Biomarkers, 2009; 14(5): 271–277

informa
healthcare

RESEARCH ARTICLE

Assessment of the total effective xenoestrogen burden in extracts of human placentas

Maria-Jose Lopez-Espinosa[1,2], Elisabete Silva[3], Alicia Granada[1], Jose-Manuel Molina-Molina[1], Mariana F. Fernandez[1], Clemente Aguilar-Garduño[1], Fatima Olea-Serrano[4], Andreas Kortenkamp[3], and Nicolas Olea[1]

[1]Laboratory of Medical Investigations, San Cecilio University Hospital, University of Granada, CIBER Epidemiología y Salud Pública (CIBERESP), Granada, Spain, [2]Center for Research on Public Health (CSISP), Conselleria de Sanitat, CIBER Epidemiología y Salud Pública (CIBERESP), Valencia, Spain, [3]Centre for Toxicology, School of Pharmacy, London, UK, and [4]Department of Food Sciences and Nutrition, University of Granada, Granada, Spain

Abstract

We have standardized a method to assess the total effective xenoestrogen burden (TEXB) in human placentas by the extraction and separation by high-performance liquid chromatography of two fractions containing lipophilic xenoestrogens (alpha) and endogenous hormones (beta), followed by assessing their estrogenicity in MCF-7 breast cancer cell-based E-Screen and Yeast Estrogen Screen (YES) bioassays. The means of TEXB alpha concentrations (in estradiol equivalent (Eeq) units) were 1.32 and 0.77 Eeq pM g^{-1} placenta in the E-Screen and YES, respectively; TEXB beta concentrations were 6.97 and 11.56 Eeq pM g^{-1} placenta, respectively. The interclass correlation coefficient was low and a fair level of agreement was observed after kappa test correction. According to the E-Screen and YES, TEXB alpha was ≥LOD in 70.0 and 55.0% of the placentas and 92.5 and 82.5% in beta, respectively. Although both bioassays can be recommended for assessing TEXB, there is greater experience with the use of the E-Screen for estrogenic assessment after extensive extraction of complex human matrices.

Keywords: *Total effective xenoestrogen burden; placenta; prenatal exposure; E-Screen; Yeast Estrogen Screen*

Introduction

Introduction of persistent chemicals into the environment has led to their widespread dissemination, and residues are now found at every level of the food chain. A large number and variety of xenobiotics are commonly found in human tissues and fluids (Skaare et al. 1988). A proportion of the 'body burden' of toxins accumulated in a woman's body may pass via the placenta to the developing fetus and via lactation to the nursing young (Olea & Olea-Serrano 1996). The immature physiological functions of the fetus and young child make them especially vulnerable to the harmful effects of toxicants (Ostergaard & Knudsen 1998), especially those that can disrupt hormonal homeostasis (Routledge & Sumpter 1996).

Mothers are not exposed to a single chemical but to a complex mixture of compounds. Therefore, investigation of toxic impact should no longer be limited to the individual effects of single agents but should consider the combined effects of chemicals (Payne et al. 2000, Rajapakse et al. 2002). Our group proposed assessing exposure to xenoestrogens by measuring the biological activity that results from the combined effects of chemicals extracted from human tissues or fluids (Soto et al. 1997, Rivas et al. 2001). Bioassay assessment of the activity of extracted xenoestrogens indicates the total effective xenoestrogen burden (TEXB), which serves as a predictable, consistent and minimally invasive biomarker of endocrine disruption (Rivas et al. 2001). Prominent among bioassays for estrogenicity are the E-Screen and

Address for Correspondence: Nicolas Olea, Laboratory of Medical Investigations, San Cecilio University Hospital, University of Granada, CIBER Epidemiología y Salud Pública (CIBERESP), 18071 Granada, Spain. Tel: +34 958 242864. Fax: +34 958 249953. E-mail: Nicolas Olea: nolea@ugr.es

(Received 24 November 2008; revised 2 March 2009; accepted 13 March 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.1080/13547500902893744

the Yeast Estrogen Screen (YES) bioassays. The E-Screen test compares cell yield between cultures of MCF-7 human breast cancer cells treated with estradiol or different concentrations of xenobiotics or extracts (Soto et al. 1995, Villalobos et al. 1995). In the YES bioassay, ER activation is discerned in the presence of estrogenic compounds after β-galactosidase-dependent colour development (Routledge & Sumpter 1996, Payne et al. 2000).

