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Effect of Carbaryl on Survival and Development in *Bombina orientalis* (Boulenger) Embryos

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Abstract *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 farming regions at Spring when the massive application of agricultural chemicals occurs. Carbaryl, carbamate chemical is a slightly to highly toxic insecticide inhibiting acetylcholinesterase. The embryotoxicity and teratogenic effects of carbaryl on *B. orientalis* embryos were investigated at 5, 10, 50 and 100 μM. The survival rates of embryos at 312 h post fertilization were decreased with concentration dependent manner. Exposure to carbaryl produced 4 types of severe external abnormalities such as bent trunk, thick-set body, bent tail and ventral blister. At 5 μM carbaryl, a dose of no observed effect on embryonic survival, developmental abnormalities were significantly increased. The developmental abnormalities showed in order of frequency with bent trunk, thick-set body, bent tail and ventral blister. This result suggests that carbaryl is detrimental for embryonic survival and teratogenic by causing the axial skeletal defects in *B. orientalis* embryos.

Keywords Carbaryl · Embryo toxicity · Teratogenicity · *Bombina orientalis*

Many amphibian species breed in aquatic habitats such as farmland, consequently, their larvae may be exposed to endocrine disruptor chemicals (EDCs) such as insecticide and herbicide at some time during development. Therefore, EDCs may advance to amphibian population declines. Carbaryl (1-naphthyl-N-methylcarbamate) is the widely used insecticide in the carbamate chemical family and it controls over 100 species of insects on fruit, cotton, forests and many other crops, as well as on poultry, livestock, and pets (Mathew et al. 1995; Bacchetta et al. 2008). Carbaryl is available in granules, dispersions, and suspensions formulations. Trade names of commercial insecticides containing carbaryl include Carbamec, Tercyl, Sevin, and Nac. Carbaryl is a General Use Pesticide (GUP). However, various formulations vary widely in toxicity. For example, it is categorized as toxicity class I—highly toxic for Tercyl; toxicity class II—moderately toxic for Sevin 803; and toxicity class III—slightly toxic for some other products. The carbaryl poisoning showed that it generally causes inhibition of acetylcholinesterase (AChE), a key enzyme in the insect nervous system (O'Malley 1997), causing the accumulation of acetylcholine (Ach) in the synaptic cleft because of impairs the hydrolysis of Ach (Ecobichon 2001).

During past decade, decrease in amphibian populations became a global phenomenon (Alford and Richards 1999; Houlahan et al. 2000; Davidson 2004). In aquatic ecosystems, a number of studies showed that carbaryl and carbamates affect amphibian health (Bacchetta et al. 2008; Bridges 1997, 2000; Davidson et al. 2007; Relyea and Mills 2001; Relyea 2003) The toxicity of carbaryl reported LC50 values to range from 2.5 to 20.6 mg L^{-1} and showed great differences among species (Bacchetta et al. 2008; Boone and Bridges 1999; Boone and Semlitsch 2001, 2002; Bridges 1997, 2000; Bridges et al. 2002; Relyea 2003, 2004; Zaga et al. 1998). Bacchetta et al. (2008) reported that carbaryl induced axial-skeletal damage in *Xenopus laevis* embryos is caused by both inhibition of AChE with consequent muscular tetanic spasms and disorders in the organization of the connective tissue matrix

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surrounding the notochord. In southern leopard frog (*Rana sphenocephala*) tadpoles, carbaryl delayed metamorphosis and decreased body size at metamorphosis (Bridges 2000) and reduced swimming performance in plains leopard frog tadpoles (*Rana blairi*) (Bridges 1997). In foothill yellow-legged frogs (*Rana boylii*), skin peptide defenses were significantly reduced and susceptibility to disease increased after exposure to carbaryl, suggesting that carbaryl may inhibit innate immune defense (Davidson et al 2007). *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 spawns in the rice field and ponds in the farming regions and uses water in lakes, ponds, swamps, streams and springs. These regions are where the massive application of carbamate insecticide occurs. Several herbicides and insecticides have been known to toxic in the embryonic, larval and adult life of this frog species (Kang et al. 2005, 2008, 2009). To date, however, embryotoxic and teratogenic effects of carbaryl has not been studied in *B. orientalis*. In the present study we examined the effects of carbaryl on the survival and development of *B. orientalis* embryos and tadpoles.

