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Screening of hormone-like activities in bottled waters available in Southern Spain using receptor-specific bioassays

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ABSTRACT

Bottled water consumption is a putative source of human exposure to endocrine-disrupting chemicals (EDCs). Research has been conducted on the presence of chemicals with estrogen-like activity in bottled waters and on their estrogenicity, but few data are available on the presence of hormonal activities associated with other nuclear receptors (NRs). The aim of this study was to determine the presence of endocrine activities dependent on the activation of human estrogen receptor alpha (hERa) and/or androgen receptor (hAR) in water in glass or plastic bottles sold to consumers in Southern Spain. Hormone-like activities were evaluated in 29 bottled waters using receptor-specific bioassays based on reporter gene expression in PALM cells [(anti-)androgenicity] and cell proliferation assessment in MCF-7 cells [(anti-)estrogenicity] after optimized solid phase extraction (SPE). All of the water samples analyzed showed hormonal activity. This was estrogenic in 79.3% and anti-estrogenic in 37.9% of samples and was androgenic in 27.5% and anti-androgenic in 41.3%, with mean concentrations per liter of 0.113 pM 17β-estradiol (E_2) equivalent units (E_2Eq), 11.01 pM anti-estrogen (ICI 182780) equivalent units (ICI 182780Eq), 0.33 pM methyltrienolone (R1881) equivalent units (R1881Eq), and 0.18 nM procymidone equivalent units (ProcEq). Bottled water consumption contributes to EDC exposure. Hormone-like activities observed in waters from both plastic and glass bottles suggest that plastic packaging is not the sole source of contamination and that the source of the water and bottling process may play a role, among other factors. Further research is warranted on the cumulative effects of long-term exposure to low doses of EDCs.

1. Introduction

Bottled water consumption has grown steadily worldwide over the past few decades, even in places where water of excellent quality is available from the tap. Mexico has the highest per capita consumption of bottled water (243 L/year), followed by Italy (187 L) and the United Arab Emirates (153 L), with Spain in eighth position (124 L) (Gleick et al., 2012). The increase in bottled water consumption has been attributed to concerns about the quality of tap water (Doria et al., 2009) and the presence of disinfection by-products in comparison to the quality of bottled water (Doria, 2010; Gopal et al., 2007). Its effective marketing and general changes in consumer habits have also been cited (Doria, 2006), but there are few scientific data on the reasons for the increased consumption of bottled water (Marcussen et al., 2013).

Some research has been conducted on the presence in bottled water of substances that interfere with the function of the endocrine system (Muncke, 2011; Yang et al., 2011), grouped under the generic name of endocrine-disrupting chemicals (EDCs). EDCs can interact with the endocrine system by mimicking or blocking natural hormones, and they may also influence the production, secretion, or metabolism of endogenous hormones and/or their nuclear receptors (Diamanti-Kandarakis et al., 2009). The diet is one of the main sources of exposure to these chemicals (Connolly, 2009), which are used as stabilizers, antioxidants, coupling agents, and pigments and can be a constituent of storage containers or added to optimize the physical and chemical properties of packaging materials (Lau and Wong, 2000). Bottled water can be

Abbreviations: Al, Aluminum; Sb_2O_3, Antimony trioxide; BP, Benzophenone; BPA, Bisphenol-A; Ce, Cerium; DCC, Dextran-coated charcoal; DIDP, Di-isodecylphthalate; DINP, Di-isononylphthalates; DMSO, Dimethyl sulfoxide; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide; 2,4-dtBP, 2,4-Di-tert-butylphenol; EDCs, Endocrine-disrupting chemicals; ESBO, Epoxidized soybean oil; E_2, 17β-estradiol; FBS, Fetal bovine serum; hAR, Human androgen receptor; hERα, Human estrogen receptor alpha; HDPE, High density polyethylene; Pb, Lead; R1881, Methyltrienolone; NP, 4-Nonylphenol; NOAEL, Non-observed adverse effects level; NRs, Nuclear receptors; OP, Octylphenol; PET, Polyethylene terephthalate; PP, Polypropylene; PTFE, Polytetrafluoroethylene; PE, Proliferative effect; SPE, Solid phase extraction; SRB, Sulforhodamine B; TCA, Trichloroacetic acid; Zr, Zirconium.

