactive chemicals in the environment has become the focus of international attention in recent years as they may pose potential threat to human health. Hormonally active chemicals (HACs) are of particular concern since they are a large group of natural and anthropogenic compounds capable to adversely affect the endocrine system of humans and animals (Diamanti-Kandarakis et al., 2009) and the exposure to HACs has been associated with reproductive disorders and hormone-related cancers (Qin et al., 2004; Ganmaa and Sato, 2005; Diamanti-Kandarakis et al., 2009). Considering the fact that food is an important route of human exposure to HACs (Courant et al., 2007; Behr et al., 2011) and that diet-regulated hormonal influences start to exert an effect *in utero*, diet may alter steroid hormone profiles and thus negatively affect human health (Harkonen and Makela, 2004). Therefore, efficient screening of overall hormonal activity in food is necessary to assess the actual health risk for consumers and to protect safety of the most susceptible population groups like pregnant women, infants or prepubertal children (Aksglaede et al., 2006).

Milk, primarily from cows is one of the most consumed food beverages worldwide and concurrently a rich source of bioactive compounds, including estrogens (Hartmann et al., 1998; Malekinejad et al., 2006). Estrogen hormones play critical roles in regulation of reproductive development and function in healthy organism but excessive or untimely exposure to estrogenic compounds can facilitate disorders or even malignancies of the hormone-dependent tissues (Harkonen and Makela, 2004; Hilakivi-Clarke et al., 2013).

In recent years much controversy exists about associations between consumption of milk and the risk of hormonally-dependent diseases. Epidemiological studies suggest a relationship between dairy consumption (especially the whole milk) and prostate cancer risk (Song et al., 2013; Qin et al., 2004; Pettersson et al., 2012). It is also hypothesized that estrogens present in milk have important role in anovulatory infertility (Chavarro et al., 2007) as well as in supporting the development of breast, endometrial/corpus uteri and ovarian cancers (Ganmaa and Sato, 2005; Ganmaa et al., 2012). Higher dairy intake was linked to an increased risk of testicular cancer (Giannandrea et al., 2013), early sexual maturation in prepubertal children (Maruyama et al., 2010) and inversely associated with sperm quality in men (Afeiche et al., 2013). To ensure the safety of milk and dairy products, assessing the actual exposure to estrogenic compounds in milk is essential for linking chemicals to observed health effects.

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Milk contains endogenous estrogens (mainly E1, E2 and E3) and their metabolites in concentrations depending on physiological stage (estrus cycle, gestation, lactation) and age of cows as well as the type of the cows diet (Malekinejad et al., 2006). Since modern dairy practices involve milking of cows during pregnancy, significantly elevated estrogen concentrations may be detected in milk (Farke et al., 2011).

The free estrogens in milk represent less than 20% of the total estrogen content in milk and most of them are inactivated in the gastrointestinal tract. The conjugated estrogen metabolites are present in milk at significantly higher concentrations (Socas-Rodriguez et al., 2013) and can be deconjugated into biologically active forms in the gastrointestinal tract by bacterial enzymes as well as *in situ*, by cells expressing high sulfotransferase activity (Macdonald et al., 1983). As a result, estrogen metabolites in milk may considerably contribute to the regulation of reproductive processes (Socas-Rodriguez et al., 2013). Until recently, most of investigations were limited to the analysis of free hormones, despite the fact that both conjugated and free hormones are necessary to monitor the effects of HACs.

In addition to endogenous hormones, milk may contain broad range of exogenous estrogens (natural and synthetic) that are illegally used to promote cattle's efficiency (Courtheyn et al., 2002). Their detection in biological samples may not be easy, especially when the natural hormones are used or when very low doses of different estrogenic compounds are used in the same illegal preparation. Since growth promoters are very potent compounds, they may considerably enhance estrogenic activity of milk.

Moreover, plant phytoestrogens (isoflavonoids, flavonoids, stilbenes and lignans) present in cattle's diet have been reported in cow's milk (Andersen et al., 2009a,b). They pass into the milk at concentrations depending on the content of soy, legumes and clover in cows feed (Krajcova et al., 2010; Nielsen et al., 2009; Andersen et al., 2009a,b) and thus may exert estrogen-like biological activity. Because of frequently reported endocrine-disrupting activity of plant phytoestrogens on reproductive health of domestic ruminants and humans, they can significantly contribute to the estrogenicity of milk (Wocławek-Potocka et al., 2013). Furthermore, it was shown that estrogenic mycotoxin zearalenone (ZEA) can pass from the cow's feed into milk (Massart et al., 2008) and recently ZEA and its metabolites were found in cow's milk-based infant formulas (Meucci et al., 2011). Since ZEA and its derivatives, are among the most potent estrogens, they may increase overall estrogenic potency of milk.

