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Effects of Endosulfan on Survival and Development of *Bombina orientalis* (Boulenger) Embryos

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Abstract The developmental toxicity of endosulfan was examined in the anuran *Bombina orientalis* embryos. Survival rates of embryos following 50 μM endosulfan treatment was significantly lower than vehicle control at 96 h onward. When the embryos develop to the tail fin circulation stage, embryonic survival was significantly decreased by 10 μM endosulfan treatment. Surviving embryos showed various developmental abnormalities including tail dysplasia at 50 μM. By hampering the embryonic development endosulfan may cause the decline in the natural populations of this frog species breeding on farmland and in the surrounding aquatic environment.

Keywords Endosulfan · Embryonic development · *Bombina orientalis*

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzodioxathiepin-3-oxide), organochlorine chemical is used extensively throughout the world, as a insecticide on vegetables, fruits, coffee, and cotton (Kullman and Matsumura 1996). Accordingly, endosulfan is distributed in the farmland, soil, and the surrounding aquatic environment. Therefore, it is a very strong possibility that could induce toxic effects by coming into direct contact with aquatic lives. Formulations of endosulfan include emulsifiable concentrate, wettable powder, ultra-low volume liquid, and smoke tablets. Trade names of commercial insecticides containing endosulfan include Cyclodan, Malix, Thiodan, and Thiolix. The parent compound is in the form of alpha and beta isomers and the major oxidation product is endosulfan sulfate. Endosulfan and its breakdown products are persistent in the environment with an estimated half-life of 9 months to 6 years. Endosulfan is a highly toxic insecticide in USEPA (U.S. Environmental Protection Agency) toxicity class l. Endosulfan is a Restricted Use Pesticide (RUP). To date, toxic effects of endosulfan have been described in a range of organisms. Endosulfan is known to produce potent effects on the nervous system of a wide variety of organisms, including man. In mammals, endosulfan affects the central nervous system in which activated metabolites of endosulfan alter and inhibit neurotransmission in the brain (Abalis et al. 1986; Gant et al. 1987) and activate cholinergic (Anand et al. 1986), dopaminergic (Anand et al. 1985), and serotonergic (Agrawal et al. 1983; Seth et al. 1986) mechanisms. Endosulfan has been implicated in gonadal toxicity (Singh and Pandey 1989; Sinha et al. 1997, 2001) and genotoxicity (Yadav et al. 1982; Pandey et al. 1990; Chaudhuri et al. 1999; Lu et al. 2000). It is extremely toxic to fishes and aquatic invertebrates (Sunderam et al. 1992). However, there has been a little investigation of the impact of endosulfan in amphibians but the probable is that may be directly effects by this insecticide as they live on farmland and in the surrounding aquatic environment. Also, endosulfan has an embryotoxic, developmental, and teratogenic effects in frog (Harris et al. 2000; Broomhall and Shine 2003).

Bombina orientalis is one of the most common amphibians in the world and comprise a large proportion of their total number in Korea. This species lives in mixed coniferous-broad-leaved forests and uses water in lakes, ponds, swamps, streams, and springs. *B. orientalis*, spawns in the rice field and ponds in the farming regions where the massive application of organochlorine insecticide occurs. Therefore use of endosulfan in farmland may potentially threaten embryonic, larval and adult life of this frog

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species. To date, however, embryotoxic, developmental, and teratogenic effects of endosulfan has not been studied in *B. orientalis*. In the present study we examined the effect of endosulfan on the survival and development of *B. orientalis* embryos and tadpoles.

Materials and Methods

Endosulfan (CAS No. 115-29-7) was obtained from Riedel-de Haën (Seelze, Germany) and dissolved in ethanol. Frogs (*B. orientalis*) were collected in Hongcheon (Gangwon-Do, Korea) and reared in animal husbandry at Hanyang University (Seoul, Korea). They were fed with mealworm three times a week and aquarium water was replaced at the time of feeding. The aquaria were maintained at a diurnal 14: 10 h light: dark cycle and at 20–22°C. Embryos were obtained from at least three different male/female pairs for each bioassay. Mature oocytes of *B. orientalis* were obtained by injecting adult females with 750 IU of human chorionic gonadotropin (hCG) in the abdominal cavity. The next day, spawning occurred and oocytes were placed into a dry Petri dish. For sperm preparation adult males were injected with 500 IU of hCG. The next day, male frogs were anesthetized by inhalation of ether (Sigma, St Louis, MO) to minimize the pain, and testes were dissected. The oocytes were mixed with fresh sperm suspension prepared by mincing of the frog testes in a 1× MMR solution. Subsequently, eggs sat for a 15 min and were then covered completely with 0.1× MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.5 mM HEPES (pH 7.8), 0.01 mM EDTA). Successful fertilization was detected a few minutes later, when the eggs were oriented with the dark animal pole side up. The healthy fertilized eggs screening performed 2 h post fertilization made it possible to remove the unfertilized and necrotic eggs. Shortly after fertilization embryos were selected for drug treatment. Totally 600 embryos are subjected to bioassay. Embryos from the same female were randomly placed in small aquarium and exposed to varying concentration of endosulfan (1, 5, 10, and 50 μM in 0.00005% ethanol) in 0.5 L of 0.1× MMR solution. In the control group, 0.00005% (v/v) ethanol was present. Experiment was replicated three times. The embryos were cultured in an incubator (MIR550, Sanyo, Japan) at 18°C for 13 days. The test medium was changed three times a week, and dead embryos were removed daily. Surviving embryos were fixed in 10% neutral formaldehyde at the end of the experiment and examined for malformations under a stereomicroscope. Staging and patterning of abnormal development were conducted as described by Rugh (1962). Statistical significance was analyzed using the chi square test and Fisher's exact test and accepted as significant when p values were lower than 0.05.

