DIFFERENTIAL *IN VITRO* EFFECTS OF SOME PESTICIDES ON CARBONIC ANHYDRASE ACTIVITIES FROM SOME FRESHWATER AND SEAWATER FISH ERYTHROCYTES

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SUMMARY

The purpose of this study was to investigate the *in vitro* effects of 4 commonly used pesticides: the fungicides Nuarimol™ [α-(2-chlorophenyl)-α-(4-fluorophenyl)-5-pyridinemethanol] and Fenarimol™ [α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyridinemethanol], the insecticide Parathion-methyl™ [O,O-dimethyl-O-(4-nitrophenyl) phosphorothioate] and the herbicide 2,4-D™ [2,4-dichloro-phenoxy acetic acid, ammonium salt] on erythrocyte carbonic anhydrases (CA) activity from *Cyprinus carpio, Scorpaena porcus, Diplodus vulgaris, Salmo gairdnerii* and *Barbus barbus.* Erythrocyte CA enzymes from different fish species were purified by using Sepharose–4B-L-tyrosine-sulphonamide affinity gel. I_{50} values of the chemicals that caused inhibition were determined by means of activity percentage [I_{50}] diagrams. The pesticides used in this study inhibited the CA activity from different fish species to various degrees. It was found that Nuarimol™ and Fenarimol™ were the most potent inhibitors for all fish species, ranging from 0.18mM to 0.59mM, whereas the others, Parathion-methyl™ and 2,4-D™, exhibited relatively low inhibitory effect with I_{50} values ranging from 1.26mM and 3.19mM for *Cyprinus carpio, Barbus barbus, Salmo gairdnerii* and *Diplodus vulgaris*. The comparison of the I_{50} values of these fish species indicates a higher carbonic anhydrase sensitivity for *Scorpaena porcus* to all pesticides. The concentrations of Nuarimol™, Fenarimol™, Parathion-methyl™ and 2,4-D™ that inhibited *in vitro* 50% of enzymatic activity (I_{50}) of *Scorpaena* CA were 0.2mM, 0.18mM, 0.62mM and 0.68mM, respectively.

KEYWORDS: *Cyprinus carpio*, *Scorpaena porcus*, *Diplodus vulgaris, Salmo gairdnerii* and *Barbus barbus*, carbonic anhydrase, pesticide and inhibition.

INTRODUCTION

Carbonic anhydrase (CA) (E. C. 4.2.1.1.) is one of the most ubiquitous enzymes found in living organisms. This metallo-protein catalyzes reversibly hydration of CO_2 to HCO_3^- and H^+. Therefore, it plays an important role in diverse processes, such as physiological pH control, and gas balance, calcification and photosynthesis [1]. Nine distinct carbonic anhydrase isozymes have been characterized from amniotes [1]. These isoenzymes can be differentiated based on the specific activity, sub-cellular and tissue distribution and their sensitivities to certain inhibitors. However, in most vertebrates, CA activity in the blood is restricted to the erythrocytes [2, 3]. In addition, most of the lower vertebrates possess only one cytoplasmic CA isozyme in their erythrocytes [4-6]. Different CA enzymes purified from organisms have been shown to be inhibited by various compounds. Sulfonamides, like acetozolamide (AZ) and heavy metals are considered as the strongest CA inhibitors [1]. In addition, some *in vitro* and *in vivo* studies showed that some antibiotics including ampicillin and gentamisin, some drugs, some chemicals like magnesium sulfate and, finally, some pesticides also inhibit CA enzyme activity to a wide range of degrees [1, 7-10].

Many pesticides are being used in agriculture in order to improve the yield. Although the use of these chemicals caused a positive effect on crop production, certain pesticides, their residues, metabolites and/or contaminants have created many unforeseen adverse effects on the environment. Pesticides may be present in very low concentrations, which may not cause immediately detectable effects. However, this small amount of chemicals can cause sub lethal damage to organism and this is more insidious and difficult to define than acute toxicity [1, 10, 11].