Studies of human placenta offer an outstanding opportunity to monitor xenoestrogens because they yield data on the exposure of both mother and fetus and no invasive procedure is required (Iyengar & Rapp 2001). The main goal of this study was to develop a method for extraction of xenoestrogens from placentas and to compare the assessment of TEXB between two bioassays. A preparative high-performance liquid chromatography method (HPLC) is used to separate xenoestrogens from endogenous hormones without destroying them (Rivas et al. 2001, Fernandez et al. 2004, 2008). Tissue extracts are eluted by a specific gradient with two mobile phases, collecting two eluates: the so-called alpha (min-1 to min-11) and beta (min-13 to min-32) fractions. These dried fractions are tested for hormonal activity in parallel using E-Screen and YES assays, comparing the cell yield between cultures of MCF-7 human breast cancer or recombinant yeast cells treated with estradiol (positive control) and those treated with different dilutions of tissue extracts. The process ends with an assessment of the proliferative effect expressed as alpha and beta TEXB in estradiol equivalent units (Eeq) per gram of placenta.

Material and methods

Study design

From August to November 2001, 40 placentas were randomly collected from healthy women (mean age 29.66 ± 4.95 years) delivering healthy babies at one hospital in Granada, Spain. The investigation was approved by the Ethics Committee of the hospital and all participants signed written informed consent.

Extraction of xenoestrogens from placenta and separation of sex steroids

Xenoestrogens were extracted from 40 placenta samples by a previously described method (Cerrillo et al. 2005, Lopez-Espinosa et al. 2007, Fernandez et al. 2007a, b). An aliquot of 400 mg of placenta homogenate was dissolved in 20 ml of hexane (Panreac, Barcelona, Spain) and eluted in a glass column filled with Alumine Merck 90 that had been dried at 600°C for 4 h and rehydrated by addition of 5% water. The process was repeated eight times (total weight used: 3.2 g of placenta) for each of the 40 placentas. The eluate obtained was concentrated at reduced pressure, dried under a stream of nitrogen, dissolved in 1600 μl of hexane and then injected (200 μl, 8 times) into the liquid chromatographer.

The HPLC procedure was performed with a Waters Model 501 Millipore apparatus (Marlborough, MA, USA) equipped with two pumps and a U6K injector of 200 μl load capacity. Ultraviolet/visible detector was a Waters Model 490 Millipore device using Millennium Chromatography Manager software. A Lichrocart column (20 × 0.4 cm) was used (Merck, Darmstadt, Germany) packed with Lichrospher Si-60 of 5-μm particle size, flow rate 1 ml min^{-1}, λ 280nm.

We used a previously described liquid chromatography method (Rivas et al. 2001, Fernandez et al. 2004, 2008) to separate xenoestrogens from natural estrogens without their destruction. The normal-phase column separated xenoestrogens according to their polarity, with the most lipophilic compounds eluting in the shortest time. Extracts were eluted by a gradient with two mobile phases: n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15) (v/v/v) (phase B) (Panreac) at a flow rate of 1 ml min^{-1}. Working conditions were: gradient $t = 0$ min, 100% phase A; $t = 17$ min, 60% phase A; $t = 22$ min, 100% phase B; $t = 32$ min, 100% phase A. Two pooled fractions (alpha and beta) were collected (alpha fraction contains first 11 HPLC min and the beta fraction min 13 to 32). Wide-ranging studies using this preparative HPLC technique have demonstrated that the alpha (most lipophilic) fraction collects the organochlorine pesticides DDT and metabolites, lindane, aldrin, methoxychlor, dieldrin, endosulfan and metabolites, PCBs, halogenated bisphenols, bromo phenylethers and bromine-*p*-nonylphenol ethoxylated acetates; the beta fraction collects progestins, androgens, estradiol esters, steroidal estrogens, bisphenol-A, phyto- and mycoestrogens, e.g. zearalenol; fractions collected between min 11 to 13 were silent and discarded in the pooling protocol (Rivas et al. 2001, Fernandez et al. 2004). Hence, non-polar xenoestrogens can be successfully separated from sex steroids, phytoestrogens, mycoestrogens and bisphenols. After preparative fractionation, alpha and beta fractions were each divided into two equal parts, one for the E-Screen and the other for the YES bioassay.

