Conference Paper

INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY IN THE TERRESTRIAL ISOPOD *PORCELLIO SCABER* AS A BIOMARKER OF ORGANOPHOSPHORUS COMPOUNDS IN FOOD*

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This paper describes the toxicity of organophosphorus pesticide diazinon in juvenile and adult terrestrial isopods *Porcellio scaber* (Isopoda, Crustacea). The woodlice were exposed to different concentrations of diazinon added to food (5, 10, 50, and 100 or 150 μg/g dry food). Weight change and food assimilation efficiency were determined two and four weeks after the exposure. The activity of acetylcholinesterase (AChE) in surviving animals was measured at the end of the experiment. The results show that woodlice exposed to diazinon do not significantly differ from controls in growth and feeding rate. The reduction of AChE activity was observed at the lowest diazinon exposure (5 and 10 μg/g dry food). These results suggest that AChE activity might prove a useful biomarker, indicating low levels of organophosphates in food.

KEY WORDS: *AChE activity, diazinon toxicity, food assimilation, hazelnut tree leaves, single-species toxicity test*

Organophosphorus compounds (OPs) are used in agriculture as insecticides. Their toxicity arises from the inhibition of the enzyme acetylcholinesterase (AChE). This inhibition is due to the phosphorylation of AChE and consequent accumulation of acetylcholine in nervous tissue and effector's organs (1).

Diazinon [*O*,*O*-diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidyl) phosphorotioate] is an organophosphorus insecticide, registered and marketed in Slovenia. It is effective against a variety of orchard, vegetable, and soil pests, and also against human ectoparasites such as flies, lice, and fleas. Diazinon is available as a technical grade product, wettable powder, emulsifiable concentrate, in granules, and in a variety of other formulations. In soils, diazinon seldom penetrates below the top 1-2 cm, but it may remain biologically available for 6 months or more in conditions combining low temperature, low moisture, high alkalinity, and lack of suitable microbial degraders (2).

Investigations of the toxicity of organophosphorus insecticides, including diazinon, on vertebrates have been carried out extensively and the effects are very well known. Only recently have the toxic effects of organophosphorus insecticides been studied on non-target invertebrates, especially on *Collembola*, lumbricids and terrestrial isopods (3).

The aim of our work was to assess the activity of AChE in juvenile and adult terrestrial isopods *Porcellio scaber* exposed to diazinon in food. We also determined their growth, faecal pellet production, consumption and assimilation efficiency. The discussion focused on the suitability of AChE activity as a biomarker of OP exposure to insecticides.

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MATERIALS AND METHODS

Test organisms

Woodlice (*Porcellio scaber* Latr., Isopoda, Crustacea) were collected in the litter layer of an uncontaminated woodland near Nova Gorica, Slovenia. In the laboratory, the animals were kept in a terrarium (20×35×20 cm) filled with a layer of moistened sand, soil (2-5 cm) and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*). Sand and soil were heated up to 80 °C for several hours to destroy predators (spiders) (4). Hazelnut tree leaves were collected in an uncontaminated woodland and air-dried at room temperature. Hazelnut tree leaves were the main source of food. Small quantities of vegetables (pieces of potato or carrot) and fruit (pieces of apple) were added weekly to provide additional vitamin supply. The culture was kept at room temperature, while the humidity was maintained by spraying water every day.

Preparation of food

Hazelnut tree leaves were collected in an uncontaminated woodland and dried between two sheets of paper. Dried leaves were cut in pieces of approximately the same size and weight (100 mg). Solutions of different concentrations of diazinon were prepared from a standard solution diluted with distilled water. Droplets of 150 μL solution were pipetted onto the lower leaf surface and evenly spread over the surface with a paintbrush. The nominal concentrations of diazinon in the leaves were 5, 10, 50 and 100 or 150 μg diazinon/g dry food. Animals in the control group were fed with hazelnut tree leaves prepared in the same way, only without diazinon.

Measurement of AChE activity

We measured AChE activity after two and four weeks of exposure to diazinon. To remove food remnants and feacal pellets from animals, each of them was cleaned with a brush-pencil before entering the analytical procedure. A single animal tissue was homogenised in 1.5 mL of 25 mM phosphate buffer (pH=7), using a homogeniser at a speed of 1400 rpm. During the homogenisation, samples were kept on ice. Homogenised samples were then centrifuged for 15 minutes at 3000 rpm. The obtained supernatant was immediately assayed for AChE activity. The AChE activity was measured by the colorimetric method as described by *Ellman and co-workers* (9). The measurements were carried out on a HP 8453 spectrophotometer at λ=412 nm for 8 minutes. To obtain a final volume of 3 mL, 20 μL of acetylthiocholine iodide 0.075 M, 100 μL of acid dithiobisnitrobenzoate 0.01 M, and 2380 μL of phosphate buffer (pH=7) were added to 500 μL of the supernatant. Two replicates were made from each animal sample. The total enzyme activity was expressed relative to the fresh weight of the animal. The AChE activity was expressed in μmol/min/g animal weight (micromoles of substrate hydrolysed per minute per gram of woodlouse).