Many chemicals, especially pesticides, at relatively low dosages affect the metabolism of biota by altering normal enzyme activity. Several researchers reported the sensitivity of CA from aquatic organisms to several heavy metals [12-14]. The detailed mechanism of toxic action of heavy metals is not clear, but many of them cause enzyme

kinetic changes, which, in turn, disrupt specific metabolic systems [13]. CA is of special concern because of physiological importance and thus could be particularly vulnerable to waterborne pollutants. However, there was not much information on CA sensitivity of aquatic organisms to pesticides. Everyone knows that pesticides widely used in agriculture are one of the major pollutants for aquatic environments. Especially this type of pollution is of great concern for freshwater organisms.

Therefore, in this study *in vitro* inhibition of some important pesticides (Nuarimol^{TM} [α-2-chlorophenyl-α-(4-fluorophenyl)-5-pyridinemethanol], Fenarimol^{TM} [α-(2-chlorophenyl)-α-(4-fluorophenyl)-5-pyridine methanol], Parathion-methyl^{TM}, [O,O-dimethyl-O-(4-nitrophenyl) phosphorothioate] and 2,4-D^{TM} [2,4-dichlorophenoxy acetic acid, ammonium salt) on erythrocyte CA enzymes was evaluated in some freshwater fish species, namely, *Cyprinus carpio*, *Barbus barbus* and *Salmo gairdneri* and seawater fish species, namely, *Diplodus vulgaris* and *Scorpaena porcus.*

MATERIALS AND METHODS

Material: All chemicals used were of analytical grade and obtained from either Sigma or Merck. All the above-mentioned pesticides (technical grade) employed in this study were obtained from local companies licensed to sell the related pesticides.

Collection of fish samples and blood collection: *Cyprinus carpio, Barbus barbus,* were collected from the lake of Selimiye, Balikesir, Turkey. *Salmo gairdnerii* was collected from the private salmon fish farm. *Scorpaena porcus* and *Diplodus vulgaris* were collected in the fall from the gulf of Edremit in the Aegean Sea, Turkey. Fish were held in aerated dechlorinated freshwater (8-15 °C) and fed a diet of crayfish and minnows. There were no signs of stress, nor mortalities among the fishes used in these experiments. Blood was collected by blind caudal puncture into a heparinized syringe and transferred into a tube containing heparin.

Purification of CA isozymes from fish erythrocytes by affinity chromatography: Erythrocytes were purified from the blood of various fish samples. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20 000 rpm for 30 min at 4 °C. The pH of hemolysate was adjusted to 8.7 with solid Tris. The hemolysate was applied to the prepared Sepharose 4B-L-tyrosine-sulfonamide affinity column equilibrated with 25 mM Tris-HCl/22mM Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/ 22 mM Na_2SO_4 (pH 8.7). The fish CAs were eluted with 0,1M $NaCH_3COO$/0,5M $NaClO_4$. Protein concentration of the eluates was determined at 280 nm by the Bradford method [15] and purities of isoenzymes were checked with SDS page [16].

Measurement of CA activity: CA activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [17]. CO_2 hydrase activity as an enzyme unit (EU) was calculated by using the equation $[(t_o-t_c)/t_c]$, where t_o and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

***In vitro* studies for pesticides:** Nuarimol ^{TM}, Fenarimol ^{TM}, methyl parathion ^{TM} and 2,4-D ^{TM} were selected as pesticides. Five different volumes (0.1, 0.2, 0.3, 0.4, and 0.5 ml) of pesticides at a constant concentration were added to the enzyme activity determination medium in a 4.2ml of total volume. CA activities with the related pesticides were assayed by following the hydration of CO_2 [17]. Activity I_{50} values of five different concentrations of each pesticide were drawn by using regression analysis graphs on a Microsoft Excell 2000 computer program. CA activity without pesticides was accepted as 100% activity. For the pesticides having an inhibition effect, the inhibitor concentrations causing up to 50% inhibition (I_{50} values) were determined from the graphs. Each inhibition effects were repeated at least three times.

