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RESEARCH ARTICLE

Assessment of cytotoxic and genotoxic potential of pyracarbolid by *Allium* test and micronucleus assay

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Abstract

The present study evaluates the cytotoxic and genotoxic potential of pyracarbolid using both micronuleus (MN) assay, in human lymphocytes, and *Allium cepa* assay, in the root meristem cells. In *Allium* test, EC_{50} value was determined in order to selecting the test concentrations for the assay and the root tips were treated with 25 ppm ($EC_{50}/2$), 50 ppm (EC_{50}) and 100 ppm ($EC_{50} \times 2$) concentrations of pyracarbolid. One percent of dimethyl sulphoxide (DMSO) and methyl methane sulfonate (MMS) were used as negative and positive controls, respectively. In the micronucleus assay, the cultures were treated with four concentrations (250, 500, 750 and 1000 μg/ml) of pyracarbolid for 24 and 48 h, negative and positive controls were also used in the experiment parallely. The results showed that mitotic index (MI) significantly reduced with increasing the pyracarbolid concentration at each exposure time. It was also obtained that prophase and metaphase index decreased significantly in all concentration at each exposure time. Anaphase index decreased as well and results were found to be statistically significant, except 24 h. A significant increase was observed in MN frequency in all concentrations and both treatment periods when compared with the controls. Pyracarbolid also caused a significant reduction in the cytokinesis block proliferation index (CBPI) in all concentration and both exposure time.

Keywords

Allium test, genotoxicity, human blood lymphocyte, micronucleus assay, pyracarbolid

History

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Introduction

Nowadays, pesticides are widely used all over the world, not only in agriculture to protect crops, but also in public health to control diseases (Ergene et al., 2007; Pastor et al., 2001). These chemicals or their derivatives could accumulate in the cell and cause risk of mutagenicity, carcinogenicity or genotoxicity (Ergene et al., 2007; Liman et al., 2011). Some pesticides' toxicity was studied by earlier researchers (Konuk et al., 2008; Liman et al., 2010, 2011; Ozakça & Silah, 2013; Sta et al., 2012; Yılmaz et al., 2013).

Plant bioassays are easy to analyze, more sensitive and easier tools to perform the genotoxicity (Grant, 1992; Türkoğlu, 2009). Additionally, these test systems are reliable and can be applied to detect the genetic damage(s). *Allium cepa* are routinely used for the determining the effects of toxic agents and this method is one of the best-established test systems (Fiskesjö, 1985; Konuk et al., 2007; Rank, 2003; Saxena et al., 2005). Moreover, *A. cepa* has been confirmed as indicator organisms for biomonitoring of environmental pollutants (Ateeq et al., 2002; Türkoğlu, 2007).

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The *in vitro* micronucleus test detects small membrane bound DNA fragments in the cytoplasm of interphase cells. The method determinate the clastogenicity (chromosome breakage) and aneugenicity (changes in chromosome number) of test substances in cells that have undergone cell division during or after exposure (İla et al., 2008; İstifli &Topaktaş, 2013; Li et al., 2012; Vrdoljak et al., 2014).

Pyracarbolid is a systemic fungicide that is a member of the anilide family of compounds. It is a broad-spectrum fungicide and used to control rusts and smuts (Shanmuganathan & Saravanapavan, 1978). In our knowledge, there has been no report on the genotoxic effects of pyracarbolid on the somatic cell of *A. cepa* and human peripheral lymphocyte in MN assay, yet. The main goal of this manuscript was to investigate the potential cytogenetic effects of pyracarbolid on both the root meristem cells of *A. cepa* using mitotic index (MI), and mitotic phases and genotoxic effects on human peripheral lymphocyte with MN assay.

Materials and methods

Chemicals

The test substance pyracarbolid (CAS No: 24691-76-7, ≥99% purity, molecular formula: $C_{13}H_{15}NO_2$) was purchased from

Fluka (Turkey), and dissolved in dimethyl sulphoxide (1% DMSO, purity 99%). Mitomycin-C (MMC, CAS No: 50-07-7) was used as positive control and dissolved in sterile double-distilled water. Cytochalasin B (CAS no: 14930-96-2), chromosome medium B (Biochrom Cat. No. F5023) and DMSO (CAS No. 67-68-5) were obtained from Sigma-Aldrich (Turkey). *A. cepa* ($2n = 16$) onion bulbs, 25–30 mm diameter, without any treatments, were purchased from a local supermarket. The other chemicals were obtained from Merck and Riedel (Turkey).

