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Regulation of Acetylcholine Esterase and Monoamine Oxidase in *Oryzias Latipes* by Carbofuran

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카보푸란에 의한 송사리 acetylcholine esterase 및 monoamine oxidase의 활성조절

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요 약

카바메이트계 농약인 카보푸란은 어류에 대한 독성이 매우 높으며, 낮은 농도에서 어류의 척추기형이나 행동이상을 유발한다. 이러한 카보푸란의 독성기전을 밝히기 위한 일환으로 신경물질대사와 관련이 깊은 acetylcholine esterase (AChE)와 monoamine oxidase (MAO)에 미치는 농약의 효과를 송사리 (*Oryzias latipes*; Medaka fish)를 이용하여 평가하였다. Medaka fish에 대한 카보푸란의 반수치사농도(LC_{50})는 2.5 ppm이었으며, 1 ppb 카보푸란에 24시간 노출된 경우, AChE 효소활성이 머리와 몸통부위에서 각각 30, 20%씩 감소되었다. 한편, MAO 효소활성은 카보푸란의 농도가 증가함에 따라 머리부위에서는 감소한 반면, 몸통부위에서는 증가하는 경향을 보였다. 특히 카보푸란의 농도가 1 ppb에서도 송사리의 MAO 효소활성이 영향을 받는 것으로 나타나, 카보푸란에 의한 송사리의 행동이상은 AChE 활성 뿐 아니라 MAO활성의 변화에 의한 복합적인 효과일 가능성이 높다.

주요어 : Medaka fish, Carbofuran, Acetylcholine esterase, Monoamine oxidase

INTRODUCTION

Carbofuran, a pesticide containing carbamate group, was known to inhibit the choline esterase and psudo-choline esterase of insects. The pesticide was also reported to be toxic to freshwater fish, posing serious environmental concern in case it contaminates soil and stays long enough to be drained into rivers. Levels of pesticides measured in superficial waters

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generally range far below the lethal concentrations for aquatic organisms, but sublethal adverse effects may result from exposure to pesticides at environmentally relevant concentrations (Bretaud, 2000; El-Alfy, 2001).

Carbofuran is one of the most extensively used pesticide in Korea and thereby deserves careful monitoring of its contamination to freshwater although it is reported to be easily degraded and will not be accumulated in environment (Wilson, 2002).

In particular, rivers or dams are vulnerable to the contamination with several kinds of pesticides, and it will affect directly the quality of drinking water.

Recently, behavioral responses to sub-lethal doses of toxic chemicals have drawn attention as a means of developing an in situ biomonitoring tool for detecting toxic chemicals in the environment (Shin 2001; Kwak, 2002). For instance, the behavioral abnormalities including sheltering, burst swimming, and nipping were reported in gold fish exposed to carbofuran (Saglio, 1996). The behavioral changes in fish exposed to carbofuran may be closely associated with the interference of neurotransmitter metabolism by the pesticide. In fact, carbamate insecticides is known to carbamilate AChE enzyme (Fleming & Grue, 1981) in insects, result in the increase of acetylcholine levels at the nerve synapse and cause stimulation of the fibers with eventual failure of the nerve to repolarize (Dembele, 1999). Furthermore, there is a possibility that the carbamate interferes with the action of enzymes involved in the metabolism of other neurotransmitters than AChE. In particular, monoamine oxidase (MAO), one of key enzymes involved in the catabolism of neurotransmitters such as serotonin, dopamine, and norepinephrine, has been reported as the potential target of many pesticides (Chen, 1994). Although the action mechanism of carbofuran is well established in insects, it remained to be confirmed whether the same mechanism is working in fish.

The Japanese Medaka fish (*Oryzias latipes*) is one of the species used extensively for monitoring freshwater contamination. In particular, this model fish has been successfully used for evaluating the acute toxicity and studying metabolism of organophosphate pesticides (Tsuda, 1997; Hamm, 1998).

This study evaluated the effect of carbofuran on the AChE and MAO to explain behavioral change observed in Medaka fish exposed to the pesticide.

MATERIALS AND METHODS

Experimental animals and chemical exposure

Medaka fish (*Oryzias latipes*) was obtained from Toxicology Research Center, Korea Research Institute of Chemical Technology (Daejeon, Korea). Fish were held in a square glass chamber (40 × 22 × 40 cm) containing 30-liter of dechlorinated water (pH 6.5 ~ 7.3) with aeration. The fish were subjected to carbofuran treatment after starvation for 24 hr. Carbofuran (purity: 99%) was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and Medaka fish was treated with appropriately diluted concentrations.