RESULTS

The inhibitory effects of some commonly used pesticides, namely, Nuarimol^{TM}, Fenarimol^{TM}, parathion- methyl^{TM} and 2,4-D^{TM} on erythrocyte carbonic anhydrase activity were investigated in different fish species, which are *Cyprinus carpio*, *Diplodus vulgaris, Barbus barbus, Scorpeana porcus* and *Salmo gairdnerii*, from different water resorts of the Aegean sea. Erythrocyte CAs from each fish species were purified by using the affinity gel with the elution buffer of 0,1M $NaCH_3COO$/ 0,5M $NaClO_4$. The purity of the enzymes was confirmed with SDS gel electrophoresis (data not shown).

Inhibition values with different inhibitor concentrations are shown in Figure 1 and I_{50} values of different CA enzymes obtained from this graph are listed in Table 1.

The I_{50} values of *Cyprinus carpio* carbonic anhydrase enzyme (CCCA) inhibited by 2,4-D^{TM}, Fenarimol^{TM}, Nuarimol^{TM} and Parathion-methyl^{TM} were found to be 2.72 mM, 0,55 mM, 0.38 mM and 2.9 mM, respectively (Table 1). The highest inhibition effect was obtained with Nuarimol^{TM}. However, Parathion-methyl^{TM} exhibited the weakest inhibitory effect with an I_{50} value of 2.9 mM, which still exerts the inhibition effect on CCCA enzyme activity.

For *Barbus barbus* carbonic anydrase (BBCA) enzyme, I_{50} values of these four above-mentioned pesticides

were 1.73 mM, 0.59 mM, 0.28 mM and 2.45 mM, respectively (Table 1).

TABLE 1 - Concentrations of pesticides needed to effect 50% inhibition of erythrocyte carbonic anhydrase activity from *Cyprinus carpio* (CCCA), *Barbus barbus* (BBCA), *Salmo gairdnerii* (SGCA), *Scorpaena porcus* (SPCA) and *Diplodus vulgaris* (DVCA).

Pesticide	CCCA (mM)	BBCA (mM)	SGCA (mM)	SPCA (mM)	DVCA (mM)
2,4-D ™	2.72	1.73	1.26	0.65	2.67
Fenarimol ™	0.55	0.59	0.51	0.18	0.37
Nuarimol ™	0.38	0.28	0.23	0.2	0.38
Parathion-methyl ™	2.9	2.45	1.77	0.62	3.19

