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Comparative Toxicities of Organotin Compounds on Fertilization and Development of Sea Urchin (*Anthocidaris crassispina*)

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Fertilization and embryo bioassays have been known as useful tools for measuring toxicities of contaminants such as metals (Kobayashi, 1984) and pesticides (Bresch and Arendt 1977). However, relatively little attention has been paid to organotin compounds. Ringwood (1992) revealed high toxicity of tributyltin (TBT) to gametes and early developmental stage of sea urchin, *Echinometra mathaei,* compared to those of cadmium and copper. The TBT-based paint leachates delayed sea urchin cell cleavage and inhibited DNA and echinochrome production at ppb levels (Ozretic et al. 1998). Effects of TBT (Hamoutene et al. 2000; Kobayashi and Okamura 2002) and butyltins (Marin et al. 2000) on fertilization and development of sea urchin embryo were studied. However, comparative toxicity of organotin compounds, including phenyltins and methyltins, has not been studied yet with sea urchin gametes and embryos. In this study, we investigated the response of sea urchin gametes and embryos to a set of structurally distinct eight organotin compounds actually found in the marine environment. The comparative toxicities of eight organotin compounds in sea urchin were discussed with their molecular structures and physicochemical properties. In addition, the toxicities of those organotins in fertilization and embryo development assays were compared with other organotin toxicity data from literatures.

MATERIALS AND METHODS

Sea urchins, *Anthocidaris crassispina*, are widely and abundantly distributed along the south coast of Korea. This species was collected on the depth of 3-6 m during the breeding season near Cheju Island from the end of July to middle of August. The collected sea urchin samples were stored in aquarium with a closed-filter system at water temperature of 22 - 24°C for less than a week prior to assay.

Eight organotin compounds, tributyltin chloride (TBT, 96%), dibutyltin dichloride (DBT, 96%), monobutyltin trichloride (MBT, 95%), triphenyltin chloride (TPT, 95%), diphenyltin dichloride (DPT, 96%), monophenyltin trichloride (MPT, 98%), trimethyltin chloride (TMT, 97%) and dimethyltin dichloride (DMT, 97%), were obtained from Aldrich Chemical and dissolved in

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dimethyl sulfoxide (DMSO). Then the stock solution was diluted to necessary concentration with artificial seawater. The range of diluted concentration was in the range of 10^{-5} - 10^{2} mg/L according to toxicity of organotins to obtain dose-response curve for each organotin in three different assays. The final concentration of DMSO in experiment was less than 1%, which did not show any toxicity on sea urchin cells.

To obtain the maturated eggs and sperm, 1-2ml of 0.5M KCl solution was injected into the sea urchin with syringe. After spawning of the female, eggs were allowed to settle to the bottom of beakers filled with seawater, and then seawater was aspirated. Final concentration of eggs was prepared as a 1000 eggs/ml of suspension. Sperms were collected without washing procedure. The standard sperm density for insemination is adjusted with a 1/1000 dilution. Isolated sperms and egg quality was checked with fertilization prior to experiment. The fertilization membrane was formed within 1-2 min after insemination in, at least, 90% of the eggs under the normal conditions. Fertilization was progressed within 12 h after isolation of sperms and eggs in all the experiment.

The sperm and egg toxicity test followed the method suggested by Kobayashi et al. (1984) with a minor modification. For sperm toxicity test, the isolated sperms were exposed to each test solution for 15 min, and approximately 1000 pre-washed eggs were added to the sperm-toxicant mixture. The fertilization was allowed to proceed for 15 min. The eggs were then preserved in 2% formaldehyde, and fertilization was scored with presence or absence of a complete fertilization membrane. For egg toxicity test, matured eggs suspension of 0.9 ml in concentration of 1000 cell/ml was added to a 24-well plate which contained 0.1 ml of each organotin solution. The suspension was mixed and plate was allowed to stand for 2 h. Then egg-toxicant suspension in each well was mixed with 0.1 ml of sperm solution. The suspension was fixed with 2% formaldehyde after 15 min and percent of fertilized eggs were counted with an inverted microscope. For developmental toxicity test, after artificial fertilization of eggs, 0.9 ml of suspension with density of 1000 cell/ml was added to each well of a 24-well plate that contains 0.1 ml of the sample solution. The plate was kept at 26 - 27°C for 2 h until eggs in control cleaved to an 8-blastomere stage. Final concentration of 2% formaldehyde solution was added to each well to stop cleavage. A hundred embryos were randomly examined with an inverted microscope to count cells developed to the 8-blastomere stage. Each concentration of toxicant was tested in triplicates for three kinds of toxicity tests.

