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

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Abstract Molinate, a thiocarbamate chemical is a slightly to moderately toxic herbicide in EPA (Environmental Protection Agency) toxicity class III, and is a registered as a General Use Pesticide (GUP). *Bombina orientalis* is one of the most common amphibians in the world and comprise a large proportion of their total number in Korea. *B. orientalis* spawns in the rice fields at spring when the massive application of agricultural chemicals occurs. In the present study, we examined the effects of molinate on embryonic survival and developmental abnormality in *B. orientalis* embryos. The difference in survival rate between vehicle control and molinate treated embryos was not observed until the blastula stage. The first statistically significant decrease in embryonic survival was observed at mouth open stage following exposure to 100 μM molinate (46.8% vs. 81.1% in control). When the embryos develop to tadpole stage survival was significantly decreased at 50 μM molinate (35.9% vs. 68.9% in control), suggesting that the lowest observed effective dose (LOED) for systemic toxicity in *B. orientalis* embryos is 50 μM. In survived embryos molinate exposure produced several types of severe developmental abnormalities in order of frequency with bent trunk, neurula with yolk plug, bent tail, tail dysplasia, ventral blister, eye dysplasia, thick-set body and cephalic dysplasia. This suggests that molinate targets multiple events in embryonic and larval development in this frog species. Together this suggested that molinate was detrimental for survival and development following zygotic transcription after midblastula transition in *B. orientalis* embryos.

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Molinate is a thiocarbamate herbicide widely used for weed control is in agricultural crops worldwide. It is toxic to germinating broad-leaved weeds and barnyard grass, possibly by inhibiting cell division during mitosis (Kuroda et al. 1992; Tomlin 2000). Molinate is available in granular and emulsifiable liquid formulations. Trade names of commercial herbicides containing molinate include Molinate, Hydram, Ordram, and Yalan. Molinate is a slightly to moderately toxic compound in EPA (Environmental Protection Agency) toxicity class III, and is a registered as a General Use Pesticide (GUP). The metabolism of molinate is primarily by three routes that carbon oxidation, sulfur oxidation, and thiocarbamate cleavage (Ellis et al. 1998). The sulfoxidation metabolite of molinate was capable of eliciting gonadal damage, suggesting that metabolic activation via sulfoxidation could be important in toxicity (Jewell and Miller 1998). A number of studies showed that molinate and its metabolites affect animal health. In mammals, molinate has an adverse effect on reproduction (Cooper et al. 1994; Stoker et al. 1996; Ellis et al. 1998; Jewell and Miller 1998; Jewell et al. 1998; Wickramaratne et al. 1998; Kavlock and Cummings 2005). Molinate was reported to have systemic toxicity larval as well as adult fishes. For example, 96-h LC50 value of molinate is 0.21–41.8 mg L^{-1} in common carp, striped bass, rainbow trout, goldfish, *Melanotenia fluviatilis*, eels (Hartley and Kidd 1983; Finlayson and Faggella 1986; Meister 1991; Pena-Llopis et al. 2001; Phyu et al. 2006). In fish larvae 9.7 mg L^{-1} of molinate caused reductions in swimming capacity (Heath et al. 1993a, b, 1997). Furthermore, laboratory and field studies showed molinate significantly

delayed development at concentrations 3.6 mg L^{-1} and above (Burdett et al. 2001).

In Korea, the use of several formulation types of molinate has sharply increased as labor shortage in the rural community decreased since 1976 and to date contributes to about 30% of the total herbicide used in Korea to date (Park et al. 2005). *Bombina orientalis* is one of the most common amphibians in the world and comprise a large proportion of their total number in Korea. *B. orientalis* spawns in the field and ponds in the farming regions where the massive application of thiocarbamate herbicide occurs. Therefore use of molinate in agricultural land may potentially threaten embryonic, larval and adult life of this frog species. To date, however, embryotoxic, developmental, and teratogenic effects of molinate has not been studied in amphibian. In the present study we firstly report the toxicity of molinate on the survival and development of *B. orientalis* embryos and tadpoles.

