

The toxicity of pyrethroid compounds in neural cell cultures studied with total ATP, mitochondrial enzyme activity and microscopic photographing

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Received 12 June 2003; accepted 12 November 2003

Abstract

Pyrethroids are important insecticides used largely because of their high activity as an insecticide and their low mammalian toxicity. Some studies have demonstrated that these products, especially compounds with an α -cyano group, show neurotoxic effects on the mammalian central nervous system (CNS). In this study, we investigate with different methods the cell toxic effects of commercial, chemically different pyrethroid compounds on neuronal cell line SH-SY5Y. Natural pyrethrin and permethrin (both with no α -cyano group) and cypermethrin (with an α -cyano group), were studied. For toxicity determinations, SH-SY5Y neuroblastoma cells were exposed to pyrethroids at 0.1–100 μ M concentrations for 1 day. The cell toxicity was evaluated by determining the total ATP with a luminescence method, the mitochondrial metabolic activity (WST-test) with a photometric method, and the morphological changes of the cell cultures with microscopic digital photographing at different dose levels of compounds. The results obtained with WST-1 method and with the measurement of total ATP were different. ATP measurement seemed to show cytotoxicity at lower concentrations than WST-1 method. There was induction of enzyme activities with WST-1 test with all pyrethroid compounds studied at low concentrations. With the ATP assay, exposure to 0.1–100 μ M of natural pyrethrin, as well as of permethrin and cypermethrin showed dose-dependent cytotoxicity. The most toxic pyrethroid was cypermethrin followed by permethrin and natural pyrethrin. Our study confirms that the cell toxicity was dependent on the chemical structure of pyrethroids and pyrethroids without an α -cyano group show the weakest physiological effect. Microscopic photographs of exposed cell cultures correlated to the toxic effects revealed by the metabolic tests.

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Keywords: Pyrethrin; Cypermethrin; Permethrin; SH-SY5Y cells; Cytotoxicity

1. Introduction

The pyrethroid insecticides represent widely used environmental chemicals. Their chief advantages are high insecticidal potency and low mammalian toxicity. The pyrethroids have a highly nonpolar nature, low water solubility and high affinity to soil and sediment particulate matter. Pyrethroids show low mobility in soil and are absorbed to the sediments of natural water systems. Despite the high attachment of pyrethroids to living organisms, their capability to bioconcentrate is mitigated by their metabolism and subsequent elimination. However, there have been cases of accidental and occupational human exposures after dermal contact

which have lead to acute pyrethroid poisonings (Altenkirch et al., 1996; Chen et al., 1991; Laskowski, 2002).

Natural pyrethrin is extracted from the flowers of *Chrysanthemum* spp., and its use was already known in China in the first century A.D. Pyrethroids, synthetic analogues of pyrethrin, have been produced since 1940 (Elliot, 1980). They are more stable in light and air than natural pyrethrin. Today there are many different synthetic pyrethroids. The increasing demand for pyrethroid products has lead chemists to focus their attention on the synthesis of new analogues with better stability in light and air, better persistence, more selectivity in target species, and lower mammalian toxicity.

The natural pyrethrins are more active poisons via dermal contacts than when ingested. Human toxicity associated with pyrethroids may stem from their allergenic properties (Ecobichon, 1996; Diel et al., 1999; Rosenberg et al., 1999). Synthetic agents show particular potency when

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ingested and are less susceptible to biotransformation by insects and mammals. According to the symptoms in animals receiving acute toxic doses, the pyrethroids form two groups. Type I pyrethroids such as pyrethrin and permethrin cause hyperexcitation, ataxia, convulsions, and paralysis. Type II pyrethroids such as cypermethrin, cause hypersensitivity, tremors, and paralysis. Despite some differences in the symptoms, the primary targets for both types are the cell membrane and the sodium channel in the neural tissue (Narahashi, 2000).

The aim of this study was to evaluate the cytotoxicity of three commercial pyrethroid products (Biospray S, a pyrethrin mixture; Biokill, a permethrin mixture; Ripcord, a cypermethrin mixture) as well as the sensitivity of the two conventional tests and microscopic photographs. The toxicity was evaluated with ATP and WST-1 tests. In parallel with these tests, microscopic photographing of cell cultures at different dose levels was used for the toxicity evaluation.

