Bull. Environ. Contam. Toxicol. (2005) 74:1199–1206

DOI: 10.1007/s00128-005-0708-x

Bulletin of Environmental Contamination and Toxicology

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

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Received: 30 October 2004/Accepted: 14 March 2005

The chloroacetanilide herbicide alachlor (2-chloro-2',6'-diethyl-N-(methoxymethyl)-acetanilide) was introduced in 1969 for the pre-emergence control of a broad spectrum of grass, sedge, and broadleaf weeds in corn, soybeans, and other crops (Hayes and Laws 1991). It is a selective systemic herbicide, absorbed by germinating shoots and by roots. It works by interfering with a plant's ability to produce protein and by interfering with root elongation. This compound is one of the most highly used herbicides in the United States. Over 70 million pounds of alachlor were used annually (EPA 1989). Trade names of commercial herbicides containing alachlor include Alanex, Bronco, Cannon, Crop Star, Lariat, Lasso, and Partner. It mixes well with other herbicides such as Bullet, Freedom, and Rasta, and is found in mixed formulations with atrazine, glyphosate, trifluralin, and imazaquin. As is the case with alachlor, overexposure to these chemicals has the potential to pose health risks to humans. Exposure to alachlor can occur via several routes, including the diet and drinking water (Holden et al. 1992; Dearfield et al. 1999). Alachlor is a restricted use pesticide (RUP). RUPs may be purchased and used only by certified applicators. The U.S. Environmental Protection Agency (USEPA) categorizes it as toxicity class III - slightly toxic. To date, toxic effects of alachlor have been described in a range of organisms. In mammals alachlor induces olfactory mucosal tumors (Wetmore et al. 1999; Genter et al. 2002a, b; Burman et al. 2003) and was associated with cancer of the thyroid (Wilson et al. 1996; Heydens et al. 2000), and stomach (Heydens et al. 1999). Also, it is selectively induces cytochrome P450 isoforms of the CYP1A and CYP2B subfamilies in rat liver microsomes (Hanioka et al. 2002), and expression of these isoforms is closely related to the toxicity of alachlor (Coleman et al. 1999). However, there has been limited investigation of alachlor-induced toxicity in amphibians, which may be directly affected by this herbicide as they

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live on farmland and in the surrounding aquatic environment. Also, alachlor has embryotoxic, developmental, and teratogenic effects in *Xenopus* (Osano et al. 2002). The frog embryo is an intact developing system, which undergoes developmental events comparable to those of other vertebrates, including mammals.

Bombina orientalis is one of the most common amphibians in the world and comprises a large proportion of their total number. *B. orientalis* is distributed in Northeastern China, Korea, Southern Japan and the Khabarovsk and Primorye regions in Russia. This species lives in mixed coniferous-broad-leaved forests and uses water in lakes, ponds, swamps, streams, and springs. *B. orientalis* spawns in rice fields and ponds in the farming regions from March to April, when the wide application of chloroacetanilides occurs. Therefore use of alachlor in farmland may threaten the reproduction of this species. After major spawning in early spring, females develop eggs and spawn throughout the summer. Males also produce spermatozoa from the spring to autumn before hibernation. In the laboratory, it is easy to rear and to collect the mature gametes by injection of gonadotropin throughout the year. Therefore this species offers potential as model organism for the study of effects of suspect environmental toxicants on an aquatic ecosystem. In an effort to develop a model species for testing the toxic effects of environmental pollutants in amphibia in Korea, we examined the embryotoxic, developmental, and teratogenic effects of alachlor in *B. orientalis* embryos.