Finally, total lipid content (mean 3.00 ± 1.64%) was gravimetrically quantified using a previously described method (Rivas et al. 2001, Fernandez et al. 2004, Lopez-Espinosa et al. 2007).

E-Screen bioassay for measuring estrogenicity

MCF-7 cells were used in the estrogenicity test according to a technique slightly modified (Villalobos et al. 1995) from the original description (Soto et al. 1995). Briefly,

cells were trypsinized and plated in 24-well plates (Limbro, McLean, VA, USA) at initial concentrations of 20 000 cells per well in 5% fetal bovine serum (FBS) in Dulbecco's modified Eagle (DME) medium. Cells were allowed to attach for 24 h and the seeding medium was then replaced with 10% charcoal–dextran-treated FBS (CDFBS)-supplemented phenol red-free DME.

Duplicated dry alpha and beta fractions obtained by preparative HPLC chromatography were resuspended in 5 ml of CDFBS-supplemented phenol red-free medium, vigorously shaken and left at rest for 30 min, then filtered through a 0.22-μm filter and tested in the estrogenicity assay at dilutions of 1:1 to 1:10. Each sample was tested in triplicate with a negative (vehicle) and positive (estradiol 100 pM) control in each plate. The assay was stopped after 144 h by removing medium from the wells, and the cells were fixed and then stained with sulphorhodamine-B (SRB). Cells were treated with cold 10% trichloroacetic acid (TCA) and incubated at 4°C for 30 min, washed five times with tap water and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was dissolved with 10 mM Tris base (pH 10.7) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan apparatus (Flow, Irvine, CA, USA) at 492 nm. The linearity of the SRB assay with cell number was verified prior to the cell growth experiments. The proliferative effect (PE) was calculated as the ratio between the highest cell yield obtained with 100 pM of estradiol and the proliferation of hormone-free control cells. The PE of alpha and beta fractions was referred to the maximal PE obtained with estradiol and transformed into estradiol equivalent units (Eeq) by reading from a dose–response curve prepared using estradiol (concentration range 0.1 pM to 10 nM) (Rivas et al. 2001, Ibarluzea et al. 2004, Fernandez et al. 2004).

Yeast Estrogen Screen for measuring estrogenicity

The YES bioassay was performed following the protocol developed by Routledge and Sumpter (1996). Briefly, 50 ml of growth medium was inoculated with 125 μl of 10× concentrated yeast stock and then grown overnight in an orbital shaker at 28°C until turbid (absorbance at 640 nm of 1.0). The assay medium consisted of 50 ml of growth medium with chlorophenol red-β-D-galactopyranoside (10 mg l^{-1}, CPRG; Boehringer Mannheim, East Sussex, UK) and 2 ml of the overnight yeast culture. Aliquots of 10 μl of estradiol and 200–400 μl of samples dissolved in ethanol were transferred to 96-well optically flat-bottomed microtitre plates and allowed to evaporate to dryness. To each well, except the blanks, 200 μl of yeast-seeded assay medium was added. Each individual plate also incorporated ethanol controls (i.e. no test agents), positive controls with estradiol (0.1 and 10 nM) and blanks without yeast cells. To minimize evaporation during the subsequent incubation time, the outer wells were not used and were filled with sterile water. Plates were sealed and shaken vigorously for 2 min on a microtitre plate shaker before their incubation at 32°C in a humid chamber for 72 h, during which time they were again shaken at 24 h and 71 h. Plates were then spectrophotometrically analysed at 540 nm (colour) and 620 nm (turbidity) using a Labsystem Multiskan Multisoft plate reader. Data were corrected for turbidity and constitutive Lac-Z expression in ethanol controls as follows:

$$\text{Corrected absorbance} = \text{test}_{540\text{nm}} - \text{test}_{620\text{nm}} + \text{control}_{620\text{nm}} - \text{control}_{540\text{nm}}$$

Samples were run in duplicate and experiments repeated three times. The PE of alpha and beta fractions was referred to the maximal PE obtained with estradiol and transformed into estradiol equivalent units by reading from a dose–response curve prepared using estradiol (concentration range 2 pM to 2 nM).