2. Materials and methods

2.1. Chemicals

Three commercial pyrethroid products Biospray S, Biokill and Ripcord were used as test compounds. Biospray S (Bioruiskute S, Kemira, Finland) contained an active compound pyrethrin 100 g/l, Biokill (Jesmond Ltd., England) contained an active compound permethrin 2.5 g/l and Ripcord (American Cyanamid Company, USA) contained an active compound cypermethrin 100 g/l and a mixture of xylene and petrol 820 g/l.

2.2. Materials

Nunc 75 cm² flasks (Cat. No. 156472) and Nunclon 96-MicroWell Plates (Cat. No. 167008) were used for the cell cultures. The cell culture reagents and solutions came from Gibco, Paisley. Minimum Essential Medium with Earle's salts, w/o L-glutamine (Gibco 21090-022), nutrient mixture F-12K Kaighn's modification L-glutamine (F-12K) (Gibco 21127-022), nonessential amino acids (Gibco 25030-024), penicillium G, streptomycin, amphotericin B as Fungizone® (Gibco 15240-035), foetal bovine serum (Gibco 10106-169), and L-glutamine (Gibco 25030-024). Black micro wells came from ThermoLabsystems (Black Cliniplate). WST-1 cell proliferation reagent came from Roche (Cat. No. 1644 807). Bioluminescent assay of ATP by ThermoLabsystems (6415000 ATP monitoring reagent) was used.

2.3. Cells

SH-SY5Y neuroblastoma cell line (ATCC Cat. No. CRL-2266) was used. SH-SY5Y is a trice cloned subline with a relatively stable neuroblastic phenotype.

2.4. Methods

2.4.1. The bioluminescent assay of ATP

The bioluminescent method utilizes the enzyme luciferase, which catalyses the formation of light from ATP and luciferin (DeLuca and McElroy, 1974; Gould and Subramani, 1988; Baldwin, 1996).

The passages of 32–33 of SH-SY5Y cells were seeded at a density of 40,000 cells/well to 96-microwell plates, 100 µl/well and grown in 1:1 MEM /Ham's F12K medium with 0.1 mM nonessential amino acids and 2 mM L-glutamine, 100 U/ml penicillium G, 25 µg/ml amphotericin B, and 10% foetal bovine serum. The cells were grown for 24 h at 37 °C and before exposure, the medium was changed to a serum-free one and incubated for 30 min at 37 °C. The test substances at 1–100 µM were added to the wells, and the cells were grown for further 24 h. The calculation of the molarity was done from the concentration of active compounds in commercial mixtures. The exposure was stopped by adding 10% TCA to the wells. The plates were frozen at –75 °C. The 25 µl of melted cell suspension was moved to black micro plates. In measuring the total ATP, 100 µl of a mixture (1:5) of Tris-acetate buffer and ATP monitoring reagent was added to the wells, and the plates were shaken and measured with ThermoLabsystems Luminoskan Ascent using a 1000 ms integration time.

2.4.2. WST-1 cell cytotoxicity test

WST-1 cell test is a calorimetric assay for the quantification of cell proliferation and cell viability. The evaluation of cell proliferation is based on the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Cook and Mitchell, 1989; Berridge, 1996).

SH-SY5Y cells were cultured in the same way as in the ATP method. After the 24 h exposure, 10 µl of WST-1 cell proliferation reagent was added to 100 µl of medium in each well and the plate was shaken for 1 min. The plates were incubated for 50 min in a humidified atmosphere at 37 °C, and the absorbances measured at 450 nm wavelength with Labsystems Multiskan MS.

2.4.3. Microscopic photographing

The cell cultures were microscopically examined and photographed at all exposure levels. The microscope was inverted phase-contrast microscope Olympus CK40 and the digital camera Olympus DP 10. The magnification was 200×.

2.5. Statistics

The determination were repeated three times at each dose level, three parallels in each determination. The results were normalized so that control values were 100%. The means and standard errors (Mean ± S.E.M.) of independent tests were calculated at each concentration. The data was statistically analyzed by the unpaired Student's *t*-test.