Allium test

EC_{50} determination

Allium root inhibition test was performed to determine suitable concentrations for the genotoxicity assay. The procedure of the root inhibition test as described by Fiskesjo (1985) was followed with some modifications (Rencüzoğulları et al., 2001). Outer scales of the bulbs and the dry bottom plates were removed without destroying the root primordia. The onions were grown in freshly distilled water for the first 24 h and afterwards exposed for 96 h to the pyracarbolid solutions (6.25, 12.5, 25, 50 and 100 ppm, respectively). In order to determine the EC_{50} values, the roots from each bundle were cut off on the fifth day and length of each root was measured from both pyracarbolid exposed bulbs and control group. EC_{50} value was considered as the concentration which retards the growth of root 50% less when compared with the control group.

MI analysis

The EC_{50} value for pyracarbolid was approximately 50 ppm. In order to demonstrate possible concentration-dependent effects of this pesticide, the root tips were treated with 25 ppm ($EC_{50}/2$), 50 ppm (EC_{50}) and100 ppm ($EC_{50} \times 2$) concentrations of pyracarbolid and all application groups were tested 24, 48 and 72 h treatment periods.

After treatment, the roots were washed in distilled water and fixed in 3:1 ethanol:glacial acetic acid for 24 h and then roots were transferred 70% alchol and stored +4 °C. Root tip cells were stained with Feulgen and five slides were prepared for each test group.

Micronucleus assay

Lymphocyte cultures

Peripheral venous blood was obtained from four healthy donors (two male and two female nonsmokers, aged 22–30 years) not exposed to any drug therapy or not known mutagenic agent over the past two years. In this part of the study, the method of Rothfuss et al. (2000) was followed. Informed consent was obtained from all donors and the study was carried out by the local ethics committee.

Experimental method

The blood from four healthy donors was added to 2.5 ml chromosome medium B and incubated at 37 °C for 68 h. The cells were treated with 250, 500, 750 and 1000 µg/ml concentrations of pyracarbolid for 24 and 48 h treatment periods. Cytochalasin B was added to the cultures at final concentration of 6 mg/mL 44 h after initiating the cultures in order to block cytokinesis. A positive (Mitomycin-C 0.20 µg/ml) and a negative control (DMSO) were also used paralelly. At the end of the incubation period, the cells were treated with a hypotonic solution (0.4% KCl) at 37 °C for 10 min and fixed once with a fixative (glacial acetic acid/methanol/0.9% NaCl = 1/5/6) for 20 min and then fixed twice with another fixative (glacial acetic acid/methanol = 1/5) for 20 min. The MN slides were dried in air overnight and stained with 5% Giemsa for 14 min.

Microscopic evaluation

For MI, the different stages of mitosis were counted in at least 5000 cells (1000 cells/slide) per concentration, and expressed as a percentage. For the MN test, 8000 binucleate lymphocytes with well preserved cytoplasm were scored from each donor only one exposure time (2000 binucleate cells per concentration) for each experiment. A total of 2000 intact cells were scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei. CBPI was calculated for cytotoxicity of pyracarbolid with using the formula below:

$$[(1 \times \mathrm{M1}) + (2 \times \mathrm{M2}) + (3 \times \mathrm{M3}) + (4 \times \mathrm{M4})]/N;$$

where M1–M4 represent the number of cells with 1–4 nuclei and N is the total number of intact cells scored (Fenech, 2000).

Statistical analyses

Dunnett *t*-test (two tailed) was performed in order to compare the frequency of micronucleus cells between negative and positive control. The same test was also used for CBPI and *Allium* test results.

Results

In this study, we aimed to determine the potential cytotoxic effect of pyracarbolid by using both *Allium* test and micronucleus assay. Table 1 summarizes the results of *Allium* root growth inhibition test. The effective concentration (EC_{50}) value which retards 50% root growth was found approximately 50 ppm.

Table 2 shows the effects of pyracarbolid on MI and mitotic phases in the root cells of *A. cepa* treated for 24, 48 and 72 h. According to results, MI significantly decreased in all concentrations of pyracarbolid and at each exposure time.

