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ORIGINAL RESEARCH

Micronucleus assay in human lymphocytes after exposure to alloxydim sodium herbicide in vitro

Dilek Akyıl · Arzu Özkara · S. Feyza Erdoğmuş · Yasin Eren · Muhsin Konuk · Esra Sağlam

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Abstract This study evaluates the cytotoxic and genotoxic potential of alloxydim sodium using micronucleus (MN) assay, in human peripheral lymphocytes. MN assay was used to investigate the genotoxic effects of alloxydim sodium in human peripheral lymphocytes treated with 250, 500, 750, 1,000 μg/ml concentrations of alloxydim sodium for 24 and 48 h. Solvent, negative and positive controls were also used in the experiments in parallel. The obtained results were evaluated in statistical analyses by using Dunnett-*t* test (two sided) and $p < 0.05$ was accepted as significant. Alloxydim sodium significantly increased the MN formation compared with the negative control, at both 750 and 1,000 μg/ml concentrations and treatment periods. We also evaluated the nuclear division index (NDI) for cytotoxicity of this pesticide in the experiment, and finally observed a significant decrease of the NDI values at all concentrations of alloxydim sodium and at both treatment periods.

Keywords Alloxydim sodium · Genotoxicity · Micronucleus assay · MN frequency · Nuclear division index

D. Akyıl (✉) · A. Özkara
Department of Biology, Faculty of Arts and Sciences, Afyon Kocatepe University, Afyonkarahisar, Turkey
e-mail: dilekakyil9@gmail.com

S. F. Erdoğmuş
Department of Veterinary Sciences, Bayat Vocational High School, Afyon Kocatepe University, Afyonkarahisar, Turkey

Y. Eren
Department of Science, Faculty of Education, Suleyman Demirel University, Isparta, Turkey

M. Konuk
Department of Molecular Biology and Genetics (English), Faculty of Engineering and Natural Sciences, Üsküdar University, Istanbul, Turkey

E. Sağlam
Department of Pharmacology and Clinical Pharmacology, Üsküdar University, Istanbul, Turkey

Introduction

Pesticides are chemicals designed to eliminate the pests, such as weeds, insects, rodents and fungi. They also improve the quantity and quality of yield in agriculture without causing much damage to non-target species (Zahm and Blair 1993). Although the benefits of conventional pesticides have been immense, humans and other living organisms are often exposed to them in the environment (WHO 1990). Widespread usage of synthetic pesticides for agricultural purpose has an adverse effect on living organisms and can lead to several hematological and neurological complications in individuals (Broughton et al. 1990; Vojdani et al. 1992; Wells and Nerland 1991).

Pesticides can be characterized on the basis of their function as herbicidal, insecticidal, fungicidal, etc. and also on the basis of chemical nature, i.e.

organophosphates, organochlorides, s-triazines and pyrethroids (Jamil et al. 2004; Whitney et al. 1995; Aksoy 1989). Herbicides which are used intensively in agriculture, are designed for the control of weeds (Bolognesi 2003). They are the most widely applied agrochemicals and their application is the most accepted and effective method for plant protection from weeds (Nikoloff et al. 2012a).

Alloxydim sodium is one of the most intensively used cyclohexene oxime herbicides in agriculture for the control graminaceous weeds in a wide range of broad-leaf crops. It is absorbed from roots and leaves of grasses, acts on active meristematic tissues and gives rise to necrotic death of the plants (Veeresakeran and Catchpole 2006; Iwataki and Hirono 1979).

Alloxydim sodium shows its toxic effect especially to aquatic organisms. LC_{50} values for *Cyprinus carpio*, *Oncorhynchus mykiss* and *Poecilia reticulata* species were found to be 191, 174, 394 µg/l after 48 h application, respectively (Svobodova et al. 1986). Although it is known that this herbicide is easily degraded under different conditions, there is little information in the literature on the fate of this compound in the environment (Sandín-España et al. 2005).