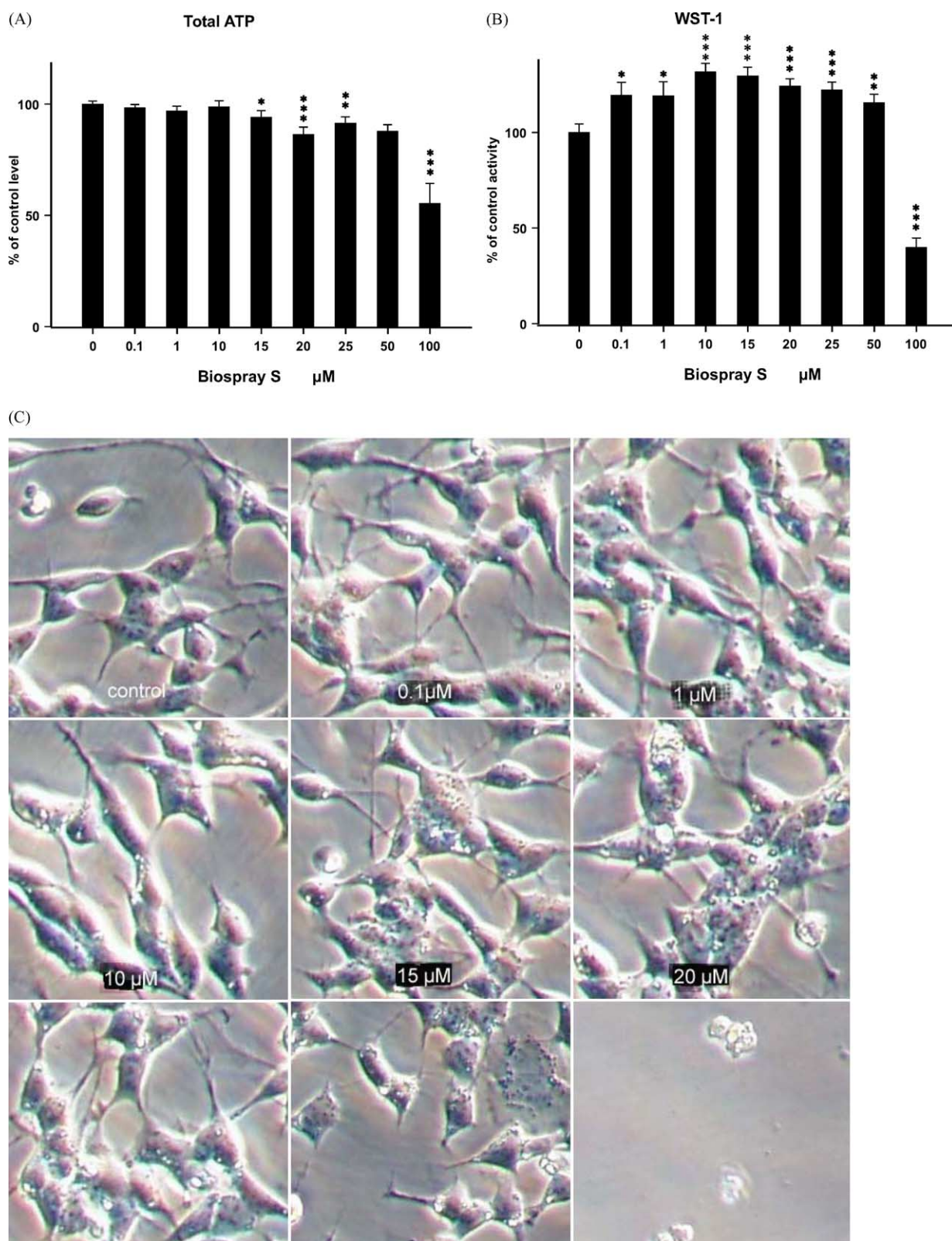


Fig. 1. The 24 h natural pyrethrin exposure to SH-SY5Y cells (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) (A) with total ATP measurement, (B) with WST-1 method, and (C) photographs of SH-SY5Y cells exposed to different concentrations of natural pyrethrin (Biospray S); magnification: 200 \times .

