

Induction of Imposex in Rock Shell, *Thais clavigera*, Exposed to Organotins and Other Endocrine Disrupting Chemicals

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ABSTRACT

Interactions of both feminizing and masculinizing chemicals inducing imposex in gastropod were investigated with a long-term exposure experiment. Imposex-free rock shell (*Thais clavigera*) was exposed to TBT and other antagonistic chemicals (nonylphenol and DDT), P450 inducer (phenobarbital) and natural sex hormone (estrogen) mixtures for 60 days. None of imposexed-females were found in control, sham (ethanol) and testosterone exposure group, on the other hand imposex was induced in the TBT and all the TBT+antagonist mixture groups. After 60-day exposure, frequency of imposexed-female ranged from 31–68%. Female mean penis length (FMPL) of imposexed-female ranged from 0.87–2.58 mm, and relative penis length indices were from 7.2 to 21.2%. However, the degrees of imposex were different among the exposure groups. The FMPLs of three exposure groups (TBT+estrogen, +DDT and +nonylphenol) were less than that of the TBT exposure group. Ethoxyresorufin-*O*-deethylase (EROD) activities in gastropod decreased in the TBT and all the TBT+antagonist exposure groups compared to the control group. A negative correlation was obtained between TBT body residue and EROD activity, while a positive relationship was obtained between TBT body residue and the degree of imposex except for the TBT+DDT exposure group. Although TBT concentration was relatively high in the TBT+DDT exposure group, the TBT+DDT exposure group demonstrated low EROD activity and low degree of imposex. These results indicate that a certain antagonistic effect of DDT occurred in induction of imposex by TBT.

INTRODUCTION

Organotin compounds are one of the most widely used organometallic chemicals. They are actively applied to antifouling paints as biocidal additives to prevent adherence of sedentary organisms to ship hull and other structural surfaces immersed in water. Since their deleterious effects on non-target organisms were revealed in the early 1980's, many industrialized countries have placed regulations of ban on use of organotin compounds, tributyltin (TBT) and Triphenyltin (TPT) compounds, in ships. Especially, TBT is considered one of endocrine disrupting chemicals (EDCs), as it is known to cause imposex (imposition of male sexual

organ on female) in marine gastropod well below ppb level. Korean government has placed the regulation on usage of TBT based paints on small ships since 2000. Antifouling System (AFS) Treaty of International Maritime Organization (IMO) requires an implementation of total ban on use of organotin based anti-fouling paints for member countries from 2003. However, TBT concentrations in the marine environment of Korea are still high enough to induce imposex in gastropod (Shim *et al.* 2002).

Many EDCs (e.g. nonylphenol) are known as feminizing chemicals, while TBT as masculinizing agent for gastropod (Shim *et al.* 2000). Actually, other feminizing chemicals as well as TBT exist together in the marine environment. However, interactions between both the feminizing and masculinizing groups in endocrine disrupting effects were not well documented yet. In this study, interactions of both the chemical groups on inducing imposex of gastropod species were investigated with a long term exposure experiment.

MATERIALS AND METHOD

Exposure experiment

Imposex-free rock shell (*Thais clavigera*) from Jeju Island was exposed to TBT and other antagonistic chemicals (nonylphenol and DDT), P450 inducer (phenobarbital) and natural sex hormone (estrogen) mixtures in seawater for 60 days. The exposure levels and combination of chemicals are given in Table 1. About 100 individual gastropods of the each exposure group were kept in 15 L glass aquarium and fed about 3 to 4 clams per day. The stock solutions of exposure chemicals and 17 β -Estradiol (E2) were made in ethanol. Nominal exposure concentrations were maintained by a static renewal everyday. During the exposure period, water temperature was about 13–16°C and salinity was 32‰.

Table 1. Summary of exposure levels of the target chemicals and 17 β -Estradiol.

