Genotoxic effects of binary mixtures of xenoandrogens (tributyltin, triphenyltin) and a xenoestrogen (ethinylestradiol) in a partial life-cycle test with Zebrafish (*Danio rerio*)

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Abstract

A partial life-cycle test with the model fish *Danio rerio* was performed in order to evaluate the genotoxic potential of binary mixtures of xenoandrogenic (tributyltin — TBT; triphenyltin — TPT) and an estrogenic compound (ethinylestradiol — EE2). Five days post-fertilisation larvae were diet-exposed to environmental relevant concentrations of TBT and TPT (25 ng/g–100 ng/g), and water-exposed to ethinylestradiol (3.5 ng/L) for a four-month period; binary mixtures of TBT plus EE2 and TPT plus EE2 were run in parallel. The erythrocytic nuclear abnormalities (ENA) assay in circulating erythrocytes was used to evaluate genotoxicity in the end of the four-month exposure period. A significant increase ($p < 0.05$, Kruskall–Wallis non-parametric ANOVA) in ENA frequency, in comparison with control animals, was observed in those animals exposed to TBT and TPT (the highest doses only), and to EE2 and the binary mixtures, although neither synergistic nor additive effects of the tested compounds were evident. Overall, the results clearly indicate that chronic exposure to low levels of TBT, TPT, EE2 and binary mixtures of TBT plus EE2 and TPT plus EE2 are genotoxic to zebrafish, which may suggest that wild fish populations may be under increased DNA damage in areas contaminated by these endocrine disrupting chemicals.
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1. Introduction

The presence of endocrine disrupting chemicals (EDCs) in the environment is a matter of great concern (Sumpter, 2005). Various man-made chemicals, such as organochloride pesticides, polychlorinated biphenyls, organotins (OTs), phthalates, alkylphenolic compounds, synthetic estrogens and some natural substances present in the environment have been shown to affect the endocrine regulatory system of several wildlife species, such as birds, reptiles, fish, amphibians, mollusc and mammals (Bortone and Davis, 1994; Sumpter, 1998; Quaglino et al., 2002; Jobling et al., 2003; Rodrigues et al., 2006). The reported effects include altered/abnormal blood hormone levels,

reduced fertility and fecundity, female masculinization and male feminization (Matthiessen, 2000; Ferreira et al., 2004; Santos et al., 2006a). One of the clearest examples of endocrine disruption in wildlife is the development of male sexual characteristics (imposex) on female prosobranch gastropods due to exposure to the antifouling agent tributyltin (TBT) (Matthiessen and Gibbs, 1998; Santos et al., 2002; Santos et al., 2006a). This has resulted in generalized masculinization of neogastropod females in the vicinity of shipping areas (Santos et al., 2002; Santos et al., 2004). In addition to the endocrine disrupting effects of TBT on mollusc, recent studies have shown that TBT, at extremely low levels, can disrupt fish sex differentiation leading to a bias of sex toward males (Shimasaki et al., 2003; McAllister and Kime, 2003). Similar to TBT, triphenyltin (TPT) has also been incorporated as antifoulant in some ship paints, but its main use is as a pesticide in several

crops (Stäb et al., 1994). The masculinizing effects of TPT on snails have recently been further confirmed (Santos et al., 2006a), although the effects of TPT on fish sex differentiation have not yet been investigated. In contrast, it is well known that a range of compounds present in the environment, such as the synthetic estrogen ethinylestradiol (EE2), are capable of inducing fish feminization (Jobling et al., 2003). As both groups of compounds exist in combination in the aquatic environment, we have recently investigated the effects of mixtures of xenoandrogenic (TBT) and estrogenic compounds (EE2) on fish sex differentiation (Santos et al., 2006b). The study shows that EE2, at environmental relevant concentrations, can block the TBT masculinizing effects on fish. However, this does not necessarily mean that exposure to EE2 will antagonise all the negative effects of TBT on fish. Several endocrine disrupting chemicals (EDCs) have been shown to induce toxicity by different mechanisms of action. Hence, in addition to the effects of mixture of OTs and EE2 on fish sex differentiation, other endpoints should be investigated.

In spite of being ubiquitous in marine/estuarine ecosystems, there are almost no available studies describing the potential genotoxic effects of TBT, TPT and EE2 on fish, when administered single or in combination (Ferraro et al., 2004). From an ecological point of view, genetic damage may lead to heritable mutations which could compromise the fitness of wild fish populations and their ability to face stress events (Würgler and Kramers, 1992; Anderson and Wild, 1994; Kirsch-Volders et al., 2002). Evidences show that TBT is genotoxic on molluscs (Jha et al., 2000a,b; Hagger et al.,2002), fish (Tiano et al., 2001; Ferraro et al., 2004) and mammals (Liu et al., 2006), whereas TPT was shown to be genotoxic on mammalian cells (Chao et al., 1999). Similarly, strong evidences indicate that EE2 may be genotoxic on mammals (Siddique et al., 2005) or interfere with the repair of DNA damage (Cargouet et al., 2006). Therefore, the present study aimed at investigating the genotoxic potential of xenoandrogens (TBT and TPT) and a xenoestrogen (EE2), single and combined, during chronic exposure, using the model fish *Danio rerio*.