Materials and Methods

Carbaryl (CAS No. 63-25-2) 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; Daesung Microbiological Labs., Kyonggi-Do, Korea) 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 was performed 2 h post fertilization to remove the unfertilized and necrotic eggs. Shortly after fertilization embryos were selected for drug treatment. Totally 463 embryos are subjected to bioassay. Embryos from the same female were randomly placed in small aquarium and exposed to varying concentration of carbaryl (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.

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

Carbaryl (μM)	No. of embryos	Surviving embryos (%)							
		Time after fertilization (h)							
		24 Lb	48 Np	72 Tb	96 Mr	144 Mo	168 Tc	192 Tc	312 Oc
0	92	91 (98.9)	87 (94.6)	84 (91.3)	81 (88.0)	66 (71.7)	66 (71.7)	64 (69.6)	59 (64.1)
5	92	87 (94.6)	80 (87.0)	74 (80.4)	70 (76.1)	42 (45.7)	42 (45.7)	42 (45.7)	35 (38.0)
10	95	93 (97.8)	83 (87.3)	74 (77.9)	65 (68.4)	31 (32.6)**	28 (29.5)**	27 (28.4)**	26 (27.4)**
50	92	87 (94.6)	71 (77.2)	58 (63.0)	52 (56.5)	26 (28.2)**	24 (26.0)**	23 (25.0)**	20 (21.7)**
100	92	86 (93.4)	67 (72.8)	51 (55.4)*	48 (52.1)*	12 (13.0)**	9 (9.7)**	9 (9.7)**	8 (8.7)**

Lb Late blastula; *Np* Neural plate; *Tb* Tail bud; *Mr* Muscle response; *Mo* Mouth open; *Tc* Tail fin circulation; *Oc* Operculum complete

* Significant different from vehicle control by Fisher's exact test ($p < 0.05$)

** $p < 0.005$

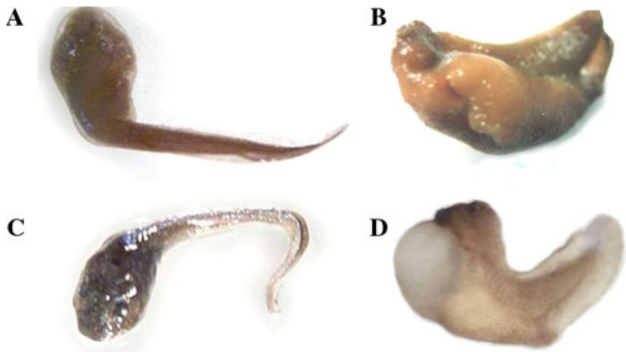

Fig. 1 Various abnormalities in embryos and tadpoles of *B. orientalis* following carbaryl treatment. **a** Bent trunk. **b** Thick-set body. **c** Bent tail. **d** Ventral blister

Results and Discussion

Four hundred sixty three embryos were used in total. The survival rates of embryos exposed to carbaryl decreased with concentration dependent manner. When exposed to carbaryl for 312 h, survival rates of embryos at concentrations of 0, 5, 10, 50 and 100 µM were 64.1, 38.0, 27.4% ($p < 0.005$), 21.7% ($p < 0.005$) and 8.7% ($p < 0.005$), respectively. The survival rates of embryos were significantly lower than vehicle control at 72 h onward in exposure to carbaryl (100 µM). In exposure to carbaryl (10 and 50 µM), embryonic survival was significantly decreased compared to vehicle control at 144 h onward (Table 1). When exposed to carbaryl, survived embryos showed various developmental abnormalities in order of frequency with bent trunk, thick-set body, bent tail and ventral blister (Fig. 1, Table 2). At the end of culture, frequency of developmental abnormalities in 5 µM carbaryl treated embryos was higher than those of vehicle control (Table 3).