No.	1	2	3	4	5	6	7
Group	Control	Sham	TBT	TBT + 17 β -Estradiol (E2)	TBT + DDT	TBT + Nonylphenol (NP)	TBT + Phenobarbital (PB)
Concn.		EtOH	0.1 ppb	0.1 ppb + 0.5 ppm	0.1 ppb + 0.1 ppb	0.1 ppb + 0.1 ppb	0.1 ppb + 0.5 ppm

Measurement of Imposex

A sample size of 30 *Thais clavigera* from each exposure group was used to measure frequency and degree of imposex. Shell length was measured with Vernier calipers. The shells were cracked open with a titanium tool and the animals were removed. *T. clavigera* were

sexed by appearance of a prominent female sperm-ingesting gland (Horiguchi *et al.* 1994). The penis of both female and male was cut, and penis length was measured to the nearest 0.01 mm using Vernier calipers. The frequency of imposex females was expressed as a percentage. Whereas the degree of imposex was expressed by index of relative penis length (RPL): [(mean length of female penis)/(mean length of male penis) × 100].

Measurement of EROD activity

EROD activity is closely correlated with cytochrome P450 system, which is actively responding to xenobiotics. Living digestive gland cells of *T. clavigera* in stead of microsome were using for EROD activity. Cells were isolated from animals with collagenase treatment, and cell suspension were adjusted to 0.5×10^6 cells/ml in minimal essential medium (MEM) with 5% fetal bovin serum albumin. EROD activity in cell suspension was measured with fluorospectrophotometer at $\lambda_{Ex} = 544$ nm and $\lambda_{Em} = 584$ nm following after 15 min incubation with 7-ethoxyresorufin at 20°C. EROD activity was quantified in unit of nM/ 0.5×10^6 cells with resorufin calibration curve (Heinonen *et al.*, 1996).

Analytical Procedure for Butyltins

Approximately 2 g of tissue samples were extracted with methylene chloride (0.1% tropolone) using a shaker for 3 hr. The extracts in hexane were derivatized with propylmagnesium bromide and then cleaned with 1 g of activated florisil. Finally, concentrated samples was analyzed with gas chromatograph (GC) equipped with a flame photometric detector (FPD). The GC temperature program and operating condition are identical to the previous study (Shim *et al.* 2003)

RESULTS AND DISCUSSION

Occurrence and degree of imposex

None of females in imposex were found in control and sham (ethanol) exposure groups, while imposex was obviously induced in all the TBT+antagonist mixture groups as well as the TBT only exposure group. Degree of imposex expressed in RPL index increased according to the exposure duration in the range of 7.2–21.5%. Female mean penis length (FMPL) of female gastropod ranged from 0.87 to 2.58 mm after 60-day exposure (Fig. 1). However, the degree of imposex in gastropod was different among the exposure groups. The FMPLs of three exposure groups (TBT+E2, TBT+DDT and TBT+NP) were less than that of TBT exposure group, on the other hand, TBT+PB exposure group was higher. When FMPLs of only imposexed-female were considered, the FMPL of TBT only exposure group was the highest after both 30- and 60-day exposure. In addition, the FMPL of TBT+E2 exposure group decreased from 30- to 60-day exposure, but the TBT+PB exposure group steeply increased.

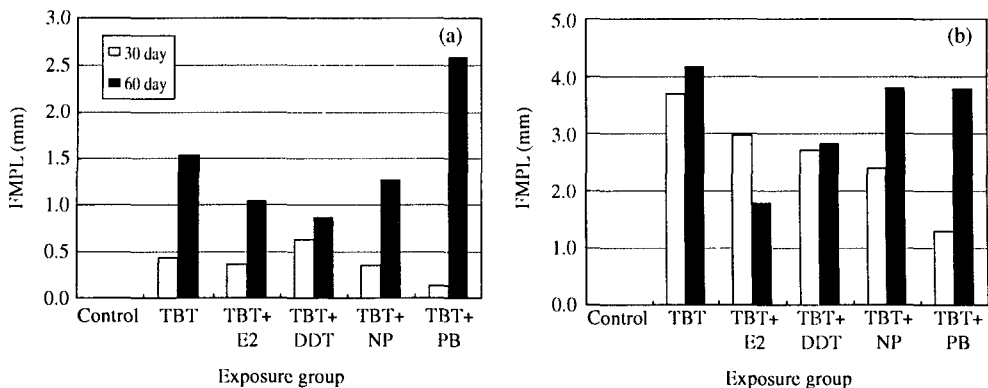


Fig. 1. Female mean penis length (FMPL) of (a) all the female *T. clavigera* and (b) only females with penis (imposexed-female) after 30 and 60 days exposure.