Table 1. *Allium* root growth inhibition test.

Test substance	Concentrations (ppm)	Mean of root length ± SD
Negative control	–	4.40 ± 0.21
Positive control	–	1.02 ± 0.12[a]
	6.25	3.63 ± 0.28[a]
	12.5	2.80 ± 0.19[a]
Pyracarbolid	25	2.74 ± 0.21[a]
	50	2.32 ± 0.37[a]
	100	2.27 ± 0.18[a]

[a]Significantly different from negative control ($p < 0.05$ Dunnet *t*-test, 2-sided).
SD: Standart deviation, negative control: 1% DMSO, positive control: 10 ppm methyl methane sulfonate and cytotoxic.

Table 2. The effects of pyracarbolid on MI and mitotic phases in the root cells of *A. cepa*.

Concentration (ppm)	Treatment time (h)	Counted cell number	Mitotic index ± SD	Mitotic phases (%) ± SD			
				Prophase	Metaphase	Anaphase	Telophase
Negative control		5039	81.30 ± 10.83	77.34 ± 10.67	1.97 ± 0.28	1.05 ± 0.57	0.93 ± 0.48
Positive control		4439	40.36 ± 5.57[a]	38.47 ± 5.36[a]	0.75 ± 0.27[a]	0.58 ± 0.24	0.55 ± 0.38
25	24	4861	49.14 ± 3.00[a]	46.16 ± 2.81[a]	1.15 ± 0.37[a]	0.75 ± 0.34	1.09 ± 0.56
50		5033	39.39 ± 5.57[a]	35.64 ± 5.09[a]	1.27 ± 0.49[a]	1.04 ± 0.69	1.32 ± 0.49
100		5250	36.28 ± 2.50[a]	34.12 ± 2.66[a]	0.98 ± 0.24[a]	0.49 ± 0.14	0.68 ± 0.17
Negative control		5092	73.31 ± 1.78	68.87 ± 2.50	1.74 ± 0.27	1.37 ± 0.36	1.32 ± 0.28
Positive control		5073	36.16 ± 2.02[a]	33.95 ± 2.12[a]	0.75 ± 0.29[a]	0.67 ± 0.40[a]	0.77 ± 0.40
25	48	5177	29.60 ± 2.76[a]	26.74 ± 2.68[a]	0.82 ± 0.19[a]	0.79 ± 0.02[a]	1.25 ± 0.10
50		5207	24.45 ± 3.00[a]	22.78 ± 3.07[a]	0.45 ± 0.03[a]	0.63 ± 0.17[a]	0.60 ± 0.27[a]
100		5186	22.42 ± 1.28[a]	20.52 ± 1.62[a]	0.38 ± 0.12[a]	0.55 ± 0.18[a]	0.97 ± 0.50
Negative control		5200	61.68 ± 2.26	57.59 ± 2.77	1.57 ± 0.42	1.53 ± 0.35	0.99 ± 0.45
Positive control		5233	32.11 ± 4.02[a]	29.26 ± 3.79[a]	1.05 ± 0.48	0.90 ± 0.25[a]	0.91 ± 0.35
25	72	5152	25.26 ± 5.88[a]	23.17 ± 5.74[a]	0.84 ± 0.09[a]	0.68 ± 0.15[a]	0.57 ± 0.22
50		4786	24.04 ± 2.42[a]	21.19 ± 1.84[a]	0.75 ± 0.21[a]	0.67 ± 0.49[a]	0.60 ± 0.22
100		5425	13.12 ± 2.89*	11.55 ± 3.00*	0.55 ± 0.26*	0.33 ± 0.20*	0.69 ± 0.51

[a]Significantly different from negative control ($p < 0.05$ Dunnet *t*-test, 2-sided).
SD: Standart deviation, negative control: 1% DMSO, positive control: 10 ppm methyl methane sulfonate and was cytotoxic.

Table 3. The frequency of MN and CBPI in cultured human lymphocytes treated with pyracarbolid.