Data analysis

Alpha and beta TEXB values were considered both as continuous variables and dichotomous variables, i.e. equal to/above or below the limit of detection (LOD), defined as the concentration needed to produce a significantly different proliferative effect from that observed in control cells. Values below the LOD were replaced by numbers from a table of randomly assorted digits (Snedecor & Cochran 1980). All continuous outcome variables were compared as raw data. The interclass correlation coefficient was used to evaluate the concordance between values of estrogenicity estimated by the two bioassays, and the Wilcoxon test was used to evaluate significant differences in the magnitude of measures between them. The chance-corrected agreement between bioassays (kappa coefficient) was calculated as $P_{obs} - P_{chance} \div 1 - P_{chance}$ (Rigby 2000). The observed agreement (P_{obs}) was estimated using the equation $(a + d) \div (a + b + c + d)$ and the chance agreement (P_{chance}) was estimated as $[a] + [d] \div (a + b + c + d)$, where (a) was the number of samples ≥LOD in both bioassays, (b) the number of samples ≥LOD in the YES bioassays or <LOD in the E-Screen, (c) the number of samples ≥LOD in the E-Screen or <LOD in the YES bioassay, and (d) the number of samples <LOD in both bioassays; [a] the product of the proportion of samples ≥LOD in both bioassays multiplied by the total number of samples, $[b] = (a + b) - [a]$, $[c] = (a + c) - [a]$ and $[d] = (c + d) - [c]$ (Landis & Koch 1977, Rigby 2000). The proportion of

Table 1. Values of total effective xenoestrogen burden (TEXB) of alpha and beta fractions of placenta samples ($n=40$) in E-Screen and YES bioassays.

	TEXB-alpha				TEXB-beta			
	Eeq pM g^{-1} placenta		Eeq pM g^{-1} lipid		Eeq pM g^{-1} placenta		Eeq pM g^{-1} lipid	
	E-Screen	YES	E-Screen	YES	E-Screen	YES	E-Screen	YES
Mean	11.87	8.87	564.37	391.01	35.46	68.65	1622.62	3294.60
GM	1.32	0.77	15.05	5.75	6.97	11.56	162.31	202.78
GSD	17.46	17.33	84.41	40.39	10.55	26.30	27.68	72.30
Median	1.62	0.43	47.44	23.60	11.86	51.89	482.11	1697.18

GM, geometric mean; GSD, geometric standard deviation; Eeq, estradiol equivalents.

samples ≥LOD detected by the bioassays was also compared. SPSS version 15 and the STATA version 9 statistical software were used for the statistical analysis.

Results

HPLC-alpha and -beta fractions were tested in the E-Screen and YES bioassays in order to estimate the TEXB. Table 1 lists arithmetic mean, geometric mean, geometric standard deviation, and median. The interclass correlation coefficient between the bioassays was low in both alpha ($p=0.048$, $p=0.382$) and beta fractions ($p=0.080$, $p=0.308$), even when values were expressed on the basis of lipid content ($p=0.134$, $p=0.200$; $p=0.117$, $p=0.230$, respectively). The Wilcoxon test did not detect differences in measures in HPLC-alpha fraction ($p=0.707$), but it detected differences by using one or other bioassays in HPLC-beta fraction ($p=0.036$). When values were lipid-adjusted these differences remained non-significant in the alpha fraction ($p=0.064$) and became more significant in the beta fraction ($p=0.027$). For the TEXB of the HPLC-alpha fraction, comparison between the bioassays showed a P_{obs} value of 0.70, considered a substantial agreement (Landis & Koch 1977, Rigby 2000). The proportion of samples identified as positive by the E-Screen ($28 \div 40 = 0.70$) and YES method ($22 \div 40 = 0.55$), i.e. $0.70 \times 0.55 = 0.385$, was then multiplied by 40, giving 15.4 as the expected frequency for the first cell [a] (see Table 2). Likewise, the expected frequency of the second cell [b] = 6.6, of [c] = 12.6, and of [d] = 5.4 (Table 2). Therefore, the value of P_{chance} was 0.52 and the kappa coefficient showed the agreement to be fair (0.38) in a range from slight to moderate (95% confidence interval (CI) 0.17–0.58) (Landis & Koch 1977, Rigby 2000). With respect to the TEXB of the HPLC-beta fraction, the P_{obs} value was 0.85, a substantial agreement, and P_{chance} was 0.78 (Table 2). The kappa coefficient again showed a fair agreement (0.33) in a range from slight to moderate (95% CI 0.08–0.56) (Landis & Koch 1977, Rigby 2000). TEXB was ≥LOD in 70.0% of the alpha fractions by E-Screen versus 55% by YES bioassay ($p=0.012$) and ≥LOD in 92.5% of the beta fractions by E-Screen versus 82.5% ($p=0.019$) by YES.