Assays of enzyme activities

Acetylcholine esterase (AChE) assay was conducted on Medaka fish which were exposed to carbofuran at concentrations of 0.001, 0.01, 0.1, 0.1, 1, 5 mg/L for 24 hrs. After treatment, fish were immediately frozen in a liquid nitrogen until use. Right before starting assay, sample were divided into head and body parts which were homogenized in 45 mM phosphate buffer (pH 8.0) using a Polytron homogenizer (Brinkmann, Westbury, NY), centrifuged at 10,000 g for 10 min. The supernatant was used for enzyme assay. AChE activities of head and body were assayed in 45 mM phosphate buffer, pH 8.0, using 0.56 mM acetylthiocholine as a substrate according to Ellman (1961).

Monoamine oxidase (MAO) activity was measured by monitoring the appearance of 4-hydroxyquinoline (4-HOQ) which arises from the spontaneous cyclization of the intermediate aldehyde formed by the oxidative deamination of kynuramine (Krajl, 1965). In brief, 1.0 ml of aqueous homogenate of tissue was

mixed with 0.5 ml kynuramine (100 μg kynuramine dihydrobromide), 0.5 ml phosphate buffer (0.5 M, pH 7.4), water in total volume of 3.0 ml. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by adding 2.0 ml trichloroacetic acid (TCA). The precipitated protein was spun down and 1 ml of supernatant was added to 2.0 ml of 1 N NaOH in a quartz cuvette. The fluorscence of the solution was measured at 315 nm of excitation wavelength and 380 nm of emission wavelength. Suitable blank and 4-HOQ standards were carried through the entire procedure.

Statistical analysis

Data were expressed as mean±s.d. and analyzed by one-way ANOVA and Duncan's multiple range test; a P-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

LC_{50} of carbofuran for *Oryzias latipes*

Acute toxicity study in which Medaka fish was continuously exposed to different concentrations of carbofuran for 24 hrs, showed that the LC_{50} of carbofuran ranged from 1 to 5 μg/ml. However, no mortality was observed in the fish exposed to 1 μg/ml carbofuran for 24 hrs.

Inhibition of acetylcholine esterase (AChE) by carbofuran

In unexposed fish, specific activities of AChE in head and body were 140±50 and 170±60 mmoles substrate hydrolyzed/min/mg protein, respectively. This result is consistent with the previous report that specific activity of AChE in muscle of goldfish was higher than that in brain. AChE activity was inhibited by exposure to carbofuran in a dose-dependent manner (Fig. 1). The lowest concentration tested (0.001 μg/ml) produced significant inhibition of the enzyme activity, with 30 and 20% reduction in the activities in head and body, respectively. Thus Medaka fish

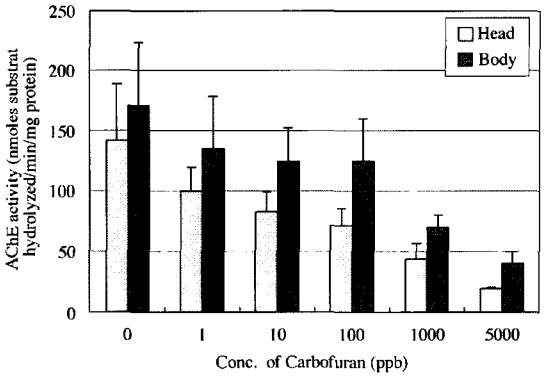

Fig. 1. Inhibition of acetylcholine esterase (AChE) of *Oryzias latipes* exposed to different concentrations of carbofuran for 24 hr. Medaka fish was exposed to 0, 1, 10, 100, 1000, and 5000 ppb concentrations of carbofuran for 24 hrs, dissected into head and body, and followed by homogenizing and AChE assay. Values are means of three measurements; error bars indicate standard deviation (SD).

appeared to be relatively sensitive to carbofuran compared to other species of fish. For instance, the AChE of goldfish exposed to 0.05 μg/ml carbofuran for 24 hrs showed 19.8% inhibition in brain while the enzyme in body muscle was not inhibited. These results suggest that Medaka fish may be a superior tool for monitoring the contamination of freshwater with carbamate pesticides.