Genotoxicity and cytotoxicity studies are frequently used to test numerous agrochemical compounds with different test systems (Ergene et al. 2007; Lin and Garry 2000; Rakitsky et al. 2000; Soloneski et al. 2007, 2008; Soloneski and Larramendy 2010; Zeljezic et al. 2006). These test systems are valuable and very well-known tools for the early and sensitive detection or estimation of genotoxic potential of chemicals. In addition, in vitro and in vivo methods focusing on test compounds provide more informative evidence about the genotoxic effects of specific pesticides (Santovito et al. 2012). The use of in vitro cell cultures for genotoxic and cytotoxic evaluation is rather economic and they are highly sensitive methods for the early detection of chemical exposure and toxicity (DiPaolo et al. 1981). Among them, one of the most used system for clastogenic and/or aneugenic screening is the micronucleus assay in human peripheral blood lymphocytes (Ali et al. 2011; González et al. 2011; Nikoloff et al. 2012b; Soloneski et al. 2008; Vera-Candioti et al. 2013).

The micronucleus (MN) assay is widely used and a good indicator of evaluation for pesticide genotoxicity estimation. Micronuclei are whole or partial chromosomes that have not been incorporated into the daughter nucleus following mitosis due to the chromosome breaking (clastogenic process) or mitotic spindle dysfunction (aneugenic process) (Fenech 2000; 2007). Micronuclei are indirect indicators of numerical and structural chromosomal aberrations (Albertini et al. 2000).

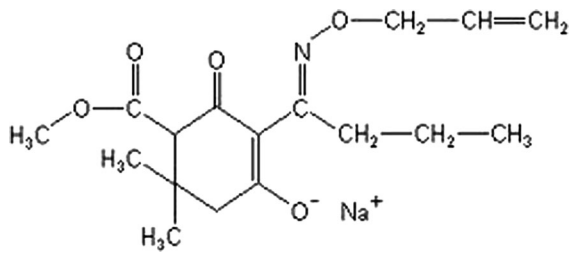

Fig. 1 The chemical structure of alloxydim sodium

The aim of this study was to determine if alloxydim sodium induces genotoxic damage in cultured human lymphocytes by using the in vitro micronucleus assay.

Materials and methods

Chemicals

The test substance alloxydim sodium was obtained from Fluka (Buchs, Switzerland; CAS No. 55635-13-7, molecular weight: 345.37 g/mol, purity of 97.2 %) and dissolved in DMSO (CAS No. 67-68-5). Mitomycin-C, cytochalasin B (CAS No. 14930-96-2) chromosome medium B (Biochrom, Cambridge, UK; cat. No. F5023) and Giemsa were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals were obtained from Merck (Darmstadt, Germany) and Riedel-de Haën (Buchs, Switzerland). The chemical structure of alloxydim sodium is shown in Fig. 1.

Lymphocyte cultures

Whole blood samples were obtained by venipuncture in heparinized tubes for genotoxicity testing. Peripheral venous blood was collected from four healthy donors (non-smokers, non-drinkers, not under drug therapy, and with no recent history of exposure to mutagens and aged 22–30 years) under sterile conditions. Informed consent was obtained from all donors and the study was carried out according to the local ethics committee.

Micronucleus assay

MN technique was carried out according to the method described by Fenech (2000) with some modification. The blood samples obtained from 4 healthy donors were added to 2.5 ml Chromosome Medium B (containing MEM Joklik with non-essential aminoacids, fetal bovine serum, heparin, penicillin G-sodium salt, streptomycin sulphate, phytohaemagglutinin L, ascorbic acid, glutathione-reduced) and incubated at 37 °C for 68 h. Cytochalasin B (final concentration 6 μg/ml) was added into the medium to arrest cytokinesis 44 h from the initiation. Mitomycin-C (MMC, 0.20 μg/ml) was used as positive control, and a negative control (untreated cultures) was also used in parallel. Different concentrations of alloxydim sodium (250, 500, 750, 1,000 μg/ml) were added 24 and 48 h after the incubation. These doses were determined based on the highest doses causing a reduction in the mitotic index of more than 50 % according to Sivikova and Dianovsky (2000). At the end of the incubation period, the cells treated with hypotonic solution (0.4 % KCl). Cells were re-centrifuged and fixed once with fixative (methanol:glacial acetic acid, 0.9 % NaCl 5:1:6) for 20 min. Fixation was repeated twice with methanol:glacial acetic acid (5:1). Microscope slides were prepared in duplicate by dropping cell samples, air-drying, and staining with 5 % Giemsa solution at pH 6.8 for 14 min. They were finally washed in distilled water, and dried at room temperature.