Preliminary toxicity test with terrestrial isopods Porcellio scaber

A preliminary four-week toxicity test was performed on juvenile woodlice. Four animals were placed in one Petri dish. The animals were fed with diazinon-dosed food (50 and 150 μg diazinon/g food) for four weeks. Food was prepared as described above. Every second day the remnants of food and faecal pellets were removed and replaced with fresh leaves. Faecal pellet production (F) and the amount of consumed food (C) were used for the calculation of assimilation efficiency (AE) according to the formula AE=(C-F)/C. AE represents the average of four animals that were exposed together. The animals were weighed before and after exposure as well as once a week. At the end of the experiment all the remaining animals were prepared for the analysis of AChE activity.

Main experiments

Male and female woodlice (juveniles with body weight of 20±5 mg, and adults with body weight of about 50 mg) were fed hazelnut tree leaves (*Corylus avellana*) dosed with diazinon (5, 10, 50 and 100 μg diazinon/g dry food) for two and four weeks. Each animal was placed individually in a Petri dish, adding pieces of leaves dosed with diazinon. There were 12 animals in each group exposed to diazinon and additional 12 animals in the control group fed diazinon-free hazelnut tree leaves. Humidity in the Petri dishes was maintained by regular spraying distilled water on the inside of the lids. All the Petri dishes were put in a large, covered plastic container, which maintained relative humidity close to 100 %. Every third day the remnants of food were removed, weighed and replaced by fresh leaf pieces. The animals were weighed every second day. Every seven days faecal pellets were removed and weighed. The assimilation efficiency was calculated as described above.

Determination of diazinon concentrations in contaminated leaves

Diazinon concentrations in food were analysed using a gas chromatograph (HP 6890 GC) with and electron capture detector. GC conditions were as follows: Supelco SPB-1 (30 m × 0.53 mm) column and helium as a carrier gas were used; the initial temperature was 80 °C; the temperature initially increased 10 °C/min up to 280 °C, keeping the final temperature for 5 min; the injection port temperature and the detector temperature were 250 °C. Samples (1 μL) were injected in a splitless mode.

Samples were prepared as follows: 250 mg of contaminated leaves were homogenised and extracted with 1 mL of acetone. After 30 min of extraction 10 mL of deionised water was added. The mixture was filtered through filter paper, extracted in solid phase extraction mode using C_{18} cartridges, activated with 1 mL of 10 % Na_2SO_4, and eluted with 500 μL of hexane as eluent. A calibration curve in the concentration range from 0 to 150 mg/L was prepared for quantification. The R^2 value for linear regression of the calibration curve was 0.9904. The relative standard deviation in all experiments was 5-15 %.

Chemicals

Diazinon [*O*,*O*-diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidyl) phosphorotioate] was provided by Pestanal, Riedel-de Haën (Seelze, Germany); ethyl acetate was purchased from Riedel-de Haën (Seelze, Germany) and acetylthiocholine iodide, dithiobisnitrobenzoic acid as well as bovine serum albumin (albumin fraction V) from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Our experimental results showed that the average growth of exposed woodlice decreased with increasing concentrations of diazinon in food relative to the control. However, due to a high variance of data, the differences were not statistically significant. During the four-week experiment, average food assimilation efficiency in juvenile animals was 48 % in the control group and 63 % and 77 % in groups exposed to 50 and 150 μg diazinon, respectively (Figure 1, left). Average food assimilation efficiency in adults was slightly higher in animals exposed to 50 and 100 μg diazinon/g dry food than in the control group. (Figure 1, right).