Inhibition of monoamine oxidase (MAO) by carbofuran

The Medaka fish exposed to carbofuran at the concentrations of 0, 1, 10, 100, 1000 ppb showed decreased MAO activity in head portion. Even in the fish treated with 1 ppb carbofuran for 24 hrs there was a significant suppression in the enzyme activity (Fig. 2). And the increase of the pesticide concentration to 10 ppb caused further decline in MAO activity in the fish head. However, the enzyme activity in body from the fish exposed to carbofuran tended to increase. Similar change pattern in MAO activity was observed for Medaka exposed to 100 ppb carbofuran for different periods. With the increase of expo-

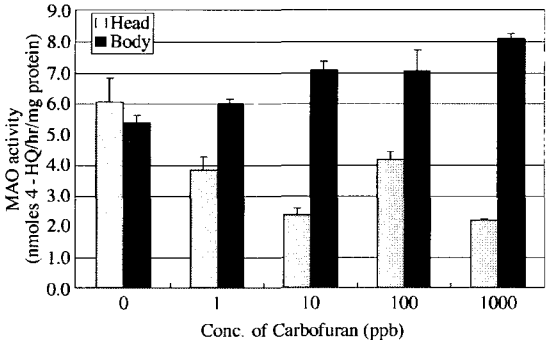

Fig. 2. Changes of monoamine oxidase (MAO) in *Oryzias latipes* by exposure to different concentrations of carbofuran for 24 hr. Medaka fish was exposed to 0, 1, 10, 100, 1000, and 5000 ppb concentrations of carbofuran for 24 hrs, dissected into head and body, and followed by homogenizing and MAO assay. Values are means of three measurements; error bars indicate standard deviation (SD).

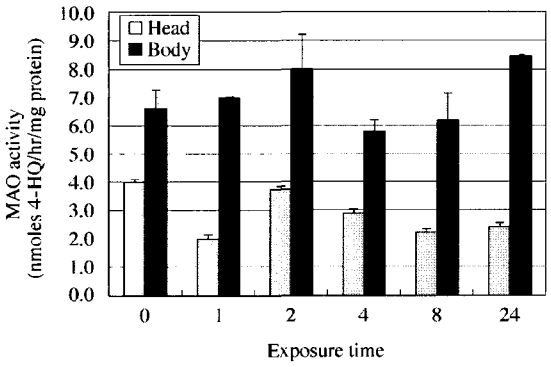

Fig. 3. Changes of monoamine oxidase (MAO) in *Oryzias latipes* by exposure to different periods at 100 ppb concentration of carbofuran. Medaka fish was exposed to 100 ppb of carbofuran for different periods, dissected into head and body, and followed by homogenizing and MAO assay. Values are means of three measurements; error bars indicate standard deviation (SD).

sure time MAO activity in head gradually decreased while the enzyme activity in body section tended to be enhanced (Fig 3).

Although there was no report on the inhibitory activity of carbamates against MAO, some pyrethroids such as deltamethrin has been suggested to enhance MAO and AChE in rat brain and make rats aggressive. Ram and coworkers (2001) found that the chronic exposure to carbofuran (4.5 ppm in static water) for six months on the gonadal histophysiology and hypothalamo-neurohypophyseal complex revealed significant inhibition of gonadal development with associated degenerative abnormalities as evidenced by ovarian and testicular histology and reduced gonadosomatic index in teleost fish. MAO is known to be involved in catabolism of dopamine and serotonin, two main neurotransmitters in brain and other tissues. Low level of dopamine and/or serotonin is associated with several psychological disorders in animals such as depression and anxiety problem. In particular, MAO inhibitor is commonly prescribed for patients with depression. However, the role of MAO in fish behavior is not clear, and what kind of behavioral changes will occur by the inhibition or stimulation by MAO remains to be evaluated. Since MAO inhibitor have a dramatic effect on mood and behavior in higher animal, we expect that carbofuran will have an influence on fish behavior to a certain extent.

CONCLUSION

Carbofuran, a carbamate pesticide, is relatively highly toxic to fish and causes vertebral malformation and behavioral change of fish at relatively low concentrations. To elucidate biochemical mechanism of the behavioral change of fish caused by carbofuran, the effects of the insecticide on acetylcholine esterase and monoamine oxidase activities were evaluated using *Oryzias latipes* (Japanese Medaka fish) as a model organism. LC_{50} of carbofuran against Japanese Medaka fish was approximately 2.5 ppm. The pesticide inhibited the acetylcholine esterase of the model fish starting from 1 ppb concentration in a dose-dependent manner. Especially a conspicuous enzymatic inhibition was observed as the pesticide concentration increased from 100 to 1000 ppb. Carbofuran treatment also affected monoamine oxidase

activities both in body and head of Medaka fish but in different pattern. In conclusion, these results suggest that carbofuran might cause altered behavior of the fish by combinational effect of the pesticide on monoamine oxidase and acetylcholine esterase, which are involved in neurotransmitter metabolism.

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