Materials and Methods

Molinate (CAS No. 2212-67-1, purity >99.0%) 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 200 IU of human chorionic gonadotropin (hCG) (Cat No. CG5, Sigma, St Louis, MO) 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 150 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 518 embryos are subjected to bioassay. Embryos from the same female were randomly placed in small aquarium and exposed to varying concentration of molinate (5, 10, 50, and 100 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

The survival of embryos exposed to molinate decreased with concentration dependent manner. The difference in survival rate between vehicle control and molinate treated embryos was not observed until the blastula stage. When exposed to molinate for 312 h, survival rates of embryos gradually decreased according to increasing concentrations of molinate (0, 5, 10, 50, and 100 μM). Following 100 μM molinate treatment the embryonic survival was significantly decreased compared with vehicle control at 144 h onward. At 50 μM molinate, embryonic survival was significantly decreased compared with vehicle control at 192 h onward (Table 1). Following molinate treatment, survived embryos showed various developmental abnormalities including bent trunk, neurula with yolk plug, bent tail, tail dysplasia, ventral blister, eye dysplasia, thick-set body, and cephalic dysplasia (Fig. 1). The incidence of developmental abnormalities increased with increasing concentration of molinate. At the end of culture the frequency of developmental abnormalities was significantly higher in 100 μM molinate treated embryos compared with vehicle control and others (Fig. 2). The malformation was in order of frequency with bent trunk, neurulae with yolk plug, bent tail, tail dysplasia, ventral blister, eye dysplasia, thick-set body, and cephalic dysplasia. Frequency of bent trunk 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 exposure to molinate, the survival of *B. orientalis* embryos decreased together with an increase in the various developmental abnormalities. When the embryos were exposed to

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

Molinate (μM)	No. of embryo	Surviving embryos (%)							
		Time after fertilization (h) and development stage							
		24 Lb	48 Np	72 Tb	96 Mr	144 Mo	168 Tc	192 Tc	312 Oc
0	106	105 (99.1)	105 (99.1)	98 (92.5)	87 (82.1)	86 (81.1)	78 (73.6)	73 (68.9)	68 (64.2)
5	103	103 (100)	101 (98.1)	97 (94.2)	84 (81.6)	78 (75.7)	68 (66.0)	63 (61.2)	54 (52.4)
10	112	112 (100)	106 (94.6)	93 (83.0)	85 (75.9)	65 (58.0)	59 (52.7)	50 (44.6)	49 (43.8)
50	103	103 (100)	99 (96.1)	89 (86.4)	76 (73.8)	56 (54.4)	55 (53.4)	37* (35.9)	34* (33.0)
100	94	94 (100)	87 (92.6)	78 (83.0)	67 (71.3)	44* (46.8)	43* (45.7)	27** (28.7)	22** (23.4)

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 (* $p < 0.05$, ** $p < 0.005$)

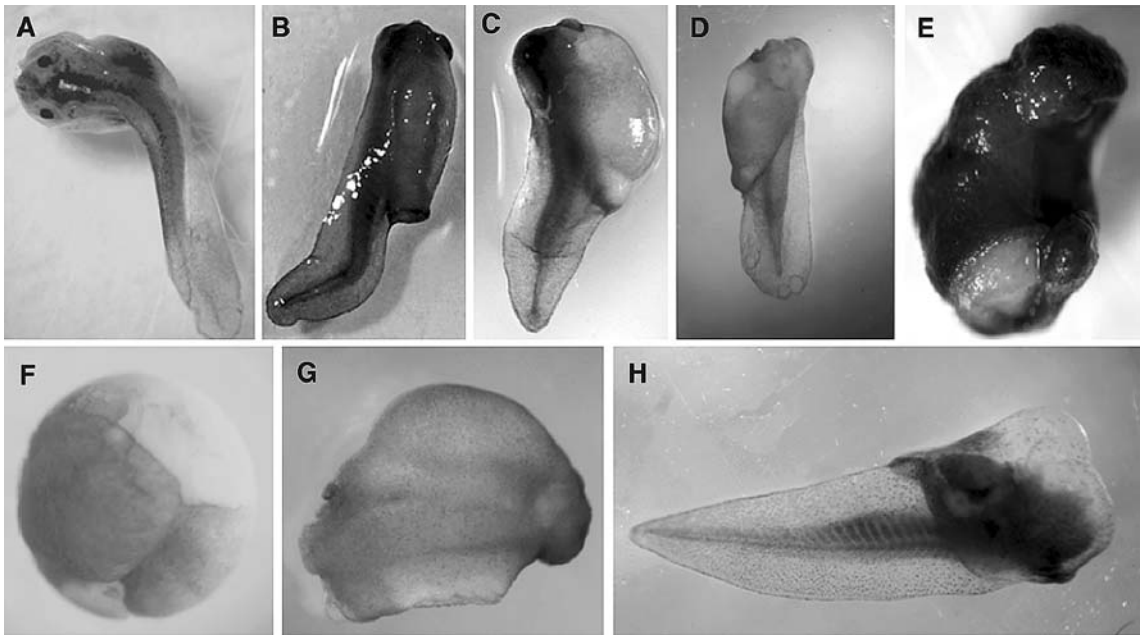

Fig. 1 Various abnormalities in embryos and tadpoles of *B. orientalis* following molinate treatment. **a** Bent trunk. **b** Bent tail. **c** Ventral blister. **d** Eye dysplasia. **e** Tail dysplasia. **f** Neurulae with yolk plug. **g** Thick set body. **h** Cephalic dysplasia