Results and Discussion

Following the endosulfan treatment the survival rates of embryos were gradually decreased in a concentration dependent manner. The survival rates of embryos following 50 μM endosulfan treatment was significantly lower than vehicle control at 96 h onward. At 10 μM endosulfan, embryonic survival was significantly decreased compared with vehicle control at 168 h onward (Table 1).

Table 1 Survival rates of *B. orientalis* embryos exposed to endosulfan

Endosulfan (μM)	No. of embryos	Surviving embryos (%)							
		Time after fertilization (h) and developmental stage							
		24 Lb	48 Np	72 Tb	96 Mr	144 Mo	168 Tc	192 Tc	312 Oc
0	120	111	101	95	92	86	86	86	86
		(92.5)	(84.2)	(79.2)	(76.7)	(71.7)	(71.7)	(71.7)	(71.7)
1	120	106	99	85	80	80	77	77	77
		(88.3)	(82.5)	(70.8)	(66.7)	(66.7)	(64.2)	(64.2)	(64.2)
5	120	102	96	86	73	68	68	65	65
		(85.0)	(80.0)	(71.7)	(60.8)	(56.7)	(56.7)	(54.2)	(54.2)
10	120	100	98	78	68	61	56*	56*	56*
		(83.3)	(81.7)	(65.0)	(56.7)	(50.8)	(46.7)	(46.7)	(46.7)
50	120	99	92	75	60*	49*	45**	42**	24**
		(82.5)	(76.7)	(62.5)	(50.0)	(40.8)	(37.5)	(35.0)	(20.0)

Note: Lb, late blastula; Np, neural plate; Tb, tail bud; Mr, muscle response; Mo, mouth open; Tc, tail fin circulation; Oc, operculum complete
*, ** Significantly different from vehicle control by Fisher's exact test (* $p < 0.05$; ** $p < 0.005$)

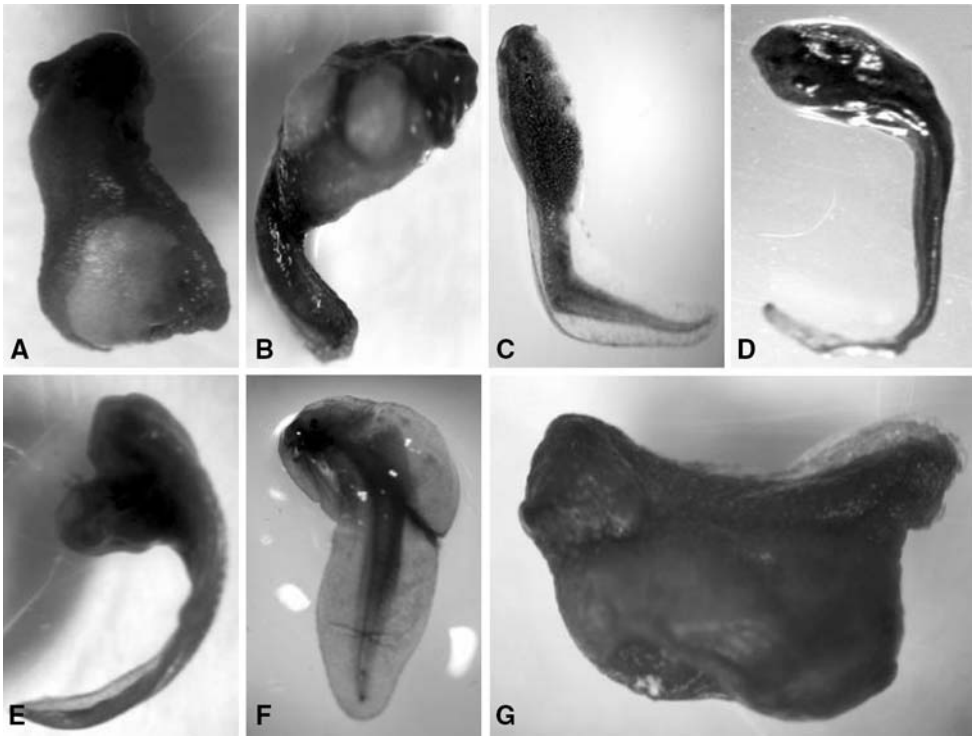

Fig. 1 Various abnormalities in embryos and tadpoles of *B. orientalis* following endosulfan treatment: (**A**) Tail dysplasia, (**B**) pectoral blister, (**C**) bent trunk, (**D**) bent tail, (**E**) cephalic dysplasia, (**F**) ventral blister, and (**G**) thick set body

