EFFECTS OF ALACHLOR ON THE EARLY DEVELOPMENT AND INDUCTION OF ESTROEN-REPONSIVE GENES IN MEDAKA, Oryzias latipes

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Introduction

Alachlor is an acetanilide herbicide used to control annual grasses and weeds in field corn, soybeans, and peanuts. It is a selective systemic herbicide, absorbed by germinating shoots and by roots[1)]. Although the specific pathways are not exactly understood, the acetanilide herbicides apparently interfere with several physiological processes including biosynthesis of lipids, proteins and flavonoids[2)]. These herbicides are widely used in agriculture and are commonly detected in surface water and groundwater[3)]. Alachlor has a relatively low acute toxicity, however, repeated exposure has been reported to cause hepatotoxicity, irreversible uveal degeneration and tumour formation in some animals[4, 5)]. Besides alachlor is one of the herbicides reported to have endocrine disrupting effects. 2,4-D, 2,4,5-T, amitrole and atrazine also belong to these types of herbicides. Alachlor is a strongly suspected endocrine disruptor in that it is listed by EPA and the World Wildlife Fund [WWF] as a potential endocrine disrupting chemical[6)]. Many mammalian and aquatic toxicological studies with alachlor were performed under the conditions of acute, subacute and chronic experiment[7)]. However, not many studies using fish have been carried out with the purpose of screening and testing of endocrine disrupting effects of alachlor.

The purpose of this study was to determine the effects of alachlor on the early morphological development of medaka (*Oryzias latipes*). Embryonic growth, deformation and hatching success were determined to see the effects of this chemical. Also, we tried to measure the estrogenic activity of alachlor using the ELISA and RT-PCR methods. By using these techniques, we

evaluated the induction of the estrogen-rsponsive genes, vitellogenin (precursor of yolk protein)[8] and choriogenin (precursor of egg envelope protein)[9] in male medaka exposed to alachlor.

Materials and Methods

Test organisms and exposure

The orange-red variety of the medaka (*Oryzias latipes*) used in this study was maintained at the Environmental Toxicology Laboratory, National Institute of Environmental Research in Korea. They were raised under constant 18:6-h (light:dark) cycles and a temperature of 25 ± 1°C. They were fed twice a day with artemia. The dechlorinated tap water was used for the maintenance of fish and the dilution of test solution. Embryos less than 24h post-fertilization were used in exposure experiment. Eggs spawned from each female fish were carefully collected within a few hours after fertilization in a petri dish, checked for normal fertilization and developmental stage under a stereoscopic microscope, and then subjected to chemical exposure. The 30 fertilized eggs in each control and treated group containing 30ml of each test solution were exposed to alachlor at nominal concentrations of 10, 20, 50, 100, 200 and 500 μg/L for 12 days. The test solution in the wells was changed every 24 hours. The development of the embryos was observed daily under stereoscopic microscope. Hatchability and time to hatching was calculated using data from all embryos. For the measuring of vitellogenin and choriogenin expression levels, sexually mature males with a mean weight of 250mg were exposed to alachlor at nominal concentrations of 10, 20, 50, 100, 200 and 500 μg/L for 7 days. Semistatic condition was applied for the exposure and 50% of the total solution volume was changed every 24 hours.

Measurement of vitellogenin protein

Vitellogenin (VTG) protein levels of male medaka were measured using enzyme-linked immunosorbent assay (ELISA) in a VTG assay kit specifically for medaka (*EnBio Corp*., Japan). The mesurement of blood VTG was performed according to manufacturer's manual.