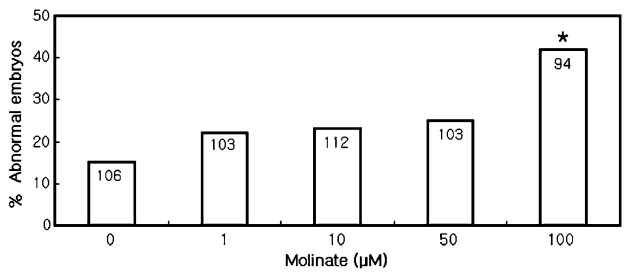

Fig. 2 Percent of abnormality in *B. orientalis* embryos after treatment of varying concentration of molinate for 13 days. Numbers inside the bars are total number of embryos examined. *Significantly different from vehicle control by Fisher's exact test ($p < 0.005$)

molinate from fertilized egg to tadpole stage systemic toxicity in *B. orientalis* embryos was not observed until the blastula stage. This suggests that molinate did not affect development to blastula stage but hampered developmental program after the onset of zygotic transcription at mid-blastula transition and that embryo becomes more sensitive to exposure to molinate. The statistically significant difference in the survival rate between vehicle control and molinate treated group was first seen at mouth open stage at 100 μM. At 50 μM molinate, embryonic survival was apparent at tail fin circulation stage onward. However below 50 μM concentration molinate did not evoked statistically significant difference in survival in embryos. Together this suggests that molinate is embryonic toxicant in amphibians and that the lowest observed effective dose (LOED) for systemic toxicity in *B. orientalis* embryos is 50 μM. To date embryotoxic effects of molinate has not been studied in amphibian. Consequently, our result on the embryonic toxicity for molinate cannot be compared with

Table 2 Frequency of abnormal embryos after molinate treatment

Abnormalities	Molinate (μM)					
	0	5	10	50	100	Sum (%)
Bent trunk	6	11	11	3	8	39*
Neurulae with yolk plug	0	4	4	12	6	26
Bent tail	2	3	1	2	8	16
Tail dysplasia	0	3	0	4	7	14
Ventral blister	2	0	6	1	5	14
Eye dysplasia	0	2	2	0	4	8
Thick-set body	2	0	2	4	0	8
Cephalic dysplasia	4	0	0	0	1	5
No. of abnormal embryos (%)	16 (15.1)	23 (22.3)	26 (23.2)	26 (25.2)	39** (41.5)	130 (25.1)
No. of test embryos	106	103	112	103	94	518

* Significantly different from other malformation type by Fisher's exact test ($p < 0.0001$)

** Significantly different from vehicle control by Fisher's exact test ($p < 0.005$)

other amphibian species. Similarly, in adult fishes, molinate was reported to have systemic toxicity. For example, 96 h LC50 values of molinate is 0.21 mg L^{-1} for the common carp, 12.1 mg L^{-1} for striped bass (Finlayson and Faggella 1986), 1.3 mg L^{-1} for rainbow trout (Meister 1991), 30 mg L^{-1} for goldfish (Hartley and Kidd 1983), 7.9–14.8 mg L^{-1} in rainbowfish (*M. fluviatilis*) (Phyu et al. 2006) and 41.8 mg L^{-1} for eels (*A. anguilla*) (Pena-Llopis et al. 2001). In newly hatched fathead minnow (*Pimephales promelas*) larvae and striped bass larvae 9.7 mg L^{-1} of molinate caused reductions in swimming capacity but not in the medaka (Heath et al. 1993a, b, 1997). In aquatic invertebrates, molinate significantly delayed development at concentrations 3.6 mg L^{-1} and above (Burdett et al. 2001).

Taken together this suggests that there is a developmental stage as well as species difference in the sensitivity to molinate among the aquatic lives and that *B. orientalis* embryos is relative sensitive to molinate. It is worth noting that the types of developmental abnormalities observed were diverse in order of frequency with bent trunk, neurulae with yolk plug, bent tail, tail dysplasia, ventral blister, eye dysplasia, thick-set body, and cephalic dysplasia following exposure to molinate. This suggests that molinate targets multiple events in embryonic development in this frog species. The pattern of malformation following molinate exposure is similar to that found in herbicide alachlor treated embryos but different from that found in the endosulfan treated embryos in which tail dysplasia is the major malformation type (Kang et al. 2005, 2008). This suggests that mechanism for developmental toxicity of molinate on *B. orientalis* embryos is similar to that of the alachlor. In my knowledge this the first report on the developmental toxicity of molinate in amphibian.

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