Test Substance	Treatment time (h)	Concentration (μg/ml)	Micronucleated binuclear cells (%) ± SD	Cytokinesis block proliferation index ± SD
DMSO			0.11 ± 0.01	1.46 ± 0.12
MMC		0.20	6.11 ± 0.10[a]	1.18 ± 0.09[a]
	24	250	0.55 ± 0.04[a]	1.34 ± 0.08
Pyracarbolid		500	0.65 ± 0.04[a]	1.31 ± 0.09
		750	1.03 ± 0.22[a]	1.27 ± 0.09[a]
		1000	1.22 ± 0.23[a]	1.22 ± 0.1[a]
DMSO			0.18 ± 0.06	1.52 ± 0.09[a]
MMC		0.20	14.46 ± 1.76[a]	1.02 ± 0.04[a]
	48	250	0.43 ± 0.10[a]	1.36 ± 0.06[a]
Pyracarbolid		500	0.68 ± 0.02[a]	1.32 ± 0.06[a]
		750	1.14 ± 0.27[a]	1.25 ± 0.09[a]
		1000	1.38 ± 0.10[a]	1.18 ± 0.07[a]

[a]The mean difference is significant at the 0.05 level Dunnett *t*-test (2-sided).
SD: Standard deviation, positive control: mitomycin-C (MMC) was cytotoxic.

The lowest MI values were obtained from 72 h applications of 100 ppm with the score of 13.12 and the highest one in 24 h applications of 25 ppm concentrations with the score of 49.14. All MI results were reduced significantly in all concentrations and at each exposure time of pyracarbolid. In addition percentage of particular phases (prophase, metaphase, anaphase and telophase) was determinated in the experiment. In general, this pesticide decreased the percentages of the both prophase and metaphase stages, significantly in all concentrations in 24, 48 and 72 h periods. The percentages of the anaphase also decreased significantly except for 24 h period. Pyracarbolid caused differentiation in the stage of telophase when compared with the control but these results were not found significant statistically except 48 h applications of 50 ppm with the score of 0.60.

MN assay and CBPI results were given in Table 3. *In vitro* MN assay, the cultures were treated with four different concentrations of pyracarbolid (250, 500, 750 and 1000 μg/ml) for 24 and 48 h. MN frequency significantly induced due to increasing pyracarbolid concentrations for the 24 and 48 h treatment periods. The highest MN value of pyracarbolid were obtained from 48 h applications of 1000 μg/ml with the score of 1.38, the lowest one from 48 h applications of 250 μg/ml with the score of 0.43. However, the frequencies of MN values were lower than the positive control. CBPI was also evaluated in this study for determinate to cytotoxicity of this substance. Pyracarbolid caused reduction in the CBPI for both 24 and 48 h treatment periods significantly when compared with negative control. Concentrations of this pesticide (250 and 500 μg/ml) decreased CBPI but these results were not found significant statistically.

Discussion

Genotoxicity tests and the determination of the carcinogenicity and mutagenicity of new chemicals are the most important part of the safety testing of these compounds. Genotoxicity tests are efficient, reliable and well-established methods for the evaluation of changes in the genetic material as a result of environmental and occupational exposure. To determinate the carcinogenic and mutagenic risks of compounds, genotoxicity testing is considered as a valuable tool (Soloneski & Larramendy, 2010). Therefore, the evaluation of the genotoxicity risks of environmental pollutants is important to provide useful information about carcinogenicity and mutagenicity. In the present study, we investigated the potential genotoxic and cytotoxic effects of pyracarbolid with two different reliable genotoxicity assays.

A lot of plant test systems such as *Tradescantia paludosa* (Mišik et al., 2006), *Vicia faba* (Sang & Li, 2004), *Pisum sativum* (Kluge & Podlesak, 1985), *Hordeum vulgare* (Amer et al., 1999; Özkara et al., 2011), *Crepis capillaris* (Gadeva & Dimitrov, 2008) and *A. cepa* (Barberio et al., 2011; Fiskesjö, 1985, Grant, 1992; Rank, 2003; Saxena et al., 2005; Yıldız et al., 2009), were developed for evaluating the genotoxicity and cytotoxicity of different pollutants.