3. Results

In this study, SH-SY5Y neuroblastoma cells were used to study the toxic effects of three commercial pyrethroid products, whose active substances were pyrethrin (Biospray S), permethrin (Biokill), and cypermethrin (Ripcord). Cell toxicity was evaluated by determining the total ATP with a luminescent method and by determining the mitochondrial activity with WST-1 method. The *in vitro* exposure of neuroblastoma cells to the test compounds lasted for 24 h. In addition, we evaluated cellular changes due to the exposures by microscopic photographing of cell cultures.

3.1. Cell toxicity obtained with ATP and WST-1 methods

Exposure to 0.1–100 μM of pyrethrin, permethrin, and cypermethrin compounds showed dose-dependent cytotoxicity. The results obtained with WST-1 method and with the measurement of total ATP were different. ATP measurement seemed to show cytotoxicity at lower concentrations than WST-1 method. Actually, there was a clear induction of enzyme activities with WST-1 test with all pyrethroid compounds studied when the concentrations were not overly toxic (Figs. 1B, 2B, and 3B).

The most toxic pyrethroid was cypermethrin, (Ripcord, Fig. 3), followed by permethrin, (Biokill, Fig. 2), and natural pyrethrin (Biospray S, Fig. 1). The detectable toxicity of cypermethrin started at 1 μM concentration, and at 15 μM concentration the total ATP was markedly decreased, to 61% of control. Decrease in enzyme activity (WST-1) was to 72% of control. At 25 μM concentration of cypermethrin, the total ATP was 7% and WST-1 about 15% of control. At 100 μM concentration almost all cells were dead (Fig. 3).

The toxicity of permethrin was detected at 25 μM concentration in the total ATP and in enzyme activity (WST-1) in both methods, and at 50 μM concentration the decrease of the total ATP was marked, about 59% of control, and WST-1 about 77% of control. At 100 μM permethrin concentration decreasing of both the total ATP and WST-1 were quite similar, about 3% of control. The result was almost the same as at 100 μM concentration of cypermethrin, and the cells appeared dead.

The toxicity of pyrethrin in the ATP test was detected at 20 μM concentration, but not in the WST-1 test. At 100 μM concentration of pyrethrin, the decrease in both tests was marked. Total ATP decreased to 55% and WST-1 to 40% (Fig. 1A and B). At the similar concentrations of cypermethrin and permethrin, almost all cells were unviable.

3.2. Microscopic evaluation of the cell cultures

Exposure to 0.1–100 μM of pyrethrin, permethrin, and cypermethrin showed dose-dependent cytotoxicity in microscopic photographs. However, the cell toxicity was detected at lower concentrations compared with the total ATP measurement. In visual examination of the cell cultures, the

toxicity was expressed first by the appearing of intracellular vacuoles and aggregates of cells, later by the disappearance of neuritis and eventually by rounding-up of the cells. At the highest concentrations only indefinite aggregates of damaged and dying cells were seen. The most toxic pyrethroid was cypermethrin, followed by permethrin and natural pyrethrin. At 15 μM cypermethrin concentration only vague aggregations of cells were seen (Fig. 3C). Interestingly, the total ATP was still about 60% of control at this same concentration (Fig. 3A). At 15 μM permethrin concentration photographs showed clear changes in cell morphology (Fig. 2C). However, the amount of total ATP at the same concentration was about 95% compared with the controls (Fig. 2A). Also, in natural pyrethrin-exposed cultures signs of toxicity was clearly seen at 15 μM concentration in microscopic photographs (Fig. 1C). In this concentration the total ATP was close to control levels.

4. Discussion

Pyrethroid products are widely used in agriculture and households. Although these insecticides cannot be considered highly toxic to mammals, their use indoors in enclosed and poorly ventilated spaces has resulted in some symptoms of toxicity in humans (Ecobichon, 1996). The main detoxification routes are oxidation and hydrolysis. The hydrolysis of ester bonds by carboxylesterases is the most efficient detoxification route. The pyrethroid esterases localize in the mammalian liver, mainly in the endoplasmic reticulum and are apparently identical with esterases acting on malathion (Enan and Matsumura, 1992). Non-specific carboxylesterases have been found in tissue homogenates in several species (Sogorb and Vilanova, 2002).