Slide evaluation

Micronuclei were scored from 2,000 binucleated cells per donor with well-preserved cytoplasm (totally 8,000 binucleated cells per concentrations). Criteria for scoring binucleated cells and MN were applied according to Fenech (2000). Cell proliferation was evaluated, using the nuclear division index (NDI), which indicates the average number of cell cycles. Totally 2,000 viable cells were scored to evaluate the percentage of cells with 1, 2, 3 and 4 nuclei. NDI was calculated using the formula: $[(1 \times M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N$; where M1–M4 represent the number of cells with one to four nuclei and N is the total number of intact cells scored (Fenech 2000). For the statistical analysis of the results, differences between treated samples and controls were tested with the Dunnett-t test (two sided).

Results

The results of the MN assay are summarized in Table 1. To evaluate the effects of alloxydim sodium on human peripheral lymphocytes, the MN frequency was investigated at different concentrations (250, 500, 750, 1,000 μg/ml) and different treatment periods (24 and 48 h) of the herbicide. In the MN assay, micronuclei were scored in 2,000 bi-nucleated lymphocytes with well-preserved cytoplasm per donor (total 8,000 bi-nucleated cells per concentration), for evaluation of MN according to Fenech (2000). A total of 2,000 lymphocytes per donor were scored to assess the percentage of cells with 1–4 nuclei. Figure 2a, b shows a binucleated cell with one and two micronuclei.

According to the results, the solvent control groups did not display any difference with the negative controls confirming that, at this low concentration (1 %), DMSO has no biological effects. Alloxydim sodium significantly ($p < 0.05$) increased the MN formation compared to the negative control, at concentrations of 750 and 1,000 μg/ml for both treatment periods. MMC was used as a positive control in this experiment and it showed a significantly increased MN formation compared with the negative and solvent controls for both treatment periods. We also evaluated the NDI for cytotoxicity of this pesticide in the experiment. Finally, we observed a significant decrease of the NDI values at all concentrations of alloxydim sodium and both treatment periods. MN formation and NDI values are shown graphically in Figs. 3, 4.

Discussion

A number of references reported on the possible genotoxic and cytotoxic effects of pesticides with different models. Therefore, we have much information about these methods but their clastogenic and aneugenic effects should be investigated.

Among the genotoxic methods, MN assay is often used to investigate the genotoxicity of chemicals due to its sensitivity and reliability as a marker of cytogenetic damage (Fenech 1993; Bolognesi 2003). MN assay in peripheral lymphocytes can be a useful biomarker for evaluating the early biologic effects of exposure to pesticides. It may arise from a whole lagging chromosome (aneugenic event leading to

Table 1 The frequency of micronucleus and nuclear division index in cultured human lymphocytes treated with alloxydim sodium

Test substance	Treatment time (h)	Concentration (μg/ml)	Micronucleated binuclear cells (%) ± SD	Nuclear division index ± SD
Solvent control	24	–	0.18 ± 0.01	1.56 ± 0.04
(−) Control (DMSO)			0.16 ± 0.21	1.58 ± 0.08
MMC		0.20	11.28 ± 2.44*	1.15 ± 0.04*
Alloxydim sodium		250	0.25 ± 0.10	1.44 ± 0.04*
		500	0.28 ± 0.05	1.42 ± 0.05*
		750	0.45 ± 0.24*	1.40 ± 0.04*
		1,000	0.68 ± 0.26*	1.36 ± 0.04*
Solvent control	48	–	0.23 ± 0.02	1.49 ± 0.05
(−) Control (DMSO)			0.20 ± 0.02	1.56 ± 0.04
MMC		0.20	12.02 ± 4.55*	1.18 ± 0.06*
Alloxydim sodium		250	0.26 ± 0.05	1.42 ± 0.05*
		500	0.30 ± 0.01	1.40 ± 0.05*
		750	0.52 ± 0.01*	1.38 ± 0.06*
		1,000	0.72 ± 0.14*	1.34 ± 0.04*

* The mean difference is significant at the 0.05 level Dunnett *t* test (2-sided)

