

In vitro aneugenic effects of the fungicide thiabendazole evaluated in human lymphocytes by the micronucleus assay

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Received: 28 July 2010 / Accepted: 28 September 2010 / Published online: 12 October 2010
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Abstract Thiabendazole is a benzimidazole-derived compound widely employed in agriculture as anthelmintic and fungicide. It is also used as a post-harvest fungicide for imported citrus fruits during transport and storage, and thus, it was found at high concentration in fruits and vegetables. Several studies have analyzed the potential genotoxic effect of thiabendazole on different prokaryotic and eukaryotic systems, but in many cases, results were contradictory. In the present study, the genotoxic potential of thiabendazole have been evaluated, by micronucleus assay in freshly isolated human peripheral lymphocytes. The cells were incubated with 0.5, 5 and 50 µg/ml concentrations of the tested substance for 48 h at 37°C. Mitomycin C at final concentration of 0.01 µg/ml culture was used as a positive control. The results indicated that the thiabendazole significantly ($P < 0.05$) increased the micronucleus frequency compared with the negative control in all treatment concentrations, indicating a potential aneugenic hazard of thiabendazole in cultured human peripheral lymphocytes. The cytokinesis-block proliferation index value, however, was not decreased significantly compared with the negative control. Significant ($P < 0.05$) differences in the micronuclei frequency were also found between the lower dose (0.5 µg/ml) and the other two analyzed doses of thiabendazole. In contrast, no differences were found between 5 and 50 µg/ml of thiabendazole and between DMSO and negative control. Finally, control cultures treated with the known mutagen MMC showed a

very consistent increase in MN with respect to the negative controls.

Keywords Pesticide · Genotoxicity · Cytogenetic study · Benzimidazole

Introduction

Pesticides are an important component of environmental pollutants as a consequence of their increased use in agriculture, and the effects to their exposure remains one of the major environmental health problem due to potential negative effects on living organisms. From a chemical point of view, pesticides are very reactive compounds forming covalent bonds with various cellular biomolecules and consequently can damage cell structures and/or interfere with metabolic processes. At the DNA level, it was showed that several pesticides induce reactive oxygen species formation which may be involved in changes or losses of nucleotidic bases and in the production of DNA single-strand breaks (Dahlhaus et al. 1995; Lioi et al. 1998; Banerjee et al. 2001; Muniz et al. 2008). Several epidemiological studies demonstrated that occupational exposure to some pesticides may be related to various kinds of cancer (Alavanja et al. 2007; Webster et al. 2002; Clary and Ritz 2003) and neurological diseases (Maggavita 2009; Abdel Rasoul et al. 2008; Thrash et al. 2007). A large body of literature has been devoted to the analysis of possible genotoxic effects of pesticides using different animal models and cellular systems (Bolognesi 2003; Çelik et al. 2004; Poletta et al. 2009). Sasaki et al. (1997), using the alkaline single-cell gel electrophoresis (Comet) assay, showed that TBZ, at the concentration of 200 mg/kg, induced DNA damage in multiple mouse tissues, indicating

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the in vivo DNA-damaging action of this compound. More recently, Watanabe-Akanuma et al. (2005) investigated the potential of DNA-damaging activity, mutagenicity and clastogenicity of TBZ by a short pulse treatment in bacterial and human cells, concluding that UVA-irradiated TBZ caused DNA damage in *Escherichia coli* and human lymphoblastoid cells.

Chromosomal aberrations (CAs), sister chromatid exchange (SCE) and micronuclei (MN) in cultured human peripheral lymphocytes have been widely analyzed as test systems (Bolognesi 2003). In particular, MN represent acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei. The MN are used as a fast and reliable assay for detecting potential clastogenic or aneugenic effects of the compound investigated (Fenech 2000).

Various experimental data clearly demonstrated that pesticides can possess genotoxic properties in animals and in vitro test systems after acute and chronic exposure (for a review see Bolognesi 2003), but the information on the genotoxic effects of some of pesticides, such as thiabendazole (TBZ), is limited. TBZ is a benzimidazole-derived anthelmintic and fungicide widely used in agriculture. It is also used as a post-harvest fungicide for imported citrus fruits during transport and storage, and thus, it was found in high concentration in fruits and vegetables (Ardito et al. 1996). The compounds inhibit fungal microtubular function (Davidse and Flash 1978; Brunner et al. 1991) and thereby cause non-disjunction of chromosomes at cell division with consequent possible aneuploidy (Mailhes and Marchetti 1994; McCarroll et al. 2002). In previous published studies, the potential genotoxic effect of TBZ on different prokaryotic and eukaryotic systems was analyzed (Carballo et al. 2006), but in many cases results, were not concordant. For example, the in vitro cytochalasin-B micronucleus (CBMN) assay on TBZ showed contradictory results, negative in human lymphocytes (Van Hummelen et al. 1995) and positive in Chinese hamster lung cell lines (Hashimoto et al. 2010).