Pyrethroids are grouped into two subclasses (types I and II) based on chemical structure and the symptoms of acute poisoning produced in animals. The type I pyrethroids, pyrethrin, and permethrin lack a cyano moiety, and the type II pyrethroid cypermethrin has an α -cyano group. Usually, pyrethroids with an α -cyano group are more toxic, but also the isomeric form has an effect on the toxic potency. Although this classification (types I and II) system is widely used, it has several defects for the identification of common toxic effects. It does not reflect the diversity of intoxication signs found following oral administration of various pyrethroids (Soderlund et al., 2002).

A well-known toxic effect associated with synthetic pyrethroids is cutaneous paresthesia observed in workers spraying esters containing an α -cyano substituent (Ecobichon, 1996). Paresthesia occurs as a result of a direct effect on intracutaneous nerve endings at very low pyrethroid doses. Pyrethroids without an α -cyano group generally show the weakest physiological effect (Wilks, 2000). Non-cyano pyrethroids, such as permethrin, are thought to produce symptoms mainly through action on the peripheral nervous system. α -Cyano pyrethroids, such

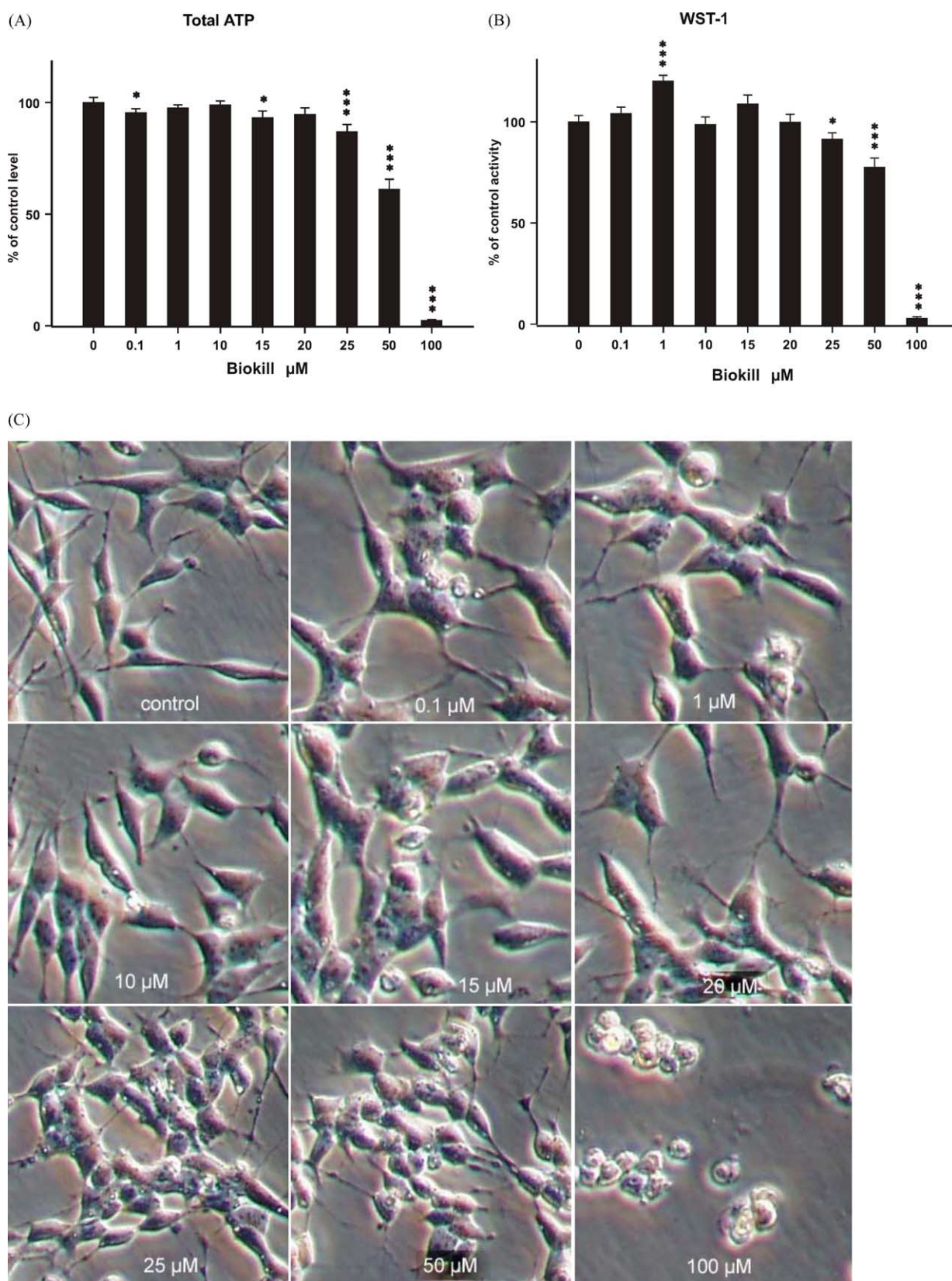


Fig. 2. The 24 h permethrin exposure to SH-SY5Y cells (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) (A) with total ATP measurement, (B) with WST-1 method, and (C) photographs of SH-SY5Y cells exposed to different concentrations of permethrin (Biokill); magnification: 200 \times .