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contaminated by EDCs at different stages of the production process, from the supply of the waters to their handling, storage, and distribution. Thus, water can become contaminated at its source, during the bottling process in the plant, by migration from the bottle or cap material (monomers, catalysts, additives, or degradation products) or by the formation of organic compounds under deficient storage conditions (Nerin et al., 2013). Exposure to low doses of chemicals leaching into the water from plastic bottles is a cause of considerable concern to consumers, regulators, and manufacturers (Guart et al., 2011, 2014a).

Despite its toxicological relevance and the importance of the dietary exposure pathway, relatively little information is available on steroid hormone activities and hormone-like chemicals in food, beverages, or water (Connolly, 2009). Klinge et al. (2003) detected a maximum of 84 ng E_2Eq/L (308.3 pM E_2Eq/L) in red wine, and Promberger et al. (2001) reported a range from 23 to 41 ng E_2Eq/L (84–150 pM E_2Eq/L) in beer. These results were confirmed by Takamura-Enya et al. (2003) and Stanford et al. (2010), who reported a maximum activity of 140 ng E_2Eq/L (513.9 pM E_2Eq/L) in beer. Hartmann et al. (1998) suggested that dairy products are the main source of estrogens, calculating a total daily intake of 80–100 ng estrogens per day for adults. Pape-Zambito et al. (2012) reported concentrations of around 10 ng/L or Kg E_2 in dairy products and Courant et al. (2008) concentrations of 34 ng/L E_2 (0.12 nM E_2Eq/L) in milk. Schilirò et al. (2011) reported values of 0.04, 1.59, 1.08, and 0.41 μg E_2Eq100g in oranges, pineapples, kiwis, and tomatoes, respectively. Although E_2 and phytoestrogens in the foods and beverages may explain their estrogenicity, it has also been attributed to anthropogenic contamination. For example, our own group associated the estrogenic burden (range 5.44–720 nM E_2Eq/L) in the liquor of vegetables packed in lacquer-coated cans with the presence of bisphenol-A (BPA) from epoxy resins (Brotons et al., 1995) and related the estrogenicity of baby food products (range 0.25–70.13 pM E_2Eq/g) to the food packaging (Pandelova et al., 2011). In drinking waters, a maximum of 8.7 ng E_2Eq/L (31.94 pM E_2Eq/L) was detected in Brazil (Bergamasco et al., 2011) and a range of 0.19–0.72 ng E_2Eq/L (0.7–2.64 pM E_2Eq/L) in the USA (Stanford et al., 2010).

Although the characteristics of plastic materials in contact with food are tightly regulated by European regulations (EU, 2012), the frequent detection in food and water of contaminants released from plastics suggests that food packaging is a source of chemicals with hormonal activity (Muncke, 2011; Yang et al., 2011). In fact, Muncke (2009) compiled a list of 50 known EDCs that are authorized for the use in food contact materials in the EU and the US.

Bottled water is usually available in glass or plastic containers. Polyethylene terephthalate (PET), a polymer derived from crude oil (Royte, 2008), is used in around 80% of plastic bottles for water. Bottles made with PET are strong, light, impact-resistant, naturally transparent, and completely recyclable, and they impart no taste to the water (Gleick, 2010). Some authors have investigated the interaction of PET bottles with the drinking water they contain, yielding analytical data on PET components and additives (Keresztes et al., 2013; Sax, 2010; Welle and Franz, 2011; Westerhoff et al., 2008).

