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Effect of tributyltin, benzo(*a*)pyrene and their mixture exposure on the sex hormone levels in gonads of cuvier (*Sebastiscus marmoratus*)

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Abstract

Tributyltin (TBT), an organometal used as an antifouling biocide, has been reported to induce masculinization of fish. Benzo(*a*)pyrene (BaP), a widespread carcinogenic polycyclic aromatic hydrocarbon, has been reported that its microsomal metabolites can produce an estrogenic response when tested in vitro. This study was therefore designed to examine the potential in vivo influence of TBT, BaP and their mixture on sex hormone levels in gonads of *Sebastiscus marmoratus*, which were given eight separate i.p. injections (a single injection every 7 days) of TBT (0.5, 1, 5 and 10 mg/kg), BaP (0.5, 1, 5 and 10 mg/kg), or both in combination (0.5, 1, 5 and 10 mg/kg); control fish received olive oil vehicle only. Six days after the first (week 1), second (week 2), fourth (week 4) and eighth (week 8) injection, gonads samples were collected and analyzed for sex hormone levels. TBT treatment alone was found to be ineffective at week 1, but significantly elevated the testosterone level in testicle of the male fish at week 4 compared to the corresponding controls. TBT treatment significantly reduced the ovarian testosterone level of the female fish at week 2 in dose-dependent manner. It was observed that TBT, BaP and their mixture significantly reduced the ovarian 17β-estradiol level of the female fish at weeks 2 and 8 in dose-dependent manner, however, the ratios of testosterone to 17β-estradiol in the ovary were elevated. This change of sex hormones levels would be one of the reasons to interpret the masculinization of fish by TBT. The present study demonstrates that BaP could influence in vivo ovarian sex hormone level of the female fish. The elevation of the ratios of testosterone to 17β-estradiol in the female fish exposed to BaP implies that BaP would have an androgenic effect on the fish in vivo, which should be deserving of further study. The joint effect of TBT and BaP at 1:1 concentration ratio on the level of 17β-estradiol in *S. marmoratus* was antagonism. TBT can antagonize bioactivation of BaP, and BaP can stimulate the Phase II metabolism of TBT and/or its biliary excretion, which were reported in previous studies, would be one of the causes that TBT and BaP had a antagonism on the level of ovarian 17β-estradiol in the present study.

Keywords: Tributyltin; Benzo(*a*)pyrene; Testosterone; 17β-Estradiol; *Sebastiscus marmoratus*; Joint effect

1. Introduction

Organotin compounds, particularly tributyltin (TBT) are widely used as biocides in a variety of consumer and industrial products. Among them, antifouling paints are the most important contributors of organotin compounds to the aquatic environment, where they are known to cause deleterious effects to non-target organisms (Alzieu, 1991). Accordingly, several countries have banned its use and restricted its application to large vessels. However, even after regulation, monitoring programmes reveal its presence in coastal and deep-sea areas (Tolosa et al., 1996; Morcillo et al., 1997; Takahashi et al., 1997); thus, organotin compounds are still a cause of concern for aquatic life. In recent years, the environmental contamination and the high toxicity of TBT have been documented. Besides the acute toxicity of TBT, some studies show that TBT have embryotoxicity (Marin et al., 2000), genotoxic (Jha et al., 2000) and TBT can produce endocrine disruptive effects. Potential reproductive

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impairment has been reported at a concentration of TBT in water as low as 1 ng/L as Sn, viz. the induction of imposex in the dogwhelk (*Nucella lapillus*) (Gibbs et al., 1988). It has been reported that TBT potentially induced masculinization, to the extent of complete sex reversal, of genetically female Japanese flounder (*Paralichthys olivaceus*) (Shimasaki et al., 2003). TBT can affect sexual behavior and reproduction in medaka (*Oryzias latipes*) (Nakayama et al., 2004). However, limited information concerning the mechanism of action of organotin compounds in fish is available.

Polycyclic aromatic hydrocarbons (PAHs) constitute one of several classes of organic molecules released into the environment largely as the result of human activities (GESAMP, 1993), resulting in the ubiquity of these toxic chemicals in the marine environment. PAHs have been demonstrated to be mutagenic and carcinogenic precursors (McElroy et al., 1991; Maccubin, 1994), as well as to impair reproductive functions of fish (Nicolas, 1999). Benzo(*a*)pyrene (BaP), a representative PAH, was found to have no direct estrogenic effect (Thomas and Smith, 1993). However, microsomal metabolites of BaP can produce an estrogenic response when tested in vitro (Bulger et al., 1985). Recent results suggest that PAHs can have a significant antiestrogenic effect through an Ah-receptor mediated mechanism (Anderson et al., 1996). Effects of BaP on reproductive functions of fish are actually still unclear.