The amphibian embryo is an intact developing system, which undergoes events comparable to those of other vertebrates, including mammals. Teratogenesis assay using amphibian embryo is useful because they can rapidly provide information on developmental toxicants. In exposure to carbaryl, the survival of *B. orientalis* embryos was decreased with concentration dependent manner. The lowest observed effective dose (LOED) for embryonic toxicity for carbaryl is 10 µM at 144 h in *B. orientalis* embryos. In the previous studies, carbaryl has been reported to have toxic effects in some frogs and showed LC50 values to range from 2.5 to 20.6 mg L^{-1} (Bacchetta et al. 2008; Boone and Bridges 1999; Boone and Semlitsch 2001, 2002; Bridges 1997, 2000; Bridges et al. 2002; Relyea 2003, 2004; Zaga et al. 1998). The 96-h LC50 values of carbaryl for frogs include 20.28 mg L^{-1} for *X. laevis* (Bacchetta et al. 2008), 2.47 mg L^{-1} for gray treefrogs (Zaga et al. 1998), 8.4 mg L^{-1} for southern leopard frog (Bridges et al. 2002) and 12.31 mg L^{-1} for boreal toad (USEPA 1999). Our results, in *B. orientalis*, 96-h LC50 value was about 100 µM (20.1 mg L^{-1}), which is similar to value found in *X. laevis*. When the embryos were exposed to 100 µM carbaryl during development from fertilized egg to tadpole stage systemic toxicity in *B. orientalis* embryos was not observed until the blastula stage. This suggests that carbaryl does not affect early development to blastula stage but hamper developmental program after the onset of zygotic transcription at midblastula transition. Thereafter *B. orientalis* embryo becomes more sensitive to exposure to carbaryl.

The types of developmental abnormalities observed were in order of frequency with bent trunk, thick-set body, bent tail and ventral blister following exposure to carbaryl. These abnormalities are most typical axial-skeletal defects. Similarly, in anurans, carbaryl reduced swimming performance in tadpoles because of notochord bending and bent tail (Bridges 1997, 2000; Venturino et al. 2003). AChE is a known target for both organophosphorous and carbamate insecticides and their toxicological effects are very similar. Organophosphorous and carbamate cause axial-muscular abnormalities and axis tail folding in several frog species (Bacchetta et al. 2008; Calumpang et al. 1997; Fulton and Key 2001; John et al. 2003; Karalliede and Henry 1993). AChE inhibition levels and malformation rate increase together by carbaryl (Bacchetta et al. 2008), suggesting that

Table 2 Frequency of abnormal embryos after carbaryl treatment

Abnormal conditions	Carbaryl (µM)					
	0	5	10	50	100	Sum
Bent trunk	10	13	3	5	0	31
Thick-set body	0	3	3	5	2	13
Bent tail	2	2	3	1	0	8
Ventral blister	1	2	2	3	0	8
No. of abnormal embryos (%)	13 (22.0)	20 (57.1)	11 (42.3)	14 (70.0)	2 (25.0)*	60 (13.0)
No. of test embryos	92	92	95	92	92	463

* Significantly different from vehicle control by Fisher's exact test ($p < 0.05$)

Table 3 Major developmental abnormalities of embryos exposed to carbaryl

Abnormal conditions	No. of abnormal embryos	% of abnormal embryos
Bent trunk	31	51.7
Thick-set body	13	21.7
Bent tail	8	13.3
Ventral blister	8	13.3
Total	60	100.0

axial-skeletal defects following carbaryl exposure may attribute to the AChE inhibition in *B. orientalis* embryos. At 10 μM carbaryl, LOED for survival of *B. orientalis* embryos and developmental abnormalities were significantly increased. However, at 5 μM carbaryl, a dose of no observed effect on embryonic survival, developmental abnormalities were significantly increased. This suggests that carbaryl can be teratogenic even below the LOED in *B. orientalis* embryos. Similarly, endosulfan, an AChE inhibitor, represents LOED for embryonic survival at 10 μM but is teratogenic at 5 μM in *B. orientalis* embryos (Kang et al. 2008). We suggest that the safety guideline for carbaryl in environmental media should be below the 10 nM, one thousandth of LOED for embryonic survival of *B. orientalis*.

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