COMPATATION STUDY OF ESTROGENIC-ACTIVITY INDUCED BY FOUR TYPICAL CHEMICALS USING *in vivo* AND *in vitro* METHODS

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Abstract

In this study, four selected chemicals, the natural estrogens 17β-estradiol (E2) xeno-estrogens 4-octylphenol (OP), 4-nonylphenol (NP), and Bisphenol A (BPA) were examined for estrogenic activity *in vivo* and *in vitro*. *In vivo* experiment, male Chinese loaches were exposed to selective chemicals using semi-static waterborne exposure system**.** Plasma vitellogenin (Vtg) was chosen as determining endpoint. The results demonstrated that all chemicals were estrogenic to male Chinese loach, and the vitellogenic responses showed in a time- and dose-related manner. The estrogenic effect was also assessed with a constructed an estrogen receptor (ER)-mediated *in vitro* bioassay system.

Introduction

Some chemicals released to the environment are able to mimic the behavior of natural endogenous estrogens. And they are suspected of being responsible for an increase in the disruption of the normal physiological functions of the endocrine systems of mammals, fish, birds, reptiles and invertebrates. These chemicals include a variety of compounds, such as the natural and synthetic steroidal estrogens, alkylphenols, organochlorine pesticides, and phthalates [1, 2].

In vitro bioassays based on the mechanism of action of estrogens provide a rapid, sensitive and inexpensive solution to some of the limitations of chemical analysis. The estrogenic or anti-estrogenic activity of any chemical is due to the ability of the compound to interact with the estrogen receptor (ER) of the cell. Some *in vitro* assays that act through the ER mode, such as the E-Screen-assay, the yeast estrogen screen (YES)-assay or receptor binding-assays, have been developed to screen for estrogenic or anti-estrogenic activity of chemicals [3].

Vitellogenin (Vtg) is an egg yolk precursor lipophosphoprotein, which is normally produced in sexually maturing females as a response to endogenous estrogens circulating in the plasma. Under natural conditions, males cannot synthesize Vtg. However, they also possess the Vtg gene, which can be expressed when exposed to estrogen or estrogen mimics. So abnormal levels of Vtg in male fish can be used as a good biomarker to demonstrate estrogenic effects of estrogens or estrogen mimics in the aquatic environment [4]. Chinese loaches are a kind of widespread freshwater fish in Asia. Moreover, the results of our previous study demonstrated that Chinese loach was sensitive to 17β-estradiol [5].

Considering quantitative comparisons of various chemical types from the literature my be confounded by differences between studies in the biological model tested, the experimental protocol and endpoints reported, a comparative study on estrogenic activity of chemicals seems to be necessary. In this study, we used a consistent set of *in vivo* and *in vitro* experimental protocols to study four different typical chemicals.

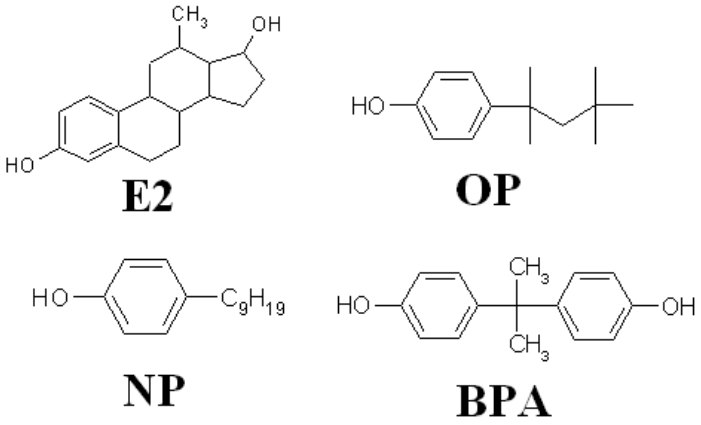

Figure 1 Structural formulae of the tested chemicals.

Methods

Bioassay of the estrogenic activity of each sample with the MCF-7 cell line was done as previously described [6]. Luciferase activities expressed as mean relative luminescence units (RLUs) of the three replicate wells, were converted to relative response units, expressed as a percentage of the maximum response observed for E2 (% E2max). The toxic equivalent was calculated according to the method described by Villeneuve et al. [7].