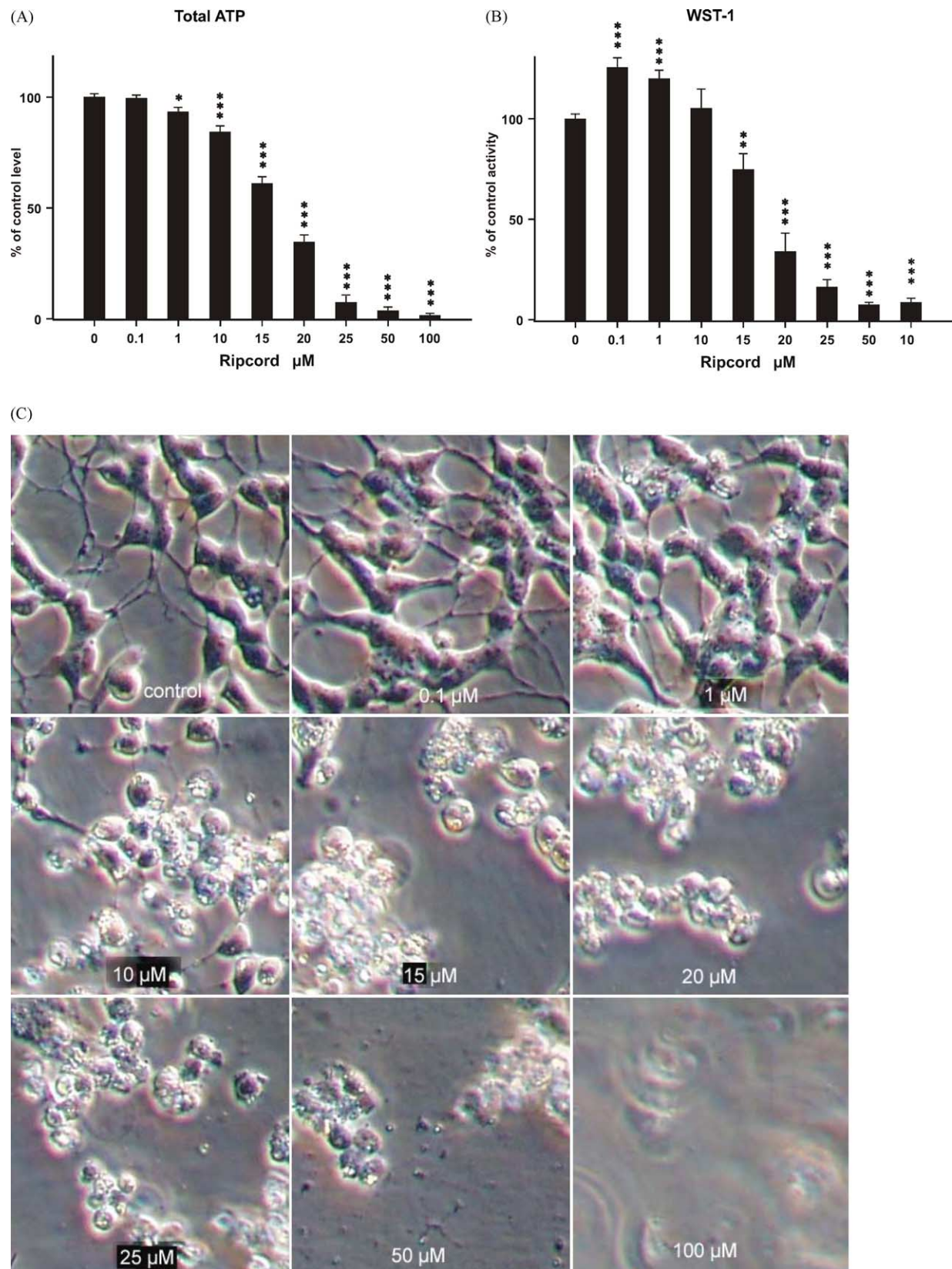


Fig. 3. The 24 h cypermethrin exposure to SH-SY5Y cells (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) (A) with total ATP measurement, (B) with WST-1 method, and (C) photographs of SH-SY5Y cells exposed to different concentrations of cypermethrin (Ripcord); magnification: 200 \times .

as cypermethrin, produce symptoms of the central nervous system (CNS) (Staatz et al., 1982; Verschoyle and Aldridge, 1980).

Due to their lipophilic nature, pyrethroids are taken up by biological membranes and tissues. At the cellular level, pyrethroids act on a variety of biochemical and physiological target sites. The important target sites for both types I and II of pyrethroids in mammals are cell membrane and the sodium channels. Mammals have multiple sodium channel isoforms that vary in their biophysical and pharmacological properties, including their differential sensitivity to pyrethroids. Effects of the pyrethroids bring about delayed and prolonged openings of sodium channels (Narahashi, 1996, 2000; Vijverberg and van den Bercken, 1990; Narahashi et al., 1995; Soderlund and Bloomquist, 1989). There is evidence that membrane ATPases may be involved in the neurotoxic actions of types I and II pyrethroids (Kakko et al., 2000; Kakko et al., 2003).

In pyrethroid metabolism reactive oxygen species are formed and pyrethroids may produce oxidative stress and alteration in antioxidant enzymes in toxication (Kale et al., 1999). Evidence of cell stress was observed in mitochondrial functions, which were reduced in mice given high doses of permethrin (Karen et al., 2001). Similarly, our WST-1 test based on the mitochondrial activity showed a considerable reduction of enzyme activities at high concentrations, but at lower concentrations, a clear activation was evident with all pyrethroid compounds studied.