SD standard deviation, *MMC* mitomycin-C

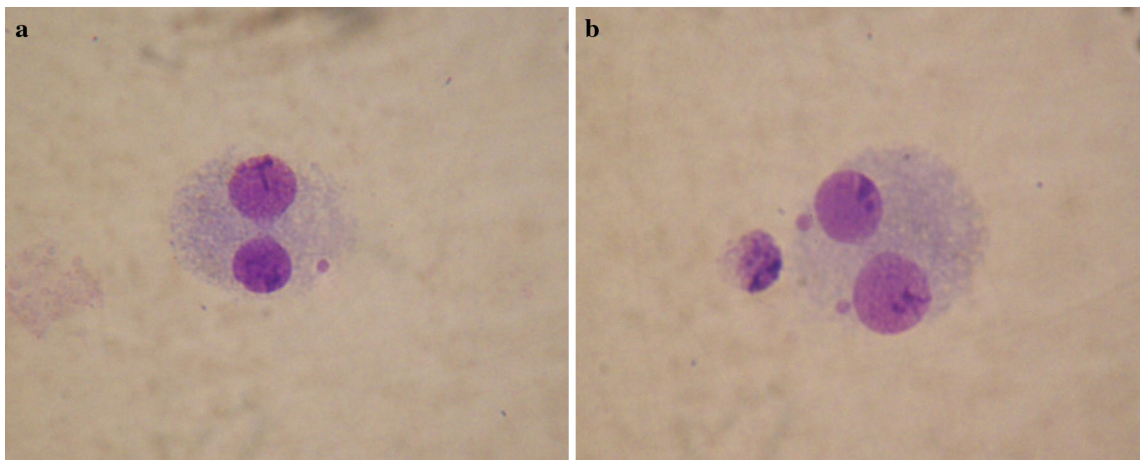

Fig. 2 A binucleated cell with one and two micronuclei

chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei (Fenech and Morley 1985; Tucker and Preston 1996).

In this study, the MN assay was chosen to investigate the potential risk for chromosome damage in cultured human lymphocytes exposed to different concentrations of alloxydim sodium (250, 500, 750, 1,000 μg/ml) for 24 and 48 h. Although alloxydim sodium is one of the most widely used herbicides in agriculture, for the control graminaceous weeds in a wide range of broad-leaf crops, the available information on the genotoxic potential of these herbicide is insufficient.

Alloxydim sodium is one of the postemergence systemic herbicides and belongs to the cyclohexanedione family of herbicides and an inhibitor of Acetyl CoA carboxylase (ACCase). This enzyme is the key enzyme in the fatty acid biosynthesis that catalyses the carboxylation of acetyl-CoA to malonyl-CoA which is necessary for the biosynthesis of fatty acids and secondary metabolites (Nikolskaya et al. 1999; Radwan 2012). Banas et al. (1993, 2000) have reported

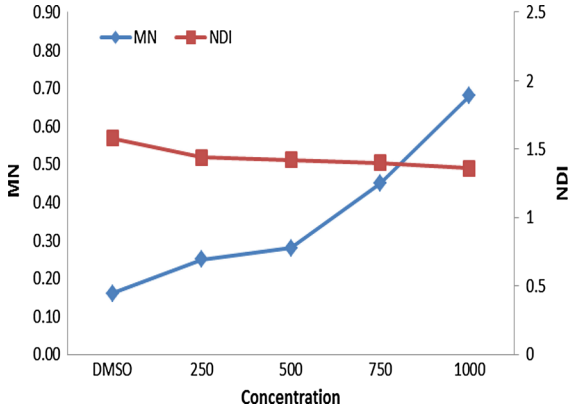

Fig. 3 Micronucleus frequency (MN) and nuclear division index (NDI) of alloxydim sodium for 24 h treatment

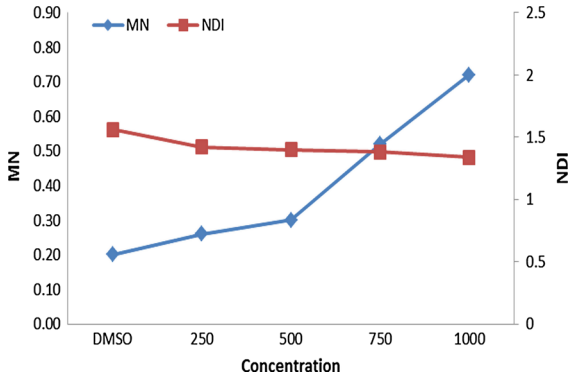

Fig. 4 Micronucleus frequency (MN) and nuclear division index (NDI) with alloxydim sodium for 48 h treatment

that ACCase inhibitor herbicides such as Haloxyfop and Alloxydim have a mode of action related to the overproduction of free radicals and oxidative stress. The application of herbicides can increase the superoxide levels and cause reactive oxygen species (ROS) accumulation which accompanied by lipid peroxidation (Feierabend and Winkelhüsener 1982). Accumulation of ROS can not only cause lipid peroxidation but also induce membrane damage and cause serious defects in the physiological metabolism of plants leading to cell death and stimulated plant senescence (Guo et al. 2006; Ogweno et al. 2009).