On the basis of results from observational studies of human exposure to TBZ, the estimate of acute reference dose was established to 0.3 mg/kg body weight for women of childbearing age and 1 mg/kg body weight for the general population (WHO 2006). In 2000, the European Economic Community (directives n. 2000/42/CE and 2000/48/CE) established the accepted TBZ maximum residual limits (MRL) for different vegetables and fruits, with values ranging from 0.05 (cereals) to 15 mg/kg (avocado and ware potatoes) and a value of 5 mg/kg for most of the commonly sold fruits and vegetables.

In this paper, we analyzed in vitro the effects of different TBZ concentrations on human lymphocytes. We

tested 0.5, 5 and 50 µg/ml of TBZ, where 0.5 and 50 µg/ml represent the sub-multiple and the multiple of the most common MRL values accepted for fruits and vegetables, respectively. Aim of the study was to determine, by means of the MN assay, whether TBZ at the tested concentration values could induce clastogenic/aneugenic damages in cultured human lymphocytes.

Materials and methods

Chemicals, media and enzymes

The chemical structure and formula of thiabendazole (CAS n. 148-79-8) is as follows: IUPAC name: 2-(thiazol-4-yl)benzimidazole or 2-(1,3-thiazol-4-yl)benzimidazole; CAS: 2-(4-thiazolyl)-1H-benzimidazole. The thiabendazole (obtained from Labservices, Bologna, Italy) was dissolved in DMSO (CAS no. 67-68-5). Gibco RPMI 1640 cell culture media supplemented with L-glutamine, fetal calf serum, phytohemagglutinin (PHA) and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Dimethyl sulfoxide (DMSO, CAS no. 67-68-5), cytochalasin B and Mitomycin C (MMC) were obtained from Sigma-Aldrich, Milan, Italy. Methanol, Acetic acid, Giemsa stain solution and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sørensen buffer were obtained from Merck S.p.A., Milan, Italy. Distilled water was used throughout the experiments.

Blood sample collection

Heparinized blood samples were obtained by venipuncture and collected into heparinized tubes, for genotoxicity testing. All blood samples were coded, cooled (4°C) and processed within 2 h after collection.

Lymphocyte cultures

Peripheral venous blood was collected from two healthy men aged 34 and 35 years, respectively, non-smoking, non-alcoholic, not under drug therapy and with no recent history of exposure to mutagens. Informed consent was obtained from the two blood donors. The study has been approved by the local ethics committee and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Cytokinesis-block micronucleus assay

Heparinized venous blood (0.3 ml) was cultured in 25-cm² flasks in 4.7 ml of RPMI-1640 medium supplemented with

20% fetal calf serum (FCS), 2% of the mitogenic agent phytohaemagglutinin (PHA), L-glutamine (2 mM), antibiotics (100 IU/ml penicillin, and 100 µg/ml streptomycin). The cultures were incubated for 72 h at 37°C under 5% of CO₂ in air in a humidified atmosphere.

After 24 h of incubation, TBZ at concentrations of 0.5, 5 and 50 µg/ml was added to the cultures. Two positive control cultures were prepared by adding mitomycin C (MMC, final concentration 0.01 µg/ml culture) and 1% of DMSO, respectively, 24 h after start of the culture. After 44 h of incubation, cytochalasin B was added to the cultures at a concentration of 6 µg/ml to block cytokinesis. Following additional 28 h of incubation at 37°C, the cells were collected by centrifugation and treated for 3 min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1 v/v). The treatment with fixative was repeated 3 times. Finally, the supernatant was discarded, and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect MN by conventional staining with 5% Giemsa (pH 6.8) prepared in Sørensen buffer. Microscope analysis was performed at 1000× magnification on a light microscope (Dialux 20, Leica, Germany). Micronuclei were scored in 1,000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 2,000 binucleated cells per concentration), following the established criteria for MN evaluation (Fenech et al. 2003). A total of 500 lymphocytes per donor (total 1,000 lymphocytes) were scored to evaluate the percentage of cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated as follows: according to Yüzbaşıoğlu et al. (2006), $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where N_1 – N_4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

Statistical analysis

Statistical analysis was carried out using the SYSTAT software statistical package program (version 10.0, Inc., Chicago, IL). The Kolmogorov–Smirnov z -test was used to compare the mean values of the percentage of cells with MN between the exposition levels. All P values were two-tailed, and the level of statistical significance was set at $P < 0.05$ for all tests carried out.

Results

To assess the effects of TBZ on human lymphocytes, MN frequency was analyzed (Table 1). Results indicated that the TBZ significantly ($P < 0.05$) increased the micronucleus frequency compared with the negative control, in all treatment concentrations. The CBPI value, however, was not decreased significantly compared with the negative control (Table 1). Significant ($P < 0.05$) differences in the MN frequency were also found between the lower TBZ dose (0.5 µg/ml) with respect to other two doses analyzed. In contrast, no differences were found between 5 and 50 µg/ml of TBZ or between DMSO and negative control. Finally, control cultures treated with the known mutagen MMC showed a very consistent increase in MN with respect to the negative controls.