Additionally milk can be contaminated with environmental pollutants that possess estrogen-like activity, such as pesticide residues and compounds leaking from food packages (Guart et al., 2011). The presence of estrogenic bisphenol-A (BPA) and alkylphenols (APs) in milk, powdered milk and infant formulas has been reported previously (Casajuana and Lacorte, 2004; Raecker et al., 2011).

In order to ensure the safety of dairy products, efficient screening of multiple groups of estrogenic compounds in milk should be performed. At present estrogens in food are commonly analyzed by instrumental techniques (Sorensen and Elbaek, 2005; Azzouz et al., 2011) which are unable to detect designer compounds, especially present in low concentrations. Moreover, high analysis costs and labor intensity negatively affect utility of analytical methods for large-scale screening. It is also important to highlight that no instrumental methods have been developed to date for the simultaneous determination of all known estrogenic substances in milk.

Alternatively, reporter gene bioassays based on estrogen receptor (ER)-mediated mechanism of action can be used to detect the overall estrogenic activity of samples (Bovee et al., 2004a,b). Although they do not measure individual concentrations of HACs, they give information on the biological response (including possible additive, synergistic, and antagonistic effects) of the sample. In this context, they can help to understand and predict the effects of chemical mixtures on the living organism. Among them, yeast-based assays are robust, relatively inexpensive and provide efficient screening strategy (Bovee et al., 2004a,b).

At present, very promising for food screening purposes is recombinant yeast-based bioassay expressing a green fluorescent protein upon exposure to estrogens (Bovee et al., 2004a). The assay is based on genetically modified yeasts containing reporter yEGFP gene under control of hormone responsive elements, stably expresses human ERα (hERα) and is able to respond to estrogenic substances in dose-, hERα-dependent and chemical-specific manner. In contrast to other yeast-based reporter systems, the yEGFP protein exhibits green fluorescence that can be measured directly without the need of adding any substrate to intact living cells.

The first step toward understanding of the risk of food-borne estrogenic compounds for human health is the application of *in vitro* methods, able to simultaneously detect all present HACs and assess the overall potential toxicity of the sample. In this perspective the aim of our study was to check the ability of the bioassay to measure estrogenic activity of 16 compounds frequently detected in milk. Among chosen compounds were natural hormones: estrone (E1), 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), estriol (E3), 17β-E2 glucuronide (17β-E2G); synthetic estrogens: 17α-ethinylestradiol (17αEE), diethylstilbestrol (DES), dienestrol (DE); phytoestrogens: equol (EQ), genistein (GEN), daidzein (DAI), apigenin (API), flavone (FLA) and environmental contaminants: bisphenol A (BPA), 4-*n*-nonylphenol (4-*n*-NP), 4-*t*-octylphenol (4-*t*-OCP). Then we measured estrogenic activity in raw and processed cow's milk samples collected in Poland. The estrogenic activities of milk were measured without and after enzymatic hydrolysis and the relationship between fat content and estrogenic activity of milk was examined. Finally simplified, theoretical dietary intake of 17β-estradiol equivalents (EEQ) with milk was calculated.

2. Materials and methods

2.1. Chemicals

Ethyl acetate, methanol, *n*-hexane and anhydrous sodium acetate (ACS grade) were obtained from POCH (POCH SA, Gliwice, Poland). Anhydrous magnesium sulfate (reagent grade, >97%) and β-glucuronidase/arylsulfatase from *Helix pomatia* were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide and dimethyl sulfoxide (DMSO, Uvasol® for spectroscopy) were purchased from Merck KGaA (Darmstadt, Germany). Water was purified and deionised by using an ultrapure water system (Milli-Q Gradient Advantage A10 system, Millipore, USA). The compounds selected for the *in vitro* assessment in the yeast estrogen bioassay, their CASRNs, purity and commercial sources are specified in Table 1. Primary stock and intermediate standard solutions of each compound were prepared in DMSO and stored in amber glass tubes at −20 °C and kept out of direct light. Working solutions were prepared in DMSO by successive 10-fold dilutions of the stock standard solutions at concentrations from 100 to 1 μg/L. They were stored at +4 °C for maximum 3 months.

For yeast media preparation, yeast nitrogen base (YNB) without amino acids and ammonium sulphate, Bacto™ Agar and D-(+)-glucose were obtained from Difco (Basel, Switzerland). Ammonium sulphate and L-leucine were provided by Sigma Aldrich. The minimal medium with L-leucine (MM/L) consisted of YNB (1.7 g/L), D-(+)-glucose (20 g/L), ammonium sulphate (5 g/L) and L-leucine

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