Measurement of vitellogenin and choriognin mRNA

Total RNA was carefully extracted from the livers of control and treated males. For tissue disruption and preparation of total RNA, QIA-Shredder and RNeasy mini-kits (*QIAgene Corp*., Hilden, FRG) were used according to the manufactures' instructions. Both vitellogenin (VTG I)

and choriogenin L (ChgL) mRNAs were measured by reverse transcription-PCR (RT-PCR) using standard protocol. cDNA was synthesized with AMV reverse transcriptase using 0.5□ total RNA per 20□ reaction. VTGI and ChgL and β-actin (internal standard) fragments from 1□ cDNA for 30 cycles with *Taq*-polymerase in 50□ reaction volume. The sequences of VTG and ChgL primers were 5'- cactcatggctctgaggaa-3' (forward) and 5'- gcagagtaaagactcagttc-3' (reverse), and 5'-gccaaacctgtagtgccatt-3' (forward) and 5'-ctgctccactgacctccttc-3' (reverse), respectively[10]. The sequences of β-actin primers were 5'-ttcaacagccctgccatgta-3' (forward) and 5'-ataccgcaggactccata ccaa-3' (reverse). RT-PCR reactions were carried out using a program temperature control system (Perkin Elmer 2400). Aliquots of each amplified cDNA fragment were separated on the 1.0% agarose gel at 70V.

Results and Discussion

The fertilized eggs of the medaka were treated with alachlor at different concentrations and the types of lesion induced in embryos were examined. Alachlor at the concentrations tested did not have severe lethal effects on the early stages of embryos. However, embryonic development, hatchability and time to hatching of medaka eggs were affected by this chemical treatment. Hatchability of fertilized eggs exposed for 12 days was significantly decreased relative to the controls in the treatment group over 100□/L. Time to hatching was also delayed compared to the controls in the treatment groups with concentrations over 100□/L. All embryos not hatched within 15 days stopped growing and finally reached to death. According to the observation of embryos and larvae, a developmental inhibition was generally seen in the embryos which were exposed to alachlor. Thus the dwarf and reduced/uncolored eyeballs were found as main symptoms and the ones with these symptoms did not succeed properly in hatching. All adverse effects on embryos were shown within 10 days of exposure. A hindrance of the yolk sac absorption and edema were observed as major lesions in larvae from the treated groups. In summary, these results indicate that alachlor may disrupt the development and inhibit the growth in fish at early life stage. Fig. 1 shows the major types of lesions observed in embryos and larvae of medaka exposed to alachlor under stereoscopic microscope. Table 1 shows the mortality, hatch success, adverse effects and major types of lesions and deformations.

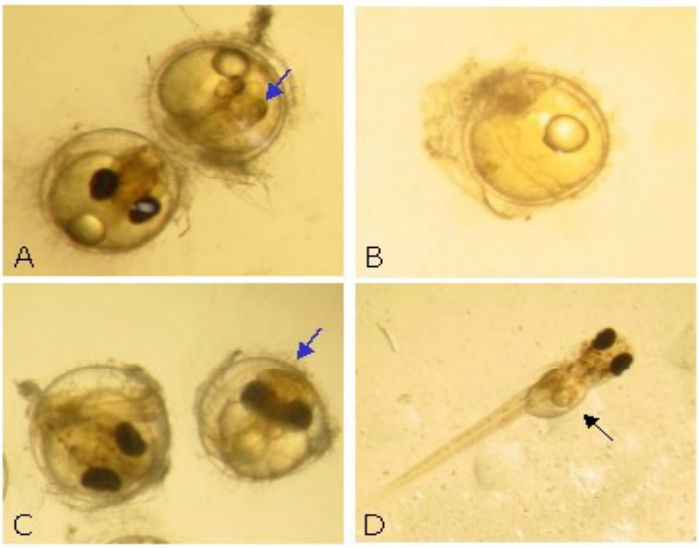

Fig. 1. Major types of abnormalities of embryos and larvae exposed to alachlor. A: uncolored eyes, B: Growth inhibition, C: Dwarf and reduced eyeballs, D: Edema and hindrance of egg yolk sac absorption

Table 1. Effects of exposure to different concentrations of alachlor on embryos and larvae