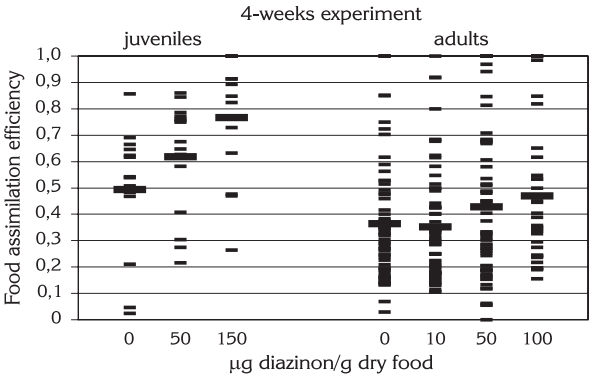

Figure 1 *Food assimilation efficiency in juvenile terrestrial isopods* Porcellio scaber *after a four-week exposure to diazinon in food (concentrations 50 and 150 µg diazinon/g dry food, N=16 in the control group and in the group exposed to 50 µg/g, N=13 in the group exposed to 150 µg/g) and in adult terrestrial isopods* Porcellio scaber *after a four-week exposure to diazinon in food (concentrations 10, 50 in 100 µg diazinon/g dry food, N=63 in the group exposed to 10 µg/g, N=62 in the group exposed to 50 µg/g, N=35 in the group exposed to 100 µg/g and N=64 in the control group). Longer lines indicate mean values.*

Another, two-week experiment with juvenile woodlice fed with hazelnut tree leaves dosed with diazinon in sublethal concentrations (5, 10, 50 in 100 μg diazinon/dry food) showed that there was no statistically significant effect on body weight and assimilation efficiency of the exposed animals compared to controls (Figure 2).

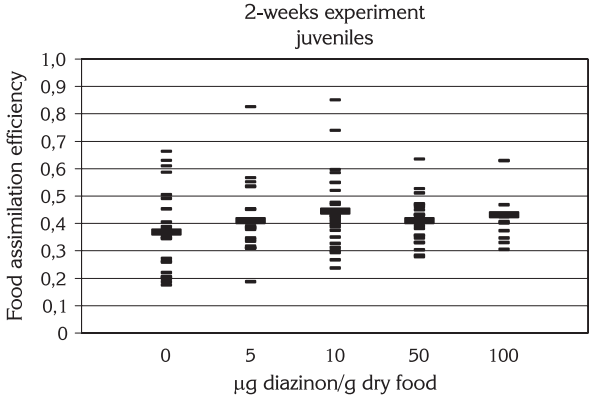

Figure 2 *Food assimilation efficiency in juvenile terrestrial isopods* Porcellio scaber *after a two-weeks exposure to diazinon in food (N=12 in each group). Longer lines indicate mean values.*

Mortality in the four-week exposure experiment was 25 % in animals exposed to 50 μg diazinon/g dry food and 94 % in those exposed to 100 μg diazinon/g dry food whereas two-week exposure to 50 μg diazinon/g dry food yielded no mortality and to 100 μg diazinon/g dry food 17 % mortality.

The results are in accordance with literature reporting that a four-week exposure to 75 μg of dimethoate/g dry food showed no significant effect on mass gain in the terrestrial isopod *Porcellio scaber* (6). In a similar experiment, terrestrial isopods of the species *Porcellionides pruinosus* were exposed to diazinon in concentrations of 8.71, 18.73, 40.73, 86.50, 186.18 and 400.2 μg diazinon/g dry food for four weeks. In the first two weeks, no clear effect was observed. In the third week, animals fed with contaminated food showed a slower body weight gain compared to controls. After three weeks the LC_{50} value was 303 μg diazinon/g dry food and after six weeks 74.15 μg diazinon/g dry food (7). However, LC_{50} of diazinon was considerably lower when isopods were exposed via the substrate. This was confirmed by *Widianarko and Van Straalen* (LC_{50}=4.4 μg/g) (8).

In our experiment, the effect of diazinon on AChE activity in juvenile woodlice was observed at as low a concentration as 50 μg diazinon/g dry food (Figure 3, left). AChE activity in adult woodlice was strongly inhibited, especially in the groups exposed to 50 and 150 μg diazinon/g dry food (Figure 3, right).

AChE activity in diazinon-fed juveniles was lowered as early as two weeks after the exposure (Figure 4).

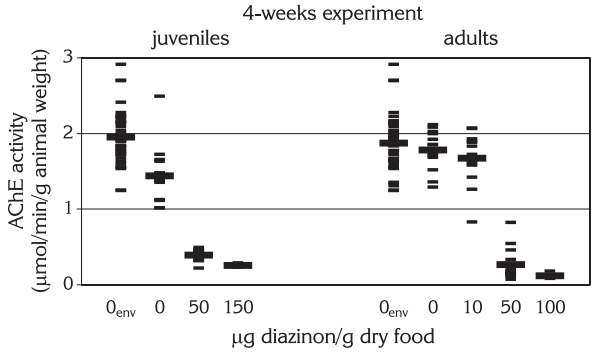

Figure 3 *Acetycholinesterase activity in juvenile terrestrial isopods* Porcellio scaber *after a four-week exposure to diazinon in food (concentrations 50 and 150 µg diazinon/g dry food, N=16 in the controls group and the group exposed to 50 µg/g, N=13 in the group exposed to 150 µg/g) and in adult terrestrial isopods* Porcellio scaber *after a four-week exposure to diazinon in food (concentrations 10, 50 in 100 µg diazinon/g dry food, N=63 in the group exposed to 10 µg/g, N=62 in the group exposed to 50 µg/g, N=35 in the group exposed to 100 µg/g and N=64 in the control group, 0_{env}=AChE activity in isopods taken from an uncontaminated area and killed immediately). Longer lines indicate mean values.*