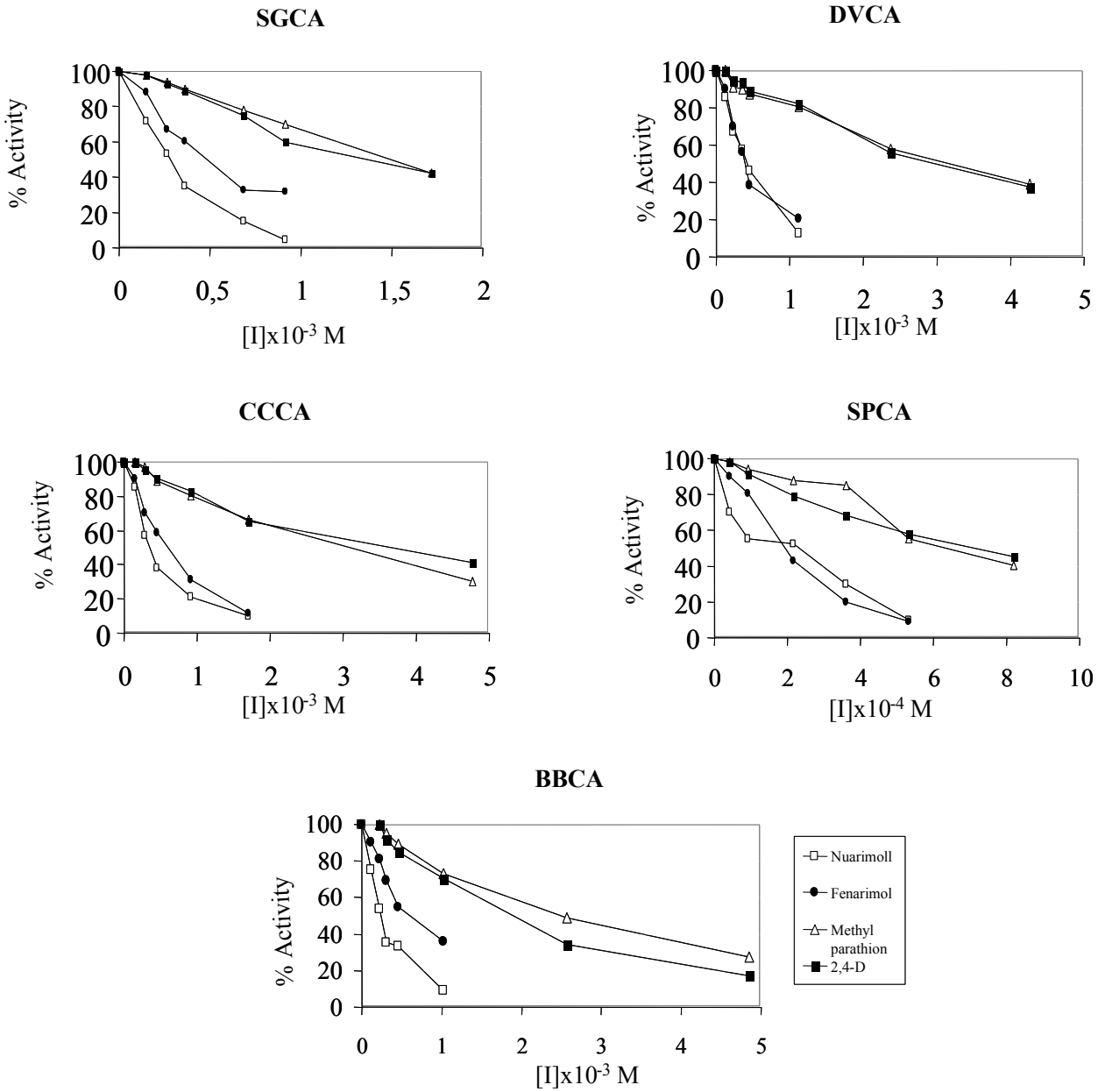

FIGURE 1 - Activity (%) of curves of CCCA (*Cyprinus carpio* carbonic anhydrase), BBCA (*Barbus barbus* carbonic anydrase), SGCA (*Salmo gairdnerii* carbonic anhydrase), SPCA (*Scorpaena porcus* carbonic anyhydrase), and DVCA (*Diplodus vulgaris* carbonic anhydrase) in different concentrations of 2,4-D™, Fenarimol™, Nuarimol™ and Parathion-methyl ™.

Salmo gairdnerii carbonic anhydrase (SGCA) or *Scorpaena porcus* carbonic anyhydrase (SPCA) enzyme were also inhibited by these pestisides to different degrees. I_{50} values of 2,4-D™, Fenarimol™, Nuarimol™ and Parathion-methyl™ were 1.26 mM, 0.51 mM, 0.23 mM and 1.77 mM or 0.65 mM, 0.18 mM, 0.2 mM and 0.62 mM, respectively (Table 1). The inhibition values of SPCA showed that erythrocyte carbonic anhydrase in this species is much more sensitive compared to the CA enzymes from other fish species. Interestingly, the strongest inhibitor was determined as Fenarimol™

The inhibition effects of 2,4-D™, Fenarimol™, Nuarimol™ and Parathion-methyl™ on *Diplodus vulgaris* carbonic anhydrase (DVCA) were found to be 2.72 mM, 0.55 mM, 0.38 mM and 2.9 mM, respectively (Table 1). Similarly, Fenarimol™, and Nuarimol™ were the pesticides exhibiting the strongest inhibitory effects on erythrocyte DVCA activity.