Fish (n = 4 for each dose) was randomly sampled of the whole exposure. Blood samples were taken from the caudal vein using heparinized syringes and transferred to 1.5 mL centrifuge tubes in the presence of aprotinin. After centrifugation (3000 r/min, 4℃, 30 min), plasma was collected, divided into aliquots and stored at −20℃ for Vtg analysis. Plasma Vtg concentrations were determined with a competitive enzyme-linked immunosorbent assay (ELISA) described elsewhere [8].

Results and Discussion

Five chemicals with known estrogenic activity, namely the natural estrogens 17β-E2, and the xeno-estrogens NP, OP and BPA, were tested in the MCF-7-luc assay. The luciferase activity was detected at the six concentrations in the bioassay and their dose-response curves are shown in Fig. 2. The most sensitive estrogenic dose-responses were found with the natural estrogens, E2. The xeno-estrogens NP and OP produced 50%-E2max-responses at a concentration of about 1×10^4 nM, and BPA was weakly estrogenic at a concentration of about 1×10^5.

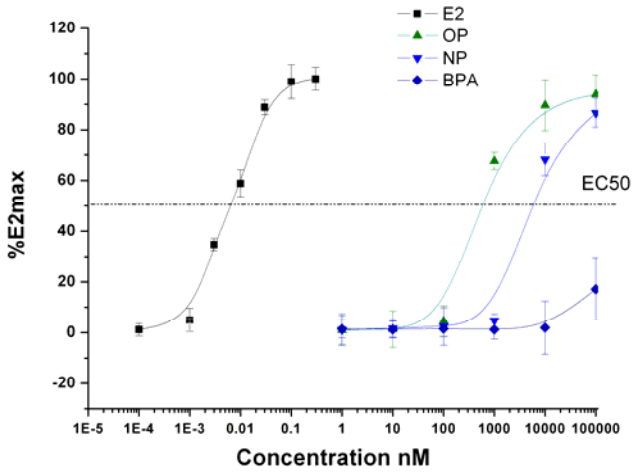

Figure 2 Response in the in vitro bioassay toward 17β-E2, NP, OP and BPA. Luciferase induction is expressed in terms of the percentage of luciferase activity relative to that of 0.3 nM E2 (% E2max). Values represent the mean±S.D.; n=3.

The relative potencies (E2=1) were determined as EC_{50}-values from the dose–response curve. The EC_{50} values and the relative potencies (REP) compared to E2 of the selected chemicals are summarized in Table 1. NP about 1×10^5 times less potent and OP about 3×10^5 times less potent than E2. The observed relative estrogenic potencies of E2, NP and OP are in agreement with reference data [9-11]. These chemicals were previously analyzed in another in vitro bioassay using a recombinant yeast cell culture. The reported relative potency of NP in the recombinant yeast culture assays was 7×10^{-3} [12] and 2.5×10^{-5} [13], and that of OP was 7.8×10^{-6} [13]. An interesting observation is the estrogenic activity of BPA in the yeast culture assay which had a relative potency of 1.1×10^{-4} and was the most potent in Rutishshauser et al.'s [13] study among NP, OP and BPA. In contrast, Gutendrof [10] reported a relative potency of 2.5×10^{-5} in the MCF-7 cells assay for BPA, but in this study, BPA showed weak estrogenic activity at a high concentration of 1×10^5 nM. As a result, the relative estrogenic potencies of selected chemicals in *in vitro* experiment descended in the order of E2>OP>NP>BPA.

Table 1 Comparison of EC_{50} and the relative potencies (REP) obtained in this study and in other studies

Chemical	Values this study		Published values	
	EC_{50}, nM	REP	EC_{50}, nM	REP
E2	4.6×10^{-3}	1	0.015[a] or 0.005[b]	1
OP	1484	3×10^{-6}	4286[c]	0.2×10^{-6c}
NP	4619	1×10^{-6}	463[a] or 400[b]	3×10^{-5a} or 1.25×10^{-5b}
BPA	LE	LE	200[b]	2.5×10^{-5b}

REP of E2 was determined as 1. LE: BPA did not reach 50% of max E2 response in bioassay.