Conc. (μg /L)	+Mortality %	Hatch success %	*Adverse effects %	Major types of lesions and deformations
0	7(2/30)	90(27/30)	13(4/30)	-
10	10(3/30)	90(27/30)	13(4/30)	-
20	7(2/30)	87(26/30)	20(6/30)	-
50	13(4/30)	80(24/30)	20(6/30)	growth delay
100	13(4/30)	70(21/30)	33(10/30)	growth inhibition (dwarf)
200	27(8/30)	53(16/30)	43(13/30)	dwarf and reduced eyeballs peritoneal/visceral edema
500	40(12/30	33(10/30)	63(19/30)	dwarf and reduced eyeballs peritoneal/visceral edema egg yolk absorption inhibition

+ Mortality was measured from the dead embryos within 12 days after the chemical treatment.

*Adverse effecrs of medaka embryos include cardiovascular defect, hemorrhage, growth inhibition and death. Adverse effects of medaka embryos were observed within 12 days after the chemical treatment

Medaka males were exposed to alachlor ranging from 10 to 500□/L for 7 days to measure the vitellogenin and the choriogenin levels. However, alachlor could not induce the expression of these genes at all concentrations (Fig. 2). In case of ELISA, the plasma vitellogenin I (VTGI) proteins in treated groups were not increased in a dose-dependent manner. The maximum plasma VTGI levels were only slightly higher than those of the control group. Also VTGI and Choriogenin L (ChgL) mRNAs could not be induced in treated groups. At a concentration of 500□/L, the amplified cDNA bands (870bp of VTGI and 860 of ChgL) were so fainted that this concentration seemed hardly to induce these both genes. The 400bp β-actin cDNA fragments were approximately expressed equally in all treated and non-treated group. Thus, no differences concerning expression of β-actin could be observed in parallel in all test fish. There are two classes of synthetic chemicals reported to have estrogenic activity[11]. The first class, represented by compounds like DES, *o,p´*-DDT and methoxychlor, and contains coplanar rings with chlorine, hydroxy, or methoxy substituents. The second class contains multichlorinated caged-ring structures like chlordecone. However, alachlor is

not structurally similar to any of these chemicals[12]. Above results in the present study indicate that alachlor may not have an estrogenic potency. They have also demonstrated that the physical and toxicological properties of alachlor are very different from the environmental estrogenic chemicals, and may disrupt endocrine system through another mechanism such as a disruption of development.

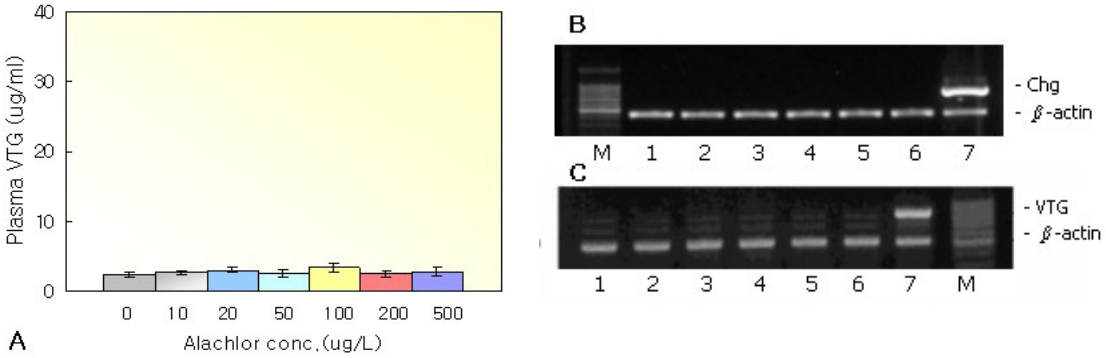

Fig 2. Vitellogenin and choriogenin gene expression in male medaka exposed to alachlor at different concentrations. A: Plasma vitellogenin levels in male medaka exposed to alachlor, B and C : Vitellogenin and choriogenin mRNA expression in medaka exposed to alachlor1: control, 2: 20µg/L, 3: 50µg/L, 4: 100µg/L, 5: 200µg/L, 6: 500µg/L, 7: spawning female

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