In our study, the cell toxicity seem to be dependent on the chemical structure of pyrethroids. Exposure to 0.1–100 μ M of natural pyrethrin, as well as of permethrin and cypermethrin showed dose-dependent cytotoxicity evaluated with the ATP assay. The most toxic ones were synthetic pyrethroids, especially cypermethrin with an α -cyano group. Permethrin and natural pyrethrin were less cell toxic. The lowest effect was shown by natural pyrethrin. Other studies have confirmed that pyrethroids without an α -cyano group generally show the weakest physiological effect (Wilks, 2000).

This dose-dependent cytotoxicity was also shown with microscopic photographing. The higher the molarity of pyrethroid compounds the more morphological damage in the cells appeared. The metabolic activity of the enzymes may continue, even the cells seem to suffer (Kale et al., 1999; Karen et al., 2001; Sogorb and Vilanova, 2002). This is why metabolic WST-1 and ATP tests showed slightly different results compared with morphological studying. Microscopic photographing with a digital camera may be a useful supplement method of recording early morphological changes in the cell cultures.

In conclusion, the results of the present study show that pyrethroids have an effect on the viability of SH-SY5Y neuronal cells, and that toxicity seems to depend on the chemical structure of pyrethroids. In the evaluation of cell toxicity, the methods used gave different results. The total ATP test revealed cytotoxicity more clearly than WST-1 method. But the result is due to the different endpoints the tests are based

on, and the activation of mitochondrial enzymes may prove to be important when evaluating the significance of the results. Microscopic photographs of exposed cell cultures correlated to the toxic effects revealed by the metabolic tests.

Acknowledgements

We are grateful to Mrs. Paula Helpiölä for skillful technical assistance.

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