Despite the aforementioned regulation of food-contact packaging in Europe (EU, 2012), PET-bottled water has been found to contain phthalates and other inorganic species that may be present as residues from the catalysts or additives used to produce PET. This is the case with antimony trioxide (Sb_2O_3), the catalyst most widely used to synthesize this polyester (Sax, 2010). With regard to the use of glass for packaging, glass bottles have been found to leach lead (Shotyk and Krachler, 2007a), and the metal closures of glass jars have been described as a source of epoxidized soybean oil (ESBO), di-isodecylphthalate (DIDP), and/or di-isononylphthalate (DINP) (Pedersen et al., 2008).

Various studies have detected hormone-like activities in commercially available water from PET and glass bottles. Thus, water stored in PET bottles has demonstrated *in vitro* estrogenic activity in MCF-7 human breast cancer cells (Wagner and Oehlmann, 2011), in recombinant cell lines (Plotan et al., 2013), and in recombinant yeast systems carrying the human estrogen receptor alpha (hERα) (Pinto and Reali, 2009; Wagner and Oehlmann, 2009), and it has shown *in vivo* estrogenic activity in a molluscan model (Wagner and Oehlmann, 2009). Anti-androgenic, progestogenic, and glucocorticoid-like activities were recently detected in bottled water, using a panel of reporter gene cell lines in a recombinant yeast system (Plotan et al., 2013), and a broader range of steroid receptor antagonists was found in bottled water by Wagner et al. (2013). However, most of the above studies on EDCs in bottled water have focused on the agonistic activity of xenoestrogens (estrogenicity).

In the present investigation, two bioassays were used to identify hormonal activity in extracts prepared from 29 waters in plastic or glass bottles commercially available in Southern Spain, after a solid phase extraction (SPE) step. The experimental approach focused on EDCs that are active *via* hERα binding and subsequent cell proliferation (E-screen bioassay, MCF-7 cells) and on those active *via* human androgen receptor (hAR) binding (PALM bioassay, human prostate cancer cells), determining the consequent gene expression.

2. Materials and methods

2.1. Chemicals, materials, and instrumentation

All reagents were analytical grade unless otherwise specified. Reference standards 17β-estradiol (E_2), methyltrienolone (R1881) and ICI 182780 (henceforth, ICI), puromycin, geneticin (G418), luciferin (sodium salt), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), sulforhodamine B (SRB), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich Inc. (St Louis, MO). Stock solutions (10 mM) of E_2, R1881, and ICI were prepared in ethanol, and successive dilutions were performed in culture medium. Stock solutions were kept at −20 °C, and dilution series were freshly prepared before each experiment. HPLC-grade solvents (methanol and acetone) were from Merck (Darmstadt, Germany) and dimethyl sulfoxide (DMSO) from Panreac (Barcelona, Spain). Culture medium and fetal bovine serum (FBS) came from Gibco (Invitrogen, Barcelona, Spain), and all cell culture plastics were supplied by Falcon (VWR International Eurolab, Barcelona, Spain).

Isolute ENV + (200 mg/3 ml) and C18 (500 mg/6 ml) SPE sorbent cartridges were supplied by Biotage (Uppsala,Sweden), while Supelclean ENVI-18 (500 mg/6 ml) and ENVI-Carb (500 mg/6 ml) SPE sorbent cartridges were obtained from Supelco (Madrid, Spain). A Supelco 12-column vacuum manifold connected to a Supelco vacuum tank and pump was used for the SPE.

For cell proliferation assays, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm, while an infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase activity in intact cells.

2.2. Bottled water samples

Twenty-nine bottled water products were purchased at local shops in Granada (Southern Spain) between March and December 2012, obtaining all bottles marketed as “natural mineral water,” i.e., derived from natural springs and not processed or altered. The characteristics of the bottled waters are given in Table 1. Twenty-six of the bottles were plastic (products 1 to 26) and three were glass (products 27 to 29). The springs of the waters are located in different geographic regions, all being in Spain except for two in France and one in Portugal. A sufficient number of bottles of each type (to obtain at least 3 L) was purchased from the same batch and stored according to the manufacturer's guidelines (in a dry place and out of the direct sunlight) until testing commenced.