[a] Values are from Van den Belt et al. (2004); [b] Values are from Gutendrof et al. (2001); [c] Values are from Legler et al. (2002).

Chinese loach (*Misgurnus angaillicaudatus*) was chosen as sentinel species. The estrogenic effects of E2, OP, BPA, and NP on plasma Vtg of male loaches were investigated. E2 showed obvious estrogenic effects. In a short time of exposure, Vtg concentrations in the plasma of E2-treated loaches increased significantly compared to the control group ($p<0.05$), and showed dose (time)-dependent. While OP and BPA were weakly estrogens, only the highest concentration (1000μg/L) of OP or BPA produced an elevated Vtg response that was significantly above the control group. NP showed the least estrogenic to male Chinese loach, and Vtg induction among four chemicals. During the whole exposure period (42 days), 20 μg/L NP did not significantly induce the production of Vtg in male Chinese loach except for day 28. Compared with the control and solvent control groups, Vtg levels were significantly induced in the samples exposed to 200 μg/L NP for 42 days ($P<0.05$), which were 77.13±5.26 μg/mL. 1000 μg/L NP can significantly induce the production of Vtg within a 21-day exposure with an average value of 26.07±5.63 μg/mL ($P<0.05$), and Vtg levels in this treatment increased from 31.47±11.92 μg/mL on day 28 to 243.27±27.30 μg/mL on day 42. As a result, the relative estrogenic potencies of selected chemicals in *in vivo* experiment descended in the order of E2>BPA>OP> NP.

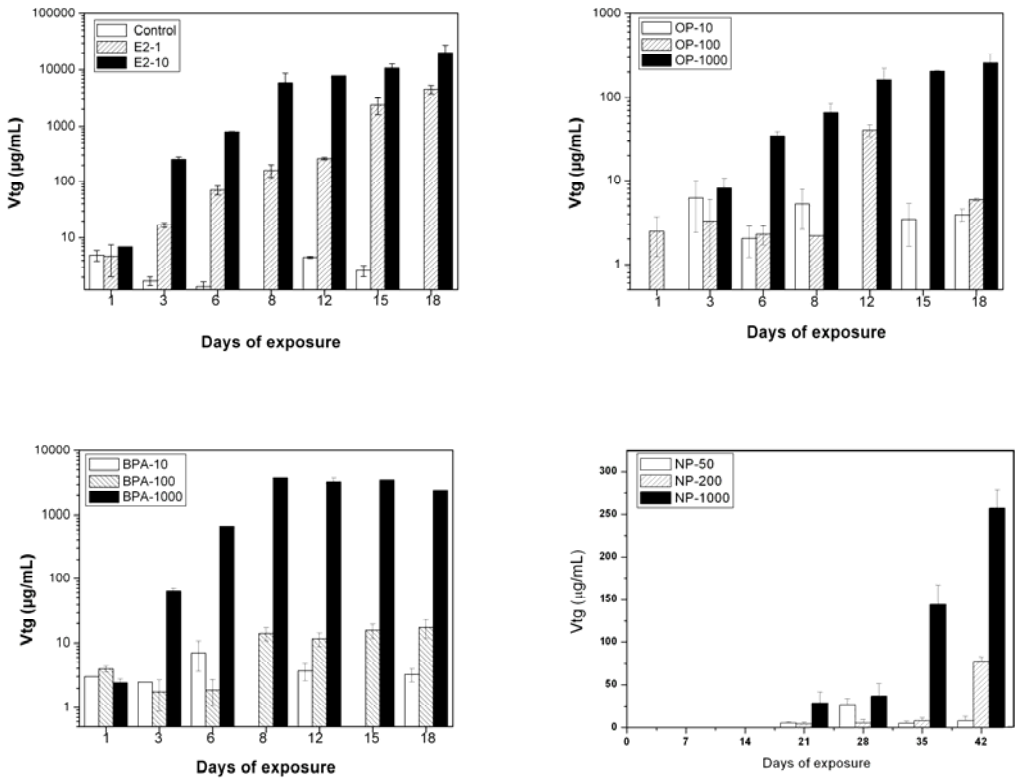

Figure 3 Vitellogenic responses of male Chinese loach exposed to selected typical chemicals.

Acknowledgements

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