The median effective concentration (EC_{50}) was estimated from a linear regression analysis between percent inhibition and logarithmically transformed exposure concentration. Other statistical parameters reported in the results are the standard error of estimate and the ANOVA ratio for regression (F). The correlation between the EC_{50} values and physicochemical parameters of organotins was analyzed by multiple regression analysis with a stepwise statistical method ($F = 4.0$)

RESULTS AND DISCUSSION

Cytotoxic effects of eight organotins on sea urchin gametes and development were occurred in a broad range of concentrations from 10^{-4} to 10^{2} mg/L. The order of sperm toxicity to eight organotins was as follows: TBT > TPT > DBT > MPT > TMT > DPT > MBT > DMT (Table 1). Among the eight organotins tested, TBT was the most toxic. Its toxicity began at 0.007 mg/L and the fertilization was completely inhibited at 0.47 mg/L. TPT showed complete inhibition at 2.5 mg/L. TBT and TPT were more toxic than TMT to the sperm.

The overall toxicity of organotins on egg was less than that on sperm. The order of egg toxicity to eight organotins was as follows: TBT > TPT > MBT > DBT > MPT > TMT > DPT ≅ DMT (Table 1). Dose-response relationship was not clear in the egg toxicity test within the test range compared to the sperm toxicity test. TBT began to show egg toxicity at 0.06 mg/L, but complete inhibition of fertilization was not found at the concentrations up to 7.45 mg/L. DPT (88.8±3.5%) and DMT (93.3±10.6%) showed even higher fertilization success at 100 mg/L in the egg toxicity test.

The development of fertilized eggs to 8-blastomere stage was also apparently inhibited with organotin compounds. Eight organotins showed typical dose-response curves. The order developmental toxicity to eight organotins was as follows: TPT > TMT > TBT > DBT > MPT > DPT > MBT > DMT (Table 1). In comparison with the sperm and egg toxicity tests, all three triorganotins showed relatively high toxicity in the developmental toxicity test. Significant inhibition started to occur at 0.078 mg/L of TPT, and development to the 8-blastomere was completely arrested at 0.625 mg/L of TPT. TBT showed complete inhibition of development at 7.45 mg/L.

There was, in general, significant correlation ($r^2 = 0.53$; $p < 0.05$) between the results of the sperm and the 8-blastomere developmental toxicities. The EC_{50} values between the sperm and the egg toxicities showed less significant correlation ($r^2 = 0.63$; $p < 0.1$), where as no significant correlation was obtained between the egg and the 8-blastomere developmental toxicities. The different toxic effects of eight organotins to sperm, egg and 8-blastomere developmental toxicity tests indicate that inhibition mechanism of organotins were influenced by such as different cell types (sperm and egg) and different processes (fertilization and development).

The response of the sea urchin gamete and embryo to organotins was compared to other *in vivo* and *in vitro* bioassays in which test organisms and cells other than sea urchin were used for various organotin toxicity (Table 1). Generally, the toxicity of eight organotins on sea urchin in this study strongly correlated with those of other toxicological tests involving whole organisms and cytotoxicity, even if the toxicological endpoints were different among the assays. The sperm toxicity showed significant correlation with 6 out of 8 assays compared. The strong significant correlations were obtained between the sperm toxicity and two