Table 2. Variations between E-Screen and YES bioassays in the number of samples equal to/above or below the limit of detection (LOD) and expected frequency for every cell.

TEXB		≥LOD	<LOD	Total
		Alpha E-Screen		
Alpha YES	≥LOD	19 (a) [15.4]	3 (b)[6.6]	22 (a + b)
	<LOD	9 (c) [12.6]	9 (d) [5.4]	18 (c + d)
Total		28 (a + c)	12 (b + d)	40 (a + b + c + d)
		Beta E-Screen		
Beta YES	≥LOD	32 (a) [30.5]	1 (b) [2.5]	33 (a + b)
	<LOD	5 (c) [6.5]	2 (d) [0.5]	7 (c + d)
Total		37 (a + c)	3 (b + d)	40 (a + b + c + d)

TEXB, total effective xenoestrogen burden.

Discussion

Most studies of xenobiotic exposure have been limited to the quantification of a single chemical or a small number of chemicals, ignoring the impact of the cumulative effects of mixtures of chemicals in the cell environment (Rasmussen et al. 2003, Fernandez et al. 2004, 2007a). There have also been very few comparative studies of the efficacy of different *in culture* bioassays to quantify the activity of xenobiotics acting via a similar mechanism, i.e. binding to nuclear receptors (Fang et al. 2000, Folmar et al. 2002). We addressed these two issues by adapting our methodology for estimating TEXB in adipose tissue to the study of placenta. A proven HPLC method (Rivas et al. 2001, Fernandez et al. 2004) was used to separate xenoestrogens from natural endogenous hormones in placenta samples, and the estrogenicity of the resulting fractions was quantified in the E-Screen and YES bioassays.

It was assumed that the estrogenicity estimated in the YES and E-Screen bioassays represented the combined effects of chemical compounds present in the extracts at variable concentrations, sometimes below proliferative effect threshold levels (Fernandez et al. 2004). The method adapted here for placentas is known to effectively extract organohalogenated xenoestrogens from human tissues, separating them from endogenous hormones (Rivas et al. 2001, Fernandez et al. 2004, 2007a). Organochlorine pesticides, halogenated alkylphenols, PCBs and halogenated bisphenols, which are collected in the most lipophilic (alpha) fraction of the preparative

HPLC, have been identified as candidates responsible for estrogenic effects in the E-Screen bioassay (Pazos et al. 1998, Rivas et al. 2001, Fernandez et al. 2004).

Residues of organochlorines pesticides were found in HPLC-alpha fraction extracts from the present series of placentas (Cerrillo et al. 2005, Lopez-Espinosa et al. 2007, Fernandez et al. 2007b). Despite its merits, chemical analysis can only be used to identify and quantify chemicals for which analytical techniques and standards are available (Hollert et al. 2005). This can result in an underestimation of potentially significant classes of contaminants. Moreover, this instrumental analysis cannot account for interactions among chemicals in complex mixtures and therefore provides only limited information on potential biological effects. In fact, Rasmussen et al. (2003), Fernandez et al. (2004) and Hollert et al. (2005) found that even the most comprehensive chemical analysis could only explain part of the biologically effective endocrine-disrupting potential evidenced in bioassay.