Discussion

In the present study, we investigated in vitro the aneugenic and clastogenic effects of TBZ, a systemic fungicide that belongs to the benzimidazoles, by MN assay. Among the cytogenetic end-points (CAs, SCE, MN) commonly used to investigate the genotoxicity of pesticides, many authors

Table 1 Induction of micronuclei produced by thiabendazole in human lymphocytes in vitro

Test substance	Treatment		BNCs scored	Distribution of BNCs according to the no. of MN				No. of MN	MN/cell (%)	Cytokinesis-block proliferation index (CBPI)
	Period (h)	Dose (µg/ml)		1	2	3	4			
Negative control	48	–	2,000	22	1	0	0	24	1.20 ± 0.25	2.32 ± 0.17
MMC	48	0.1	2,000	226	21	2	0	274	13.7 ± 0.85*	1.85 ± 0.32
1% DMSO	48	–	2,000	23	2	0	0	27	1.35 ± 0.28	2.15 ± 0.20
TBZ	48	0.5	2,000	35	4	0	0	43	2.15 ± 0.35*	2.02 ± 0.21
TBZ	48	5.0	2,000	55	5	1	0	68	3.40 ± 0.45*	1.95 ± 0.25
TBZ	48	50.0	2,000	51	7	2	0	71	3.55 ± 0.49*	1.71 ± 0.31

BNCs binucleated cells, CBPI cytokinesis-block proliferation index = $\{(1 \times N_1) + (2 \times N_2) + [3 \times (N_3 + N_4)]/N\}$ where N_1 – N_4 = cells with 1–4 nuclei, respectively, SD standard deviation, MMC mitomycin C, TBZ thiabendazole

* Significantly different from the negative control $P < 0.05$ (z -test)

have utilized the MN test for its reliability and sensitivity as marker of cytogenetic damage (Bolognesi 2003). Indeed, the MN assay is a valuable approach for identifying the ability of a chemical to induce chromosome loss and non-disjunction because it allows to detect both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (Elhajouji et al. 1998; Parry et al. 2002).

The induction of MN formation by exposition to different pesticides has been reported by different authors in different test systems (Bolognesi 2003). Nevertheless, available data on the genotoxic effects of TBZ are scanty. In a previous set of experiments (Dolara et al. 1994; Van Hummelen et al. 1995), the effects on CA and MN of different pesticide mixtures containing also TBZ were tested, but no significant differences were found with respect to the controls. In these experiments, the concentration of TBZ was 3 µg/ml (Dolara et al. 1994) and 300 µM (Van Hummelen et al. 1995). Ardito et al. (1996), analyzing the effect on human lymphocytes of three different concentrations of TBZ (0.6, 20 and 60 µg/ml, respectively) by a SCE in vitro test, found a significant increase in SCE frequency and a decrease in proliferating rate index with respect to negative control culture only for the cultures treated with the higher concentration. More recently, Vindas et al. (2004) failed to observe effects on human lymphocytes of four different TBZ concentrations (25, 50, 75 and 100 µg/ml, respectively), while Watanabe-Akanuma et al. (2005) observed that TBZ at 50–100 µg/ml with UVA irradiation significantly induced micronuclei in human lymphoblastoid WTK1 cells.

In the present study, the concentrations of 0.5, 5 and 50 µg/ml of TBZ were used to evaluate the potential risk of chromosome damage. Our results showed that TBZ significantly induced micronuclei formation in treated cells, indicating a potential aneugenic hazard of the commercially used fungicide TBZ. Moreover, while in the SCE assay performed by Ardito et al. (1996), the cytogenetic effects of the TBZ on lymphocyte cultures were significant only at the highest concentration (60 µg/ml), in our MN assay, these effects resulted significant also at the lowest concentration (0.5 µg/ml). These discordant results could be explained by the fact that MN assays reveal alterations in either chromosome number or chromosome structure, while the SCE analysis can detect alterations in the chromosome structure only (Bolognesi 2003). One of the mechanisms of action of aneugenic chemicals is the induction of alterations to centromeric DNA (Fenech 2000), with consequent failure of the damaged chromosomes to attach to the mitotic spindle (Parry et al. 2002). As postulated for other pesticides (Yüzbaşıoğlu et al. 2006), our data seem to indicate that TBZ may also provoke some kind of damage to chromosomes centromeres,

thus determining MN formation. Finally, a significant reduction in the CBPI value in cultures treated with TBZ was not observed. This result is congruent with that obtained after treatment with another pesticide, the afugan (Yüzbaşıoğlu et al. 2006). Conversely, Carballo et al. (2006) showed a significant decrease in the mitotic index and changes in the replication index of lymphocytes exposed to 100 µg/ml of TBZ, but not in those exposed to 50 µg/ml, which represents the highest concentration of TBZ utilized in the present study. On the basis on these results, we can conclude that TBZ does not seem to produce effects on the proliferation/mitotic index when the concentration is equal or less than 50 µg/ml.

Acknowledgments This work was supported by grants from the Italian Ministry of University and Scientific Research. We are grateful to Natascha Rogge for her valuable help and criticism.

Conflict of interest The authors declare that they have no conflict of interest.

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