2. Material and methods

The experimental setup including details on chemical analysis has been described in detail in Santos et al. (2006b). Briefly, adult zebrafish kept in our laboratory for two generations were used as breeding stocks. At 1 h past the start of the light phase the following day, viable eggs were collected and allowed to hatch in running water. Larvae (5 days post-fertilization — pf) were assigned to 5 l aquaria provided with dechlorinated tap water at a flow rate of 50 l/day, using a flow-through design. At day 21, larvae were transferred to 30 l aquaria. Water was maintained at a temperature of 27 °C, and photoperiod at 14 h light: 10 h dark. Larvae were exposed to TBT or TPT incorporated in the diet (casein based) at nominal concentrations of 25 and 100 ng/g wet weight (real concentrations for TBT diets of 90 and 150 ng/g TBT, respectively). Actual TBT concentrations were checked in animal's tissue after the exposure period (Santos et al., 2006b). EE2 was dissolved in DMSO and delivered to a mixing chamber by a peristaltic pump (ISMATEC IP-N 16) where it was further diluted in dechlorinated tap water to a final nominal aquarium concentration of 3.5 ng/l (actual concentration of 3.85 ± 0.95 ng/l). The aquaria were allowed to equilibrate for two weeks before the beginning of the experiment, and the actual EE2 concentrations were confirmed twice during the experiment by GC-ITD-MS (Santos et al., 2006b), at the Institute for Environmental Studies (Amsterdam, The Netherlands). Four

treatments with the mixture of androgenic and estrogenic compounds (TBT 25 ng/g+EE2 3.5 ng/l; TBT 100 ng/g+EE2 3.5 ng/l; TPT 25 ng/g+EE2 3.5 ng/l; TPT 100 ng/g+EE2 3.5 ng/l) were also performed, together with a solvent control (DMSO at 20 ng/l). Each treatment was run in duplicate for a four-month period. At the end of the experiment, blood was collected by cardiac puncture in 6–12 animals from each replicate and immediately used for slides preparation. The evaluation of erythrocytes nuclear changes due to genotoxic chemical's exposure has been widely applied in the assessment of aquatic genotoxicity. The most popular technique used has been the determination of micronucleus formation (MN) (Al-Sabti and Metcalfe, 1995). However, recent studies from several research groups have shown that genotoxic chemicals induce other erythrocytic nuclear abnormalities in addition to MN formation. These findings have resulted in an increased use of the erythrocytic nuclear abnormalities (ENA) assay in the detection of genotoxicity of a wide range of compounds, both in the field and under laboratory exposures (Carrasco et al., 1990; Pacheco and Santos, 1996; Çavas and Ergene-Gözükara, 2005; Bolognesi et al., 2006; Teles et al., 2006; Matsumoto et al., 2006; Barsiene et al., 2006a,b,c; Oliveira et al., 2007; Çavas and Könen, 2007; Ergene et al., 2007; Talapatra and Banerjee, 2007; Van Ngan et al., 2007). Therefore, for the present study, we have selected the ENA assay to evaluate the genotoxicity of TBT, TPT and EE2. The ENA test was performed according to Carrasco et al. (1990) and Pacheco and Santos (1996). Approximately 5 µl of blood per specimen was collected and smeared in clean slides and allowed to air-dry. After fixation in absolute methanol for 10 min, slides were allowed to air-dry and stained with 5% Giemsa for 45 min. 1000 erythrocytes per fish were scored for the presence of ENA under 1000× magnification. Slides were coded and scored blind by the same observer. ENA were identified into one of the following categories: micronuclei, small (<1/3 of the main nucleus) non-refractive, circular or ovoid chromatin bodies, showing the same pattern as the main nucleus (Al-Sabti and Metcalfe, 1995); cells with two nuclei were considered as binuclei; nuclei with two lobes were classified as segmented nuclei; nuclei with a central and unilateral constriction were classified as kidney shaped nuclei. The final results were expressed as sum for all individual lesions per 1000 erythrocytes. As no sex differences (Mann–Whitney *U* test) on ENA induction were found on those groups displaying sufficient number of males and females to perform the assay (control, TBT 25, TBT 100, TPT 25, TPT 100, TBT 25+EE2), the data from males and females was pooled before analyses. The Kruskall–Wallis non-parametric ANOVA followed by Dunn's test (all pairwise multiple comparison) was used to compare ENA frequencies among treatments, using the software SigmaStat 3.0.

3. Results

Fig. 1 displays the ENA frequency in zebrafish erythrocytes after the four-month exposure period. In comparison with control animals, all treatments lead to a significant increase ($p < 0.05$) in the average ENA per group (Fig. 1), the only exception being the TBT 25 and TPT
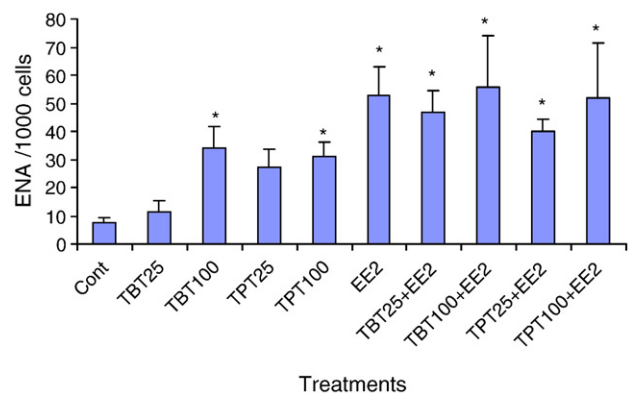

Fig. 1. Average erythrocytic nuclear abnormalities frequency (ENA/1000 erythrocytes) in zebrafish erythrocytes at the end of the four-month exposure period. Values are mean ± S.E. ($n = 12$–24).*Significantly different from solvent control ($p < 0.05$, Kruskal–Wallis non-parametric ANOVA and Dunn's test).