cytotoxicity assays (NR; $r^2 = 0.96$, $p < 0.01$ and MTT; $r^2 = 0.90$, $p < 0.05$)), while no significant ($p > 0.05$) correlation was found with LC_{50} of fish and hemolysis of rat erythrocyte. TBT and TPT showed relatively high toxicity not only in the sperm toxicity test, but also in the other assays (Table 1). Among the organotins tested, MBT and DMT were less toxic in many assays except for LC_{50} of fish and hemolysis. Toxicity of MPT on sea urchin sperm was much higher than those of seven other assays in which MPT was tested (Table 1). The egg toxicity of organotins in this study significantly ($p < 0.05$) correlated with toxicity on whole organisms, particularly fish and algae, and with erythrocyte hemolysis. The 8-blastomere developmental toxicity in this study also showed significant ($p < 0.05$) correlation with those of six assays referred in Table 1 except for Microtox and erythrocyte hemolysis assays. TMT in the 8-blastomere developmental toxicity test was more toxic than those of five other bioassays except for *Daphnia magna* toxicity.

MBT and MPT were relatively toxic on these sea urchin toxicity tests compared to most of other assays (Table 1). Exceptionally, the higher EC_{50} values of MBT and MPT in neutral red (NR) and tetrazolium salt reduction (MTT) (Brüschweiler et al. 1995) indicate that MBT and MPT toxicities are not strongly related to cell membrane integrity and mitochondrial activity. Uncertain coagulation of sperm was observed in only MBT and MPT treated-sperm suspension in this study. The sperm cells were aggregated with some mucous materials. The mode of toxic action of MBT and MPT on sea urchin sperms could be different from the other di- and tri-substituted organotin compounds.

All the eigenvalues, which include hydrophobic, electronic and steric parameters, applied to quantitative structure active relationship (QSAR) are shown in Table 2. Log ($1/EC_{50}$) was adopted as an indication of toxicity on the three sea urchin toxicity tests. Linear correlation was obtained between each descriptor of the tested organotin compounds as an independent variable and log ($1/EC_{50}$) values of sperm, egg and 8-blastomere toxicity tests as the dependent variable.

Total surface area (TSA) of six organotins showed a strong significant correlation with log ($1/EC_{50}$) of the sperm toxicity test ($r^2 = 0.76$; $p < 0.05$). Significant ($p < 0.05$) correlation was also found between log ($1/EC_{50}$) of the sperm toxicity test and octanol-water partition coefficient (log P), sum of fragment constants (Fr), electro negativity (EN), complementary information content (CIC), and valence connectivity index ($^1X_p^v$). In the egg toxicity test, only EN ($r^2 = 0.76$; $p < 0.05$) and CIC ($r^2 = 0.82$; $p < 0.05$) showed significant ($p < 0.05$) correlation with log ($1/EC_{50}$) of the test. Log ($1/EC_{50}$) of the 8-blastomere developmental toxicity test showed significant correlation with Fr ($r^2 = 0.62$; $p < 0.05$) and pKa ($r^2 = 0.69$; $p < 0.05$), whereas low correlation coefficients were obtained with the other parameters.

Multiple regression analyses were carried out with a stepwise method to predict organotin toxicity on each sea urchin test in this study. In the sperm toxicity test, the regression equation obtained with variable is shown as in Eqn[1]:

$$\text{Log } (1/EC_{50sperm}) = 0.339(Fr) - 1.377 \quad (r^2 = 0.99) \qquad \text{Eqn [1]}$$

The sperm toxicity was well predicted with the Eqn[1] except for MPT that showed relatively high toxicity compared to other di- and mono-organotin compounds (Fig. 1a). A good regression formula to predict the egg toxicity was obtained with the following equation:

$$\text{Log } (1/EC_{50egg}) = 0.399(Fr) + 6.1(SIC) - 4.490 \quad (r^2 = 0.99) \qquad \text{Eqn[2]}$$

The egg toxicity of mono- and tri-organotin compounds could be well predicted with the Eqn[2] (Fig. 1b). A multiple regression between the 8-blastomere developmental toxicity and the physicochemical parameters produced the following equation:

$$\text{Log } (1/EC_{508\text{-blastomere}}) = 0.522(pKa) + 0.009(IP) - 0.022(EN) - 4.088 \ (r^2 = 1.0) \qquad \text{Eqn[3]}$$

The 8-blastomere developmental toxicity of MPT could not be predicted, because pKa value of MPT was not applicable. However, the 8-blastomere developmental toxicity of the other seven organotins could be well estimated with three electric parameters (pKa, IP and EN) (Fig. 1c).