When exposed to endosulfan, surviving embryos showed various developmental abnormalities including tail dysplasia, pectoral blister, bent trunk, bent tail, cephalic dysplasia, ventral blister, and thick-set body (Fig. 1). The incidence of developmental abnormalities increased with increasing concentration of endosulfan. At the end of culture the frequency of developmental abnormalities was significantly higher in 50 μM endosulfan treated embryos compared with that of vehicle control. The malformations showed in order of frequency with tail dysplasia, pectoral blister, bent trunk, bent tail, cephalic dysplasia, ventral blister, and thick-set body. Frequency of tail dysplasia was significantly higher than that of other malformations (Table 2).

The frog embryo is an intact developing system, which undergoes events comparable to those of other vertebrates, including mammals. Amphibian embryo teratogenesis assay is useful because they can rapidly provide information on developmental toxicants. Following the endosulfan treatment, the survival of *B. orientalis* embryos decreased together with an increase in the developmental abnormalities in a concentration dependent manner. The lowest observed effective dose (LOED) for embryonic toxicity for endosulfan is 10 μM in *B. orientalis* embryos. Similarly, in previous studies, endosulfan was reported to have embryotoxic, teratogenic or growth retardation effects in some frog species. In American toad (*Bufo americanus*) embryos, 2.35 mg/L endosulfan affects the survival of embryos following 96 h treatment (tail bud stage) (Harris et al. 2000). Consequently, our results consent with LOED for embryonic toxicity for endosulfan in other amphibian species. Furthermore, Australian treefrog (*Litoria freycineti*) tadpoles exposed to 0.03 or 1.3 μg/L endosulfan grew more slowly than control tadpoles (Broomhall and Shine 2003). In salamander (*Ambystoma barbouri*) 10 μg/L of endosulfan decreased larval survival, growth rates and larval activity and induced limb deformity and nervous system malfunction (Rohr et al. 2003). The types of developmental abnormalities observed were diverse in order of frequency with tail dysplasia, pectoral blister, bent

Table 2 Frequency of abnormal embryos after endosulfan treatment

Abnormalities	Endosulfan (μM)					
	0	1	5	10	50	Sum (%)
Tail dysplasia	12	14	11	13	29	79 (40.9)**
Pectoral blister	8	7	8	7	12	42 (21.8)
Bent trunk	0	5	10	5	5	25 (12.9)
Bent tail	0	2	7	6	5	20 (10.4)
Cephalic dysplasia	0	0	0	6	4	10 (5.2)
Ventral blister	2	3	0	4	0	9 (4.7)
Thick-set body	2	3	3	0	0	8 (4.1)
No. of abnormal embryos (%)	24	34	39	41	55**	193 (100)
No. of test embryos	120	120	120	120	120	600

* Significantly different from other malformation type by Fisher's exact test ($p < 0.0001$); ** significantly different from vehicle control by Fisher's exact test ($p < 0.005$)

trunk, bent tail, cephalic dysplasia, ventral blister, and thick-set body following embryonic exposure to endosulfan. This suggests that endosulfan targets multiple events in embryonic and larval development in this frog species. Because simultaneous exposure to multiple agricultural chemicals is most likely unavoidable to frog embryos and tadpoles in the contaminated aquatic habitat, it should be emphasized that even at a no observable effect concentration (NOEC) endosulfan may be harmful for embryonic survival and development of this frog species when other chemicals are also present in environment (Rajapakse et al. 2002). Indeed, agricultural chemical such as alachlor, benomyl, and diuron which hampered embryonic survival and increased developmental abnormalities in amphibians including *B. orientalis* (Schuytema and Nebeker 1998; Osano et al. 2002; Yoon et al. 2003; Kang et al. 2005). Therefore it is tempting to speculate that endosulfan even below at 10 μM concentration was detrimental for survival and development of *B. orientalis* embryos. We suggest that the safety guideline for endosulfan in environmental media should be below the 10 nM, one thousandth of LOED for embryonic survival. Furthermore, present study may provide additional clues on factors causing the decline in the populations of amphibians in worldwide and may explain the increased incidence of abnormalities in the natural populations of frog species. In the near future, it should be also studied whether parental exposure to endosulfan could affect developmental potency of gametes or embryos in next generation.

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