MATERIALS AND METHODS

Alachlor (CAS No. 15972-60-8) was obtained from Riedel-de Haen (Seelze, Germany) and dissolved in ethanol. *B. orientalis* adults were collected in Hongcheon (Gangwon-Do, Korea) and reared 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: 10h light: dark cycle and at 20-22℃. 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 testis in a 1×MMR solution. Subsequently, eggs sat for 15 minutes and were then covered completely with 0.1×MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO4, 0.2 mM CaCl2, 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 575 embryos are subjected to bioassay. Embryos from the same female were randomly placed in a small aquarium and exposed to varying concentration of alachlor (5, 10, 20, and 40 μM in 0.00004% ethanol) in 0.5 L of 0.1×MMR solution. In the control group, 0.00004% (v/v) ethanol was present. Experiment was replicated three times. The embryos were cultured in an incubator (MIR550, Sanyo, Japan) at 18℃ 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 rates of embryos exposed to alachlor decreased in a concentration-dependent manner ($P<0.0001$). Alachlor at 5 μM significantly decreased survival of the embryos at 72 h post-exposure initiation when most of embryos had reached the neurula stage. 40 μM alachlor was lethal to all test embryos at 312 h exposure, when the normal control embryos had reached the tadpole stage (Table 1). When exposed to alachlor, the incidence of developmental abnormalities increased with increasing alachlor concentration (Table 2). These abnormalities are bent trunk, ventral blister, bent tail, tail dysplasia, eye dysplasia and thick-set body in order of frequency in the test embryos (Table 3 and Fig. 1). Following exposure to alachlor, the types of abnormalities were diverse, suggesting alachlor targets multiple events in embryonic and larval development in this species.

Alachlor is embryotoxic and teratogenic in *Xenpous*, with 23 μM alchlor causing 50% embryo lethality at 96 h of exposure, and the most frequent developmental disorders were edema, axial flexures and eye abnormalities (Osano et al. 2002). It has been proposed that the genotoxicity of many organic pesticides could be mediated by their alkylating potentials. Since alachlor has alkylating radicals, it may act as a DNA alkylating agent (Surralles et al. 1995). Alchlor has been known to be oncogenic and form adducts with DNA (Hoberg 1990; Nesnow et al. 1995). Alachlor strongly induces excision repairable DNA lesions. It was also found, by use of fluorescence in situ hybridization and an antikinetochore antibody, that alachlor is a clastogen acting in the S phase (Surralles et al. 1995). Consistent with earlier observations, the present study shows that alachlor is a suspect teratogen in *B. orientalis*.

The use of environmental xenobiotics is increasing every day. We are now all dependent on synthetic substances in agriculture, pharmacy, petrochemical and food industries. The use of herbicides in agriculture may lead to contamination of surface and ground waters by drift, runoff, drainage and leaching. Surface water contamination may have ecotoxicological effects for aquatic fauna and for human health. Alachlor is one of the most widely used herbicides in agriculture in Korea and worldwide. In US, alachlor was detected in ground water (Iowa, Nebraska, Pennsylvania, and Maryland) at concentrations between 1 (0.0037 μM) and 10 ㎍/L (0.037 μM) (Cohen et al. 1986). In Portugal, 13 ㎍/L (0.048 μM) of alachlor was detected from ground water (Cerejeira et al. 2003). Alachlor has been classified as human carcinogen and its maximum contaminant level (MCL) was established at 2 ㎍/L (0.0074 μM) by the U.S. Environmental Protection Agency (USEPA) in 1992. Accordingly, alachlor in soil and the surrounding aquatic environment could induce lethal or malformation by coming into direct contact with amphibian embryos. Moreover, frog embryos may be exposed to metabolites of alachlor maternally during oogenesis via the food chain. Therefore, the use of alachlor for agricultural purposes has the potential to cause direct harm during early embryogenesis in amphibians inhabiting contaminated aquatic habitats in the vicinity of farmlands. Until present, environmental contamination of surface water by alachlor was not studied in Korea. However, the present study revealed that alachlor even at 5 μM concentration was detrimental for survival and development of *B. orientalis* embryos. Given the surface water is contaminated by alachlor much more than the ground water, the criteria for establishment of