DISCUSSION AND CONCLUSION

Some investigations have reported the sensitivity of CA from aquatic organisms (some fish species, crabs and teleosts) to some inhibitors including several heavy metals and AZ [12-14, 18-20]. However, there was not much information available on inhibition of CA enzymes from aquatic organisms by pesticides. In this respect, the inhibition effects of several commonly used pesticides on different fish erythrocyte carbonic anyhydrases were investigated. These fish species are *Cyprinus carpio*, *Barbus barbus* and *Salmo gairdneri* obtained from regional freshwater sources and *Diplodus vulgaris* and *Scorpaena porcus* from regional seawater. These fish species were chosen because they are economically important in the western part of Turkey and widely used as meat consumption.

From the inhibition studies, it was found that erythrocyte CA enzymes belonging to different fish samples showed differential inhibitions by these pesticides. Nuarimol™ and Fenarimol™ were found to be the strongest inhibitors for all fish types under investigation. (Figure 1). This finding is not surprising since our previous work also indicated that the inhibition effects of Nuarimol™ on human CAI and CAII enzymes were 1,84 mM and 0.0136 mM suggesting the strongest effect for human CAII [7]. In addition, Fenarimol™ more and less, exhibits similar strong inhibition effects as Nuarimol™ for all fish carbonic anhydrases. Fenarimol™ was also found to be the strongest inhibitor for HCAII enzyme [7]. 2,4-D™ and Parathion-methyl™ were generally found as the weakest inhibitors for all fish species, except for SPCA. Among the five fish types, the most sensitive CA enzyme was found to be SPCA. Its activity was inhibited to similar degrees by all pesticides used in this study (Figure 1; Table 1). The reason for this might be that this fish species, which lives in seawater, does not have any tolerance to pesticides and other pollutants. However, this possibility cannot be applied to all seawater organisms, since DVCA, which is another seawater fish, shows similar inhibition patterns like the other fish species.

Differential sensitivity to chemical inhibition may be a more common and widespread feature of the differences in CA structure among organisms. For instances, heavy metals were known to inhibit CA from a variety of aquatic organisms, and CA from different tissues in the same organism have been shown to have different sensitivities to these metals. Erythrocyte CAs in the catfish *Ictalurus punctatus,* the most abundant pool in fish was reported as having Ki values between 35 and 900µM for Ag^{+}, $Cd^{+,}$ $Cu^{2+}$and Zn^{2+} [18]. In the eel, *Anguila anguilla,* CA was more sensitive to metal inhibition than was CA from intestinal brush border [21]. Also, the concentration of different metals and acetazolamide (AZ) that inhibited gill CA of fish *Ictalurus punctatus* is markedly higher than that presented by estuarine crab *C. granulata,* suggesting that CA activity of the latter species could be relevant biomarker for monitoring environmental pollution by heavy metals [13].

Differential sensitivity of fish CAs might be depending on a number of factors. It is possible that differences in inhibitions, rooted in the differences in binding affinity of the pesticides to the enzyme, are a result of species-specific isoforms. Differences in the sensitivity of CA to these pesticides may also have an impact on the ability of the intact organism to interact with its environment.

In conclusion, it was determined that some pesticides, which are widely used for agricultural benefits, dramatically inhibit the erythrocyte CA enzymes of some fresh water and seawater fish species. This finding is important, because pesticides generally contaminate not only the soil, but also water resources. Consequently, the inappropriate use of pesticides endangers the balance of aquatic environment and organisms, and they are also potentially a risk to animals and human health too. In addition, the inhibition studies of these pesticides against these fish species may suggest the use of them as biomarker for monitoring environmental pollution by pesticides. Further studies about this matter would be beneficial in terms of the prevention of water pollutions.

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