The log ($1/EC_{50}$) values of organotins on water flea (Nagase et al. 1991) and fish hepatoma cells (Brüschweiler et al. 1995) showed good correlation with the corresponding log P values. However, in this study, the EC_{50} values of the three sea urchin tests correlated well with sum of fragment constant (Fr), index of hydrophobic behavior, than log P. Lauglin *et al.* (1985) showed that the log ($1/LC_{50}$) values of 15 organotin compounds on the marine crab larvae could be predictably explained using TSA. In this study, only the sperm toxicity test produced significant correlation with TSA. A correlation between the EC_{50} values and hydrophobic parameters of organotin molecules implies that either more favorable incorporation of lipophilic compounds to cell membrane or more efficient interaction with hydrophobic functional cell unit may produce higher toxicity.

There have been attempts to estimate and predict toxicity of organotin compounds with molecular descriptors. Toxicity of 16 organotins on water flea was well predicted with three parameters, log P, pKa and ^1X_p or one steric parameter, $^1X_p{}^v$ (Vighi and Calamari 1985). The LC_{50} values of 29 organotins in red killfish were adequately estimated with only steric parameters such as the information contents and the molecular connectivity indices (Nagase et al. 1992). In this study, the EC_{50} value of each organotin on sea urchin sperm toxicity was predictable with sum of fragment constant. The sum of fragment constant accounted for 99% of the total variability of the toxic effect excluding MPT compound. The sum of fragment constant and one steric parameter, structure information content, accounted for 99% of the variability of the toxic effects on sea urchin egg, while almost 100% of the variability of 8-blastomere

Table 1. Median effective concentration (EC_{50}) values for organotin compounds determined by the sperm, egg and 8-blastomere developmental toxicity test and toxicity data, taken from literature, for various bioassays.

	Toxicity (mg/L)										
Organotin	Sperm EC_{50}	Egg EC_{50}	8-blastomere EC_{50}	Fish[a] LC_{50}	Plankton[b] EC_{50}	Algae[c] IC_{50}	Microtox[d] EC_{50}	Cells[e] IC_{50}	Cells[f] NR_{50}	Cells[f] MTT_{50}	Subcells[d] EC_{50}
TMT	3.1	75	0.6	5.62	0.48	5.5	29.9	381	-	-	6.58
TBT	0.06	1.25	0.8	0.04	0.01	0.02	0.01	2.43	0.04	0.20	0.03
TPT	0.31	2.4	0.27	0.06	0.02	0.01	0.02	5.74	0.07	0.15	0.01
DMT	29	> 100	41	6.00	0.65	21	703	80.0	483	571	110
DBT	2.5	19	2.2	5.80	0.90	6.8	0.18	65.6	6.08	9.11	0.33
DPT	3.4	> 100	19	30.6	0.65	8	1.65	131	5.16	5.50	2.51
MBT	9.0	19	34	38.1	49.1	25	762	-	>2822	>2822	734
MPT	1.0	61	10	109	-	19	-	268	>30200	>30200	-

[a] *Oryzias latipes* (Nagase et al. 1991), [b] *Daphia magna* (Vighi and Calamari 1985), [c] *Ankistrodesmus falcatus* (Wong et al. 1982) [d] Sub-Mitochondira article (Argese et al. 1998), [e] Hemolysis of erythrocytes (Hamasaki et al. 1995), [f] NR = neutral red; MTT = tetrazolium salt reduction (Brüschweiler et al. 1995)

Table 2. Physicochemical and topological parameters of organotin compounds.