Our results showed that alloxydim sodium induced significant MN formation in cells treated with concentrations of 750 and 1,000 µg/ml for both treatment periods (24h, 48h). MN frequency also increased in the two other concentrations (250 and 500 µg/ml), however, the results were not found statistically significant. Besides, a significant reduction of the NDI value was observed in all cultures treated with alloxydim sodium for all exposure time. These results indicate that this herbicide can inhibit ACCase and increase MN frequency.

The cyclohexanedione family of herbicides has been studied by previously investigators with different test systems except the micronucleus assay and different results were obtained. Clethodim is also one of the postemergence systemic herbicides and belongs to the cyclohexanedione family of herbicides (Prostko et al. 2001; Clewis et al. 2002). Radwan (2012) applied this herbicide in maize and Clethodim application to maize leaves caused leaftip yellowing, browning or drying in some parts of the leaf. These injuries were probably due to enhanced production of ROS and/or accumulation of H_2O_2 and oxidative stress leading to death of some leaf parts. In a different study Sethoxydim which is an herbicide belonging to same group was investigated with the Salmonella assay and was not found mutagenic in this method (Claxton et al. 2004). In addition, Alloxydim was investigated with a standard microtest procedure based on the decrease of light emission by the marine bacterium *Vibrio fischeri*. The results indicated that the toxicity of the photoproducts was higher than the toxicity of the parent compound (Sandín-España et al. 2013).

Although there was no study with micronucleus assay in cyclohexanedione herbicides, MN test system was used by many investigators for other pesticide groups and they reported increases in MN formation (Zeljezic and Garaj-Vrhovac 2004; Yüzbasioglu et al. 2006; Ergene et al. 2007; Ali et al. 2008; Revankar and Shyama 2009). These results support our findings. However, some investigators reported that different pesticides did not lead to MN formation (Surrales et al. 1995; Gollapudi et al. 1995; Villarini et al. 1998). These results contradict our findings. On the other hand, Alloxydim sodium has cytotoxic effect due to decreasing the NDI and similar results have been reported by Ila et al. (2008) and İstifli and Topaktaş (2013).

The emergence of so many different results can be a result of contact time with the pesticides, the dose of the substance, or the way of how it is metabolized – directly or indirectly. Organophosphates, pyrethroids, organochlorines, and carbamates have been reported to be genotoxic, generating free radicals that react with

cell membranes. The accumulation of these radicals can cause oxidative stress, depending on the antioxidant capacity of individuals exposed to these pesticides (Hérnandez et al. 2005; Salvador et al. 2008). Binucleated cells were also found to be more frequent in the group exposed to pesticides. They might be indicative of the failure of cytokinesis due to aneuploidy (Bonassi et al. 2011). The genotoxicity of the investigated compounds could depend on the chemical structure, biological activity, having rings in the structure and the positions of the binding location etc. (Kutlu et al. 2011). Moreover, it might be related to differences in lifestyle, climate and environmental conditions, and the pesticides are used at different quantities, at different durations of exposure and individual eating habits (Omenn 1991). Therefore, it can be explained, why some studies find an increase of genetic damage whilst in others the results are negative. Due to these reasons, genotoxic evaluation using different test systems are useful and necessary to determinate genotoxic effects of chemicals. By assessing genotoxic modifications in individuals, those who are at risk to develop diseases such as cancer may be identified and greater attention may be recommended.

In conclusion, alloxydim sodium was found to be cytotoxic and genotoxic due to decreasing of NDI and increasing of MN frequency. Micronucleus assay is highly sensitive in the detection of hazards arising from pesticides; further investigations are needed to determine the toxicity of these pesticides with in vivo and in vitro test systems.

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