The *A. cepa* test is one of the most sensitive and easier method in order to evaluate the toxicity of different substances and this test have been successfully carried out in previous research for pesticides (Barberio et al., 2011, Liman et al., 2010, 2011; Yıldız & Arıkan, 2008). In *Allium* test, EC_{50} (50 ppm) value was firstly determined before setting up the experiments. The effective concentration value was used to be useful parameter for selecting the test concentrations for the genotoxicity assays (Chauhan et al., 1999; Ma et al., 1995). In *Allium* root growth inhibition test, dose-dependent significant inhibition referred to the potential cytotoxicity of substance which was used in the experiment. The inhibition of root growth in *A. cepa* test can be related with apical meristematic activity (Liu et al., 2003; Webster & MacLeod, 1996), the inhibition of cell elongation in the extension regions (Fusconi et al., 2006; Voutsinas et al., 1997) and the inhibition of protein synthesis (Seth et al., 2007).

MI is proved to be a useful parameter that allows one to evaluate the frequency of cellular division (Marcano et al., 2004). Estimation of potential cytotoxicity of substances is generally related to the inhibition of mitotic activities (Smaka-Kincl et al., 1996). In our study, the used concentrations of pyracarbolid also caused significant inhibition of MI. In the previous studies, a number of researchers have reported the inhibitions in MI by pesticides (Marcano et al., 2004; Pandey, 2008; Yıldız & Arıkan, 2008).

The significant decline of MI could be due to the inhibition of DNA synthesis or blocking of G1 suppressing DNA synthesis or the effect of test compound at G2 phase of the cell cycle (El-Ghamery et al., 2000; Majewska et al., 2003; Sudhakar et al., 2001). When a pesticide penetrates the cells and reach a critical concentration, it could be in active form, causing lesions during several following cellular cycles (Marcano et al., 2004; Rank, 2003). It is thought that the decline of MI in our study can be related to these reasons.

In this study, all concentrations of pyracarbolid caused the changes in the percentage of particular phases' distribution when compare with the control group. Generally, this pesticide decreased the percentages of the prophase and metaphase stages in *A. cepa* significantly in all concentration and at each exposure time. Beside this anaphase stage was also decreased but the data was not found significant. Pyracarbolid caused differentiation in the stage of telophase when compared with the control.

Pesticides accumulate in the cell and cannot step out of the cell easily after once penetrate the cell and may be highly toxic in the cell (Antunes-Madeira & Madeira, 1979). The accumulation of the pesticides into the cell may affect the phases of mitotic division. The similar results have been reported with different pesticides in *Allium* test (Andrioli et al., 2012; Liman et al., 2010; Pandey, 2008; Srivastava & Mishra, 2009; Yıldız & Arıkan, 2008).

The *in vitro* micronucleus assay is generally used routinely for the assessment of chemicals' safety. This assay defines clastogenic (compounds directly react with DNA) and aneugenic activity (compounds interact with the components of the mitotic and meiotic cell division cycle) of environmental pollutants (Kirkland, 2005).

In this study, increasing pyracarbolid concentrations caused to significant increase in MN frequency both treatment periods when compared with negative control. Our results showed that pyracarbolid induced significant micronucleus formation in cells treated with concentrations. The similar results have been reported from the other investigators in MN assay with pesticides (Ali et al., 2008; Ergene et al., 2007; Graillot et al., 2012; Revankar & Shyama, 2009; Zeljezic & Garaj-Vrhovac, 2004).

CBPI was also evaluated in this study. A significant reduction was obtained in CBPI by pyracarbolid for both 24 and 48 h treatment periods when compared with negative control. Concentrations of this pesticide (250 and 500 µg/ml) decreased CBPI, but these results were not found to be statistically significant. In accordance to these results, pyracarbolid has cytotoxic effect due to decreasing of CBPI and the similar results have been reported from the other researchers with our work (Ila et al., 2008; İstifli & Topaktaş, 2013).

The tested substances with different test systems can be genotoxic or not genotoxic depending on a number of factors such as chemical structure and biological activity, having rings in the structure and the positions of the binding location (Kutlu et al., 2011). In addition to these, it might be related to differences in test conditions, such as exposure time, cell types, concentrations of substances, the dispersal of the materials and physico-chemical characteristics of the compounds (Ema et al., 2012).Therefore, it could be explained why some studies find an increase of genetic damage while in others result as negative.

In conclusion, pyracarbolid was found to be cytotoxic due to the decrease of MI in *Allium* test and CBPI in MN test. This pesticide also induced MN frequency. However, we suggest that further ongoing assays, using other *in vitro* and *in vivo* systems, to test the genotoxicity of the same pesticide will improve our knowledge on the base of estimating the genotoxic risks and the main characteristics of their toxicity.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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