Organotin	MW[a]	Log (P)[b]	Fr[c]	TSA[d]	pKa	IP[e]	EN[f]	Log (W)[g]	Iwd[h]	CIC[i]	SIC[j]	IC[k]	$^{1}X_{p}{}^{m}$	$^{3}X_{p}{}^{m}$	$^{1}X_{p}{}^{vn}$
TMT	199.3	-2.3	2.31	174	6.6	176.5	31.2	1.2	3.25	2.71	0.288	1.09	2	11.69	0
TBT	325.5	2.6	7.53	360	6.99	290.7	91.5	2.54	6.31	4.09	0.236	1.26	6.68	13.83	3.4
TPT	385.5	2.65	5.7	337	5.2	427	81.3	3.08	7.78	3.64	0.291	1.49	9.82	12.33	7.52
DMT	219.7	-3.1	-0.1	157	3.54	137.5	25.4	1.2	3.25	2.36	0.317	1.1	2	9.81	0
DBT	303.8	1.49	3.38	274	3.8	368.2	65.6	2.26	5.57	3.23	0.233	1.13	5.12	11.24	2.41
DPT	343.8	1.9	2.16	290	2.72	304.5	58.8	2.55	6.52	3.13	0.326	1.51	7.21	10.24	5.32
MBT	282.2	0.35	-0.77		2	213.8	39.7	1.85	4.62	2.93	0.284	1.16	3.56	8.65	1.28
MPT	302	1.15	-1.38			181.9	36.3	2.16	5.33	2.37	0.392	1.53	4.61	14.97	2.83

[a] MW, molecular weight, [b] n-octanol/water partition coefficient from Wong *et al.* (1982), [c] Hansch fragment constant [d] TSA, Total surface area from Laughlin *et al.* (1985), [e] IP, ionization potential; [f] EN, electronegativity; [g] Wiener number from Hamasaki *et al.* (1995), [h] Information index; [i] CIC, complementary information content; [j] SIC, Structural information content; [k] IC, information content from Nagase *et al.* [12], [m] Connectivity indices; [n] Valence connectivity index from Hamasaki *et al.* (1995)

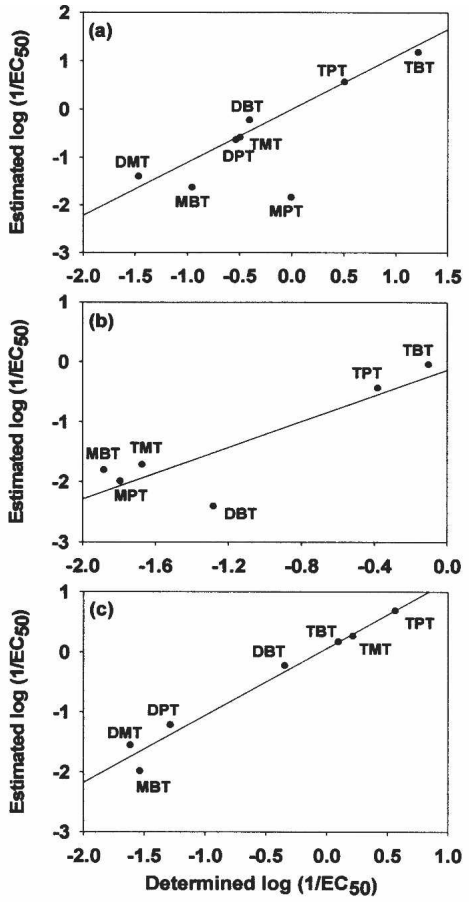

Figure 1. Relationship between determined and estimated log (1/EC50) on three sea urchin toxicity tests. (a) sperm toxicity from log ($1/EC_{50}$sperm) = 0.339 (Fr) - 1.377, $r^2 = 0.99$ (Note: MPT was not included in a regression analysis), (b) egg toxicity from log ($1/EC_{50}$egg) = 0.399(Fr) + 6.1(SIC) - 4.490, $r^2 = 0.99$ and (c) 8-blastomere developmental toxicity from log ($1/EC_{50}$8-blastomere) = 0.522(pKa) + 0.009(IP)

developmental toxicity was explained with only three electric parameters, pKa, IP and EN.

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