Concentrations of lipophilic xenobiotics are commonly expressed in terms of lipid content in order to compare concentrations from different sources (blood, adipose tissue, etc.). Accordingly, TEXB is expressed in this study as both Eeq pM g^{-1} of placenta and Eeq g^{-1} of lipid. For the HPLC-alpha fraction, geometric mean values of 1.3 Eeq pM g^{-1} placenta (15.1 Eeq pM g^{-1} lipid) and 0.8 Eeq pM g^{-1} placenta (5.8 Eeq pM g^{-1} lipid) were observed with the E-Screen and YES bioassays, respectively. This means that each gram of extracted placenta tissue contained a chemical mixture that produced an effect equivalent to 1.3 or 0.8 fmol of estradiol in 1 ml of MCF-7 or yeast culture medium, respectively.

These were considerably lower than the mean values 7.0 Eeq pM g^{-1} placenta (162.3 Eeq pM g^{-1} lipid) and 11.6 Eeq pM g^{-1} placenta (202.8 Eeq pM g^{-1} lipid) estimated by E-Screen and YES bioassays, respectively, for the HPLC-beta fraction. When estrogenicity values were expressed as arithmetic means in Eeq g^{-1} of lipid, results were similar to values reported in adipose tissue samples from women in the same geographical area (Rivas et al. 2001, Fernandez et al. 2004).

The YES and E-Screen bioassays measure different endpoints of estrogenic action at distinct levels of biological complexity, i.e. expression of a reporter gene and cell proliferation. Our comparative study allows conclusions to be drawn about the characteristics and performance of these bioassays. First, the Wilcoxon test detected differences ($p = 0.036$) by using one or the other method in HPLC-beta fractions, but found no significant differences ($p = 0.707$) in the HPLC-alpha fractions. This can probably be explained by the small size sample, as the interclass correlation coefficient between bioassays was low for alpha ($\rho = 0.048$, $p = 0.382$) and beta ($\rho = 0.080$, $p = 0.308$) fractions. Second, the agreement in estrogenicity detection between the methods in the detection of estrogenicity was estimated to be substantial when based on raw data (alpha fraction: 0.7; beta fraction: 0.9) but was downgraded to a fair agreement (ranging from slight to moderate) when corrected for chance (alpha fraction: 0.4; beta fraction: 0.3) using the kappa test (Landis & Koch 1977, Rigby 2000). Finally, the number of samples ≥LOD was significantly higher by E-Screen versus YES assay in both alpha ($p = 0.012$) and beta ($p = 0.019$) fractions.

Account should be taken of some differences between human breast cancer and yeast cells in their ability to detect xenoestrogens (Fang et al. 2000). It is known that MCF-7 cells can metabolize chemicals that are not active binders to ER (Soto et al. 1995) but whose metabolites are. Thus, methoxychlor must be converted to the active metabolite HPTE to become estrogenic (Shelby et al. 1996), and the beta isomer of hexachlorohexane was estrogenic according to *in vivo* testing and the E-Screen but was negative in the YES bioassay (Coosen & Van Velsen 1989, Soto et al. 1995, Steinmetz et al. 1996, Payne et al. 2000). Besides bioavailability and metabolism issues, differences in the assessment of pure chemicals between *in vitro* bioassays may also be due to variations in their uptake (Routledge & Sumpter 1996). In the YES bioassay, for example, some chemicals cannot cross the cell membrane and are therefore unable to bind to the nuclear ER.

For low-potency chemicals, the YES assay has a lower resolving power than the E-Screen (Fang et al. 2000), which has been suggested to be inherent to the reporter gene construct. Moreover, chemicals considered antiestrogens are commonly identified as outliers in the E-Screen/YES comparison, explained by differences in the mechanisms to induce a hormonal response, in impurities among batches of chemicals or in the sensitivity of different species, or by limitations of the bioassays (Fang et al. 2000). The YES system does not detect antiestrogenic effects of tamoxifen but can detect antiestrogenic effects of its hydroxy metabolite under some circumstances (Liu et al. 1999). In fact, the YES assay cannot distinguish the partial agonist activity of type I antiestrogens from that of strong or weak agonists. This impediment of the YES assay to measure directly antagonist activity can result in antiestrogens being considered weak agonists. Folmar et al. (2002) reported interesting discrepancies between the bioassays in their study of the estrogenic potencies of natural estrogens, pharmaceutical estrogens and xenoestrogens in MCF-7 breast cancer cells and yeast cells. They found that the EC_{50} values of pharmaceuticals were approximately one order of magnitude less sensitive in the YES bioassay than in the MCF-7 bioassay and that ethinylestradiol was slightly less potent than estradiol in the YES bioassay