TBT and BaP are widespread pollutants that occur simultaneously in many aquatic environments under both dissolved and particulate forms. Because animals inhabiting polluted areas are exposed to mixtures of chemicals, additive, synergistic, or antagonistic effects of endocrine-disrupting chemicals are possible. Padrós et al. (2003) reported combined effect of TBT and BaP on a suite of biomarkers, but there are not any previous study reporting the combined effect of the mixture of TBT and BaP on the level of sex hormones in fish. This study was therefore designed to examine the potential in vivo influence of TBT, BaP and their mixture on sex hormone levels in gonads of *Sebastiscus marmoratus*. The selection of the test species was based on its availability, commercial importance (fisheries and aquaculture), and distribution throughout the coastal waters of China.

2. Materials and methods

2.1. Chemicals

TBT was obtained from Fluka AG, Switzerland, with a purity of greater than 97%. BaP was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grades and were obtained from commercial sources.

2.2. Experimental species and estimation of LD_{50}

Cuvier (*S. marmoratus*) weighing 25–50 g, were captured from a pristine coast in Xiamen city, Fujian province, China. Before the exposure experiment, the fish were acclimated in tanks (30 fish in each tank) containing 60 L of aerated sand-filtered seawater, under flow-through conditions with natural photoperiod for 7 days.

Short-term static toxicity tests were performed. Median lethal doses LD_{50} were estimated by the Bliss method (1938). The 95% confidence limits (UCL, LCL) of the LD_{50} values were determined according to Sokal and Rohlf (1995). A series of degressive doses with a ratio of 1.2, from 48, 40, 33.33, 27.78 to 23.15 mg TBT/kg body weight, were employed for intraperitoneally (i.p.) injection. A single i.p. injection of BaP at 200 mg/kg BW had no effect upon the fish survival for up to 7 days post-injection.

2.3. Treatments and sample collection

Thirty fish per dose group were i.p. injected with TBT (0.5, 1, 5 and 10 mg/kg), Bap (0.5, 1, 5 and 10 mg/kg) or both in combination (0.5, 1, 5 and 10 mg/kg); control fish received an equal volume of the olive oil (injection volume in each case 1 ml/kg). Injections were repeated every 7 days over a 49-day period. The fish were fed with fresh clam *Meretrix meretrix* flesh for 2 h to satiation before replacing the water, the clam was collected from a pristine coast and previously maintained in aerated marine water for up to 7 days. This process was repeated every other day until the third day before sampling. The water temperature was maintained at $14 \pm 2\,^{\circ}C$ and salinity 22–24.

The fish were randomly sampled from each treatment group 6 days after the first (week 1), second (week 2), fourth (week 4) and eighth (week 8) injection. Sampling was between 8:00 a.m. and 11 a.m. in order to minimize diurnal variability. At weeks 1 and 4, three fishes were sampled; at week 2, four to six fish were sampled and five to six fish at week 8. Gonads were frozen in liquid N_2 immediately after collection and stored at $-80\,^{\circ}C$ until analyzed.

2.4. Sex hormone analysis

The gonads were individually cut into small pieces of 0.25 g wet weight for analyzed. Subsamples were homogenized in ethanol (5 ml) and frozen at $-80\,^{\circ}C$ for at least 24 h. Homogenates were then extracted with diethyl ether (8 ml), followed with two further extractions with 4:1 diethyl ether:ethanol (2×10 ml) as described in Bettin et al. (1996). The organic extract was evaporated under nitrogen and redissolved in 2 ml of 80% methanol. This solution was then washed with petroleum ether (2×5 ml) to remove the lipid fraction and evaporated to dryness. For 17β-estradiol (testosterone) analysis, the dry residue was dissolved in 0.05 M phosphate buffer (pH 7.6) containing 0.1% gelatine, and assayed for 17β-estradiol and testosterone titres using a commercial radio-immunoassay (RIA) kit (Furui Biological Engineering Co., Beijing, China).