The enzyme activity was inhibited in animals exposed to 5 and 10 μg diazinon/g dry food and significantly inhibited in animals exposed to higher concentrations of diazinon (50 and 100 μg diazinon/g dry food).

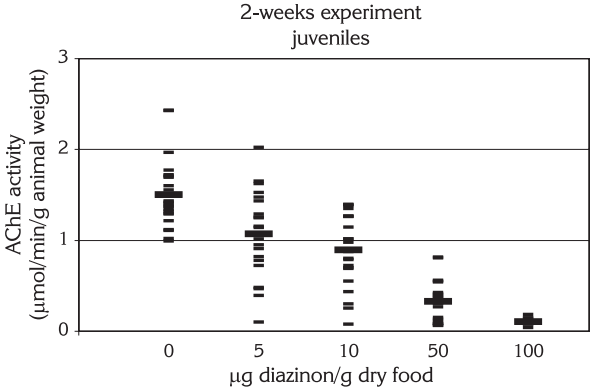

Figure 4 *Acetycholinesterase activity in juvenile terrestrial isopods* Porcellio scaber *after a two-week exposure to diazinon in food (N=12 in each group). Longer lines indicate mean values.*

Literature reports similar results for the isopod *Porcellio dilatatus* exposed to a wide range of concentrations of parathion (0.1, 1, 10, 25, 50, 100, 250 in 500 μg parathion/g dry food). After 21 days of exposure, the activity of AChE and lactate dehydrogenase (LDH) were determined. Parathion induced a significant drop in both AChE and LDH activitiy. This decrease in AChE activity was observed at sublethal concentrations as low as 0.1 μg parathion/g dry food (9).

CONCLUSIONS

Present results provide a biological tool for evaluating pesticide toxicity with the terrestrial isopod *P. scaber* under standardised laboratory conditions. Exposure to diazinon led to the reduction of AChE activity in juvenile and adult woodlice, but did not affect their wet body weight. For that reason both juveniles and adults are suitable for toxicity testing. AChE activity was strongly inhibited by the ingestion of diazinon at concentrations above 10 μg diazinon/g dry food, but the reductions were observed even at low concentrations of diazinon. Our results suggest that AChE activity could be useful in detecting low levels of exposure to organophosphates.

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Sažetak

INHIBICIJA AKTIVNOSTI ACETILKOLINESTERAZE U ZEMALJSKOG JEDNAKONOŠCA *PORCELLIO SCABER* KAO MOGUĆI POKAZATELJ PRISUTNOSTI ORGANOFOSFORNIH SPOJEVA U HRANI

Autori su istraživali toksičnost organofosfornog pesticida diazinona za nezrele i odrasle forme zemaljskog jednakonošca *Porcellio scaber*. Kukci su eksponirani različitim koncentracijama diazinona dodanog lišću od lijeske, hrani kojom se ti kukci hrane. Kontrolna skupina hranjena je jednakom hranom bez dodatka diazinona. Primijenjene koncentracije diazinona bile su 5, 10, 50 i 100 ili 150 μg/g suhog lista. Autori su mjerili promjenu tjelesne težine i učinak asimilirane hrane, i to nakon 2 i 4 tjedna trajanja ekspozicije. Na kraju pokusa u preživjelih kukaca mjerili su aktivnost acetilkolinesteraze Ellmanovom metodom. Rezultati su pokazali da se kukci eksponirani diazinonu ne razlikuju značajno od kontrolne skupine u pogledu rasta i hranjenja. Aktivnost acetilkolinesteraze, međutim, bila je smanjena već u kukaca eksponiranih najnižim koncentracijama diazinona (5 i 10 μg/g suhe hrane). Ovi rezultati upućuju na to da bi aktivnost acetilkolinesteraze u zemaljskih jednakonožaca mogla biti dobar biološki pokazatelj ekspozicije organofosfornim spojevima u hrani.

KLJUČNE RIJEČI: *aktivnost AChE, asimiliranje hrane, lišće lijeske, testiranje toksičnosti, toksičnost diazinona*

REQUESTS FOR REPRINTS:

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