(0.7) and nearly twice as potent (1.9) as estradiol in the MCF-7 bioassay. Comparisons of the activity of estradiol activity among different human cell lines often yield very distinct EC_{50} values, and E-Screen results can even vary among MCF-7 cell stocks from different laboratories (Villalobos et al. 1995). Given these discrepancies, laboratories should use the bioassay system with which they have most experience and that best fits their working protocol.

Studies on environmental exposure during pregnancy and childhood have recommended the use of biomarkers to quantify individual exposure and to evaluate interindividual variability (Whyatt & Perera 1995). The use of E-Screen and YES-measured estrogenicity of the placenta as biomarkers of exposure appears to be a reasonable strategy to assess maternal exposure to xenoestrogens and to estimate exposure of the fetus, and it may be more valuable than the study of individual residues. Results indicate that both bioassays can be used as endpoints for assessment of xenoestrogen exposure using the TEXB approach, as the number of samples ≥LOD were high and the kappa test showed a fair agreement between them. Nevertheless, the E-Screen bioassay can be recommended because of the greater experience with its use and its higher validity to estimate estrogenicity in human placenta samples.

Acknowledgements

We are indebted to all participants, without whom this work would not have been possible. The authors are also grateful to the nursing staff of the maternity wards and special care nursery for their cooperation. The authors are grateful to Richard Davies for editorial assistance. This work was supported by grants from the Spanish Ministry of Health (INMA G03/176), the Spanish Ministry of Science and Innovation (Juan de la Cierva Programa-FSE) and the European Union Commission (QLK4-1999-01422, QLRT-2001-00603 and FP7-ENV-2007-1-212502).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Cano A, Olea N, Fatima Olea-Serrano M. (2005). Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. *Environ Res* 98:233–9.

Coosen R, Van Velsen FL. (1989). Effects of the beta-isomer of hexachlorocyclohexane on estrogen-sensitive human mammary tumor cells. *Toxicol Appl Pharmacol* 101:310–18.

Fang H, Tong W, Perkins R, Soto AM, Prechtl NV, Sheehan DM. (2000). Quantitative comparisons of in vitro assays for estrogenic activities. *Environ Health Perspect* 108:723–9.

Fernandez MF, Aguilar-Garduño C, Molina-Molina JM, Arrebola JP, Olea N. (2008). The total effective xenoestrogen burden, a biomarker of exposure to xenoestrogen mixtures, is predicted by the (anti)estrogenicity of its components. *Reprod Toxicol* 26:8–12.

Fernandez M, Molina-Molina JM, Lopez-Espinosa MJ, Freire C, Campoy C, Ibarluzea J, Torne P, Pedraza V, Olea N. (2007a). Biomonitoring of environmental estrogens in human tissues. *Int J Hygiene Environ Health* 210:429–32.

Fernandez MF, Olmos B, Granada A, Lopez-Espinosa MJ, Molina-Molina JM, Fernandez JM, Olea-Serrano F, Olea N. (2007b). Human exposure to endocrine disrutping chemicals and prenatal risk factors for cryptorchidism and hypospadias. A nested case–control study. *Environ Health Perspect* 115 (Suppl. 1):8–14.

Fernandez MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, Martinez-Vidal JL, Olea N. (2004). Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. *Anal Bioanal Chem* 379:163–70.

Folmar LC, Hemmer MJ, Denslow ND, Kroll K, Chen J, Cheek A, Richman H, Meredith H, Grau EG. (2002). A comparison of the estrogenic potencies of estradiol, ethinylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. *Aquatic Toxicol* 60:101–10.

