CYTOLOGICAL AND CYTOGENETIC EFFECTS INDUCED BY THIABENDAZOLE ON *ALLIUM CEPA* ROOT MERISTEMS

Andrioli Nancy B.[1]; Mudry Marta D.[1,2]

[1]Grupo de Investigación en Biología Evolutiva (GIBE), Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

nandrioli@hotmail.com

ABSTRACT

The toxicity and genotoxicity of thiabendazole (TBZ) in its commercial formulation Foldan® was studied in Allium cepa meristematic cells at concentrations ranging between 10 and 250 µg / ml. Our results show that exposure to TBZ induces a significant increase in the frequency of anaphase telophase chromosomal aberrations, micronuclei and binucleated cells at all the concentrations of TBZ assayed. The frequency of anaphase telophase aberrations was observed to reach a plateau at the concentration of 50 µg / ml of TBZ, which could be probably due to TBZ mitotic toxicity manifested at concentrations of 100 and 250 µg / ml. An increase was also observed in the rate of metaphase and anaphase, which was, in turn, indicative of alterations in chromatid segregation. Results from the present study indicate that exposure to TBZ induces toxicity and genotoxicity, both being consistent with mechanisms that interfere with microtubule formation.

Key words: Genotoxicity, cytotoxicity, mitotic index, micronuclei, binucleates, thiabendazole

RESUMEN

Se estudió la toxicidad y genotoxicidad de tiabendazole (TBZ) en su fórmula comercial Foldan® en células meristemáticas de *Allium cepa* para un rango de concentraciones que oscilaban entre 10 y 250 µg/ml. Nuestros resultados muestran que la exposición a TBZ induce un incremento significativo en la frecuencia de aberraciones cromosómicas anafase telofase, células binucleadas y micronúcleos en todas las concentraciones de TBZ analizadas. La frecuencia de aberraciones anafase telofase alcanzó un *plateau* en la concentración de 50 µg/ml de TBZ, probablemente a causa de la toxicidad mitótica manifestada en las concentraciones de 100 y 250 µg/ml de TBZ. También se observó un incremento en el índice de metafase y anafase, indicando alteraciones en la segregación de las cromátides. Los resultados del presente estudio muestran que la exposición a TBZ induce tanto toxicidad como genotoxicidad, ambas consistentes con la presencia de mecanismos que interfieren en la formación de los microtúbulos.

Palabras clave: Tiabendazol, genotoxicidad, índice mitótico, células binucleadas, micronúcleos.

INTRODUCCIÓN

Failure in the migration of chromosomes to poles as a result of the absence of a functional spindle, an event known as mitotic slippage, can lead to either aneuploidy by chromosome loss or polyploidy (Elhajouji *et al.*, 1998; Tsuiki *et al.*, 2001). Microtubule arrays are essential for functional mitosis and require the perfect balance between assembly and disassembly of tubulin molecules (Sammak and Borisy, 1988; Cassimeris *et al.*, 1988). As several therapeutic drugs are mitotic spindle poisons which inhibit microtubule polymerization, they are used to carry out research studies on the processes and mechanisms occurring through microtubules (Riffell *et al.*, 2009). On the other hand, benzimidazole drugs, which seem to cause a mitotic slippage effect (Albertini, 1990; Pisano *et al.*, 2000; Kiso *et al.*, 2004), are used as fungicides and anthelminthic in humans, animals and plants (Grover *et al.*, 2001).

The 2-(4 thiazolyl) benzimidazole or thiabendazole (TBZ) is a chemical compound widely used as an anthelminthic in human and veterinary medicine, as well as a fungicide for the control of post-harvest diseases on fruits and vegetables (Lankas *et al.*, 2001). *In vitro* tests have shown that TBZ binds to tubulin monomers, thus inhibiting microtubule formation (Pisano *et al.*, 2000). In contrast, *in vivo* analyses have reached inconclusive results (Mudry de Pargament *et al.*, 1987; Carballo *et al.*, 2006). Recent research suggests that TBZ affects microtubule dynamics, altering the balance between tubulin monomer pools and microtubule polymers and thus inhibiting the autoregulatory control of tubulin synthesis (Kiso *et al.*, 2004; Carballo *et al.*, 2006). On the other hand, several parameters used in genotoxic analysis may provide information about the underlying mechanisms behind these effects, thus allowing the characterization of possible clastogenic or aneugenic agents.

Plants are widely used in laboratories all over the world to analyze mutagenicity and meristematic tissues are adequate for this type of studies (Rank and Nielsen, 1997). In addition, plants have many structural and functional analogs of the CDK-cyclin complex involved in the control and regulation of mitosis in fungi and animals (De la Torre, 2005).

However, mitotic processes in higher plants show additional microtubule arrangements, different from animal mitotic cells (Hepler and Hush, 1996). Microtubule damage in these cells may result in cytological and cytogenetic changes that can be quantified to assess the effect of exposure to different chemicals and environmental conditions on account of the fact that they are also the target of microtubule disrupting agents. Furthermore, apart from the mitotic spindle, microtubule arrays are formed during the plant cell cycle which, in turn, give rise to preprophasic bands and phragmoplast. Cytokinesis requires the formation of microtubule phragmoplast and root meristems and the inhibition of phragmoplast formation gives rise to binucleate cells (Giménez-Abián *et al.*, 2004).

Allium cepa is an excellent plant model for the study of chromosome aberrations after any chemical treatment. This model is sensitive and cytological studies are easy to perform, thus giving information on the effects on cell division and chromosomes (