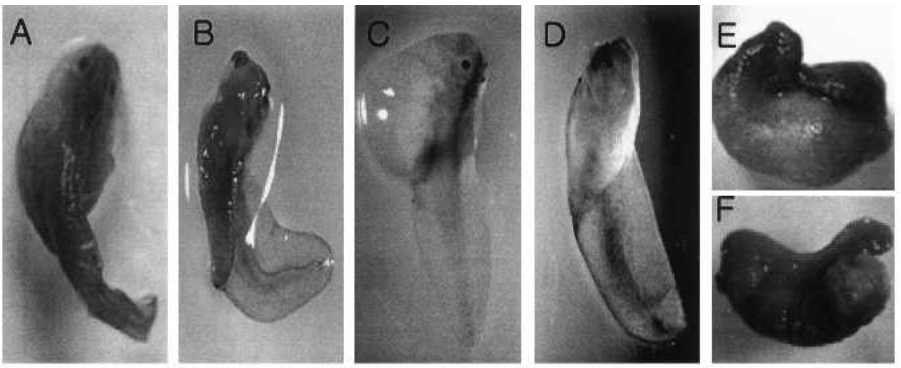

Figure 1. Various abnormalities in embryos and tadpoles of *B. orientalis* following alachlor treatment. (A) Bent trunk, (B) Bent tail, (C) Ventral blister, (D) Eye dysplasia, (E) Thick-set body, (F) Tail dysplasia.

alachlor safety should be considered at both acute and chronic exposures in frogs. Furthermore, the present study may provide additional clues on factors causing the decline in populations of amphibians worldwide and may explain the increased incidence of abnormalities in the natural populations of frog species.

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

Alachlor (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	119	118 (99.2)	111 (93.3)	109 (91.6)	105 (88.2)	101 (84.9)	101 (84.9)	99 (83.2)	97 (81.5)
5	116	112 (96.6)	104 (89.7)	94* (81.0)	89* (76.7)	87 (75.0)	87 (75.0)	84 (72.4)	79* (68.1)
10	107	103 (96.3)	94 (87.9)	84* (78.5)	78* (72.9)	76* (71.0)	75* (70.1)	71* (66.4)	60* (56.1)
20	119	108* (90.8)	93* (78.2)	85* (71.4)	56* (47.1)	50* (42.0)	50* (42.0)	47* (39.5)	32* (26.9)
40	114	106* (93.0)	94* (82.5)	57* (50.0)	39* (34.2)	31* (27.2)	30* (26.3)	24* (21.1)	0* (0)

Survival of embryos are significantly different among the groups by chi square test ($P<0.0001$). * Significantly different from control by Fisher's exact test ($P<0.05$). Lb: Late blastula, Np: Neural plate, Tb: Tail bud, Mr: Muscle response, Mo: Mouth open, Tc: Tail fin circulation, Oc: Operculum complete.

Table 2. Frequency of abnormal embryos after alachlor treatment.

Abnormal conditions	Alachlor (μM)					
	0	5	10	20	40	Sum
Bent trunk	0	9	9	27	16	61
Ventral blister	1	7	7	13	12	40
Bent tail	1	0	5	18	9	33
Tail dysplasia	1	3	3	8	8	23
Eye displasia	1	2	2	4	4	13
Thick-set body	0	1	0	2	2	5
No. of abnormal embryos (%)	4 (3.36)	22* (18.97)	26* (24.3)	72* (60.5)	51* (44.74)	175* (30.43)
No. of test embryos	119	116	107	119	114	575

Frequency of abnormal embryos are significantly different among the groups by chi square test ($P<0.0001$). * Significantly different from control by Fisher's exact test ($P<0.05$).

Table 3. Major developmental abnormalities of embryos exposed to alachlor.

Abnormal conditions	No. of abnormal embryos	% of abnormal embryos
Bent trunk	61	34.9
Ventral blister	40	22.9
Bent tail	33	18.9
Tail dysplasia	23	13.1
Eye dysplasia	13	7.4
Thick-set body	5	2.9
Total	175	100.0

Acknowledgment. This study was supported by grants from the Korean Research Foundation (KRF-2002-005-C00019).

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