Hollert H, Durr M, Holtey-Weber R, Islinger M, Brack W, Farber H, Erdinger L, Braunbeck T. (2005). Endocrine disruption of water and sediment extracts in a non-radioactive dot blot/RNAse protection-assay using isolated hepatocytes of rainbow trout. *Environ Sci Pollution Res Int* 12:347–60.

Ibarluzea JJ, Fernandez MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, Exposito J, Lorenzo M, Torne P, Villalobos M, Pedraza V, Sasco AJ, Olea N. (2004). Breast cancer risk and the combined effect of environmental estrogens. *Cancer Causes Control* 15:591–600.

Iyengar GV, Rapp A. (2001). Human placenta as a 'dual' biomarker for monitoring fetal and maternal environment with special reference to potentially toxic trace elements. Part 2: essential minor, trace and other (non-essential) elements in human placenta. *Sci Total Environ* 280:207–19.

Landis JR, Koch GG. (1977). The measurement of observer agreement for categorical data. *Biometrics* 33:159–74.

Liu JW, Jeannin E, Picard D. (1999). The anti-estrogen hydroxytamoxifen is a potent antagonist in a novel yeast system. *Biol Chem* 380:1341–5.

Lopez-Espinosa MJ, Granada A, Carreño J, Salvatierra M, Olea-Serrano F, Olea N. (2007). Organochlorine pesticides in placentas from Southern Spain and some related factors. *Placenta* 28:631–8.

Olea N, Olea-Serrano F. (1996). Oestrogens and the environment. *Eur J Cancer Prev* 5:491–6.

Ostergaard G, Knudsen I. (1998). The applicability of the ADI (Acceptable Daily Intake) for food additives to infants and children. *Food Add Contam* 15 (Suppl.):63–74.

Payne J, Rajapakse N, Wilkins M, Kortenkamp A. (2000). Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environ Health Perspect* 108:983–7.

Pazos P, Perez P, Rivas A, Nieto R, Botella B, Crespo J, Olea-Serrano F, Fernandez MF, Exposito J, Olea N, Pedraza V. (1998). Development of a marker of estrogenic exposure in breast cancer patients. *Adv Exp Med Biol* 444:29–40.

Rajapakse N, Silva E, Kortenkamp A. (2002). Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. *Environ Health Perspect* 110:917–21.

Rasmussen TH, Nielsen F, Andersen HR, Nielsen JB, Weihe P, Grandjean P. (2003). Assessment of xenoestrogenic exposure by a biomarker approach: application of the E-Screen bioassay to determine estrogenic response of serum extracts. *Environ Health* 2:12.

Rigby AS. (2000). Statistical methods in epidemiology. Towards an understanding of the kappa coefficient. *Disability Rehab* 22:339–44.

Rivas A, Fernandez MF, Cerrillo I, Ibarluzea J, Olea-Serrano MF, Pedraza V, Olea N. (2001). Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue. *Acta Pathol Microbiol Immunol Scand (APMIS)* 109:185–97.

Routledge E, Sumpter J. (1996). Estrogenicity activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem* 15:241–8.

Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. (1996). Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ Health Perspect* 104:1296–300.

Skaare JU, Tuveng JM, Sande HA. (1988). Organochlorine pesticides and polychlorinated biphenyls in maternal adipose tissue, blood, milk, and cord blood from mothers and their infants living in Norway. *Arch Environ Contam Toxicol* 17:55–63.

Snedecor GW, Cochran WG. (1980). *Statistical methods*. Iowa: Iowa State University Press, 1980.

Soto AM, Fernandez MF, Luizzi MF, Oles Karasko AS, Sonnenschein C. (1997). Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ Health Perspect* 105 (Suppl. 3):647–54.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 103 (Suppl. 7):113–22.

Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, Bigsby RM. (1996). Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. *Cancer Res* 56:5403–9.

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V. (1995). The E-screen assay: a comparison of different MCF7 cell stocks. *Environ Health Perspect* 103:844–50.

Whyatt RM, Perera FP. (1995). Application of biologic markers to studies of environmental risks in children and the developing fetus. *Environ Health Perspect* 103 (Suppl. 6):105–10.