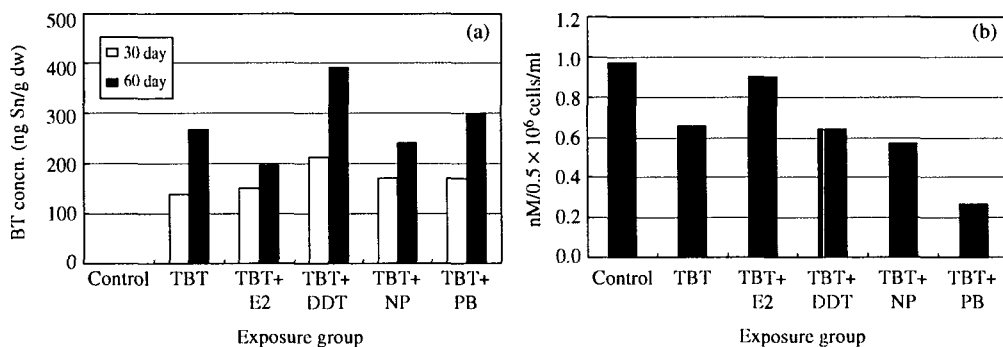


Fig. 2. Total butyltin residues and Ethoxyresorufin-*O*-deethylase (EROD) activity of *T. clavigera* after 30 and 60 days of exposure.

Butyltin Residues and EROD

Tributyltin (TBT) and its degradation product, dibutyltin (DBT), were detected in TBT and TBT+antagonist exposure groups. Body residue of butyltin (BTs, sum of TBT and DBT) increased according to the exposure duration. The residue of BTs in gastropod of the TBT+DDT exposure group was the highest on 30 and 60 days of exposure (214 and 391 ng Sn/g dw, respectively), and TBT+E2 was the least (152 and 196 ng Sn/g) (Fig. 2). EROD activity on 60-day exposure depended on the exposure groups. The activities in control, sham, androgen and TBT+E2 groups were relatively high, but the other groups demonstrated lower activities.

Relationship between BT residues vs EROD activity or FMPL

Relationship between EROD activities and BT residues in gastropod demonstrated a negative correlation except for the TBT+DDT exposure group, which showed high BT concentration and mid-level EROD activity. A significant positive correlation was obtained between BT residues and FMPLs of gastropod except for the TBT+DDT exposure group on

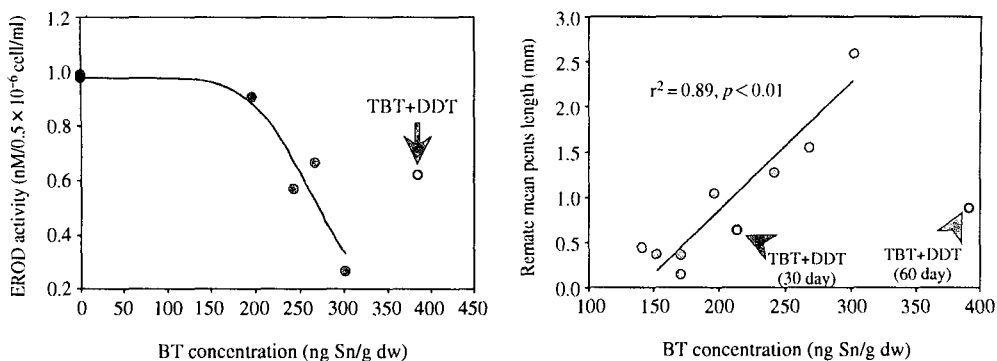


Fig. 3. Relationship between butyltin body residue and Ethoxyresorufin-*O*-deethylase activity (EROD) or female mean penis length (FMPL) of *T. clavigera* after 30 and 60 days of exposure.

30- and 60-day exposure (Fig. 3). Although the BT residue was the highest in the TBT+ DDT exposure group (Fig. 2), the degree of imposex was relatively low in the group. Therefore, it could be concluded that a certain antagonistic effect on induction of imposex in gastropod was occurred by DDT.

CONCLUSION

The degree of imposex in gastropod was relatively low in the TBT+E2, DDT and NP exposure groups compared to the TBT only exposure group. EROD activity was reduced in gastropod exposed to the TBT+antagonist as well as the TBT exposure group. A certain antagonistic effect of DDT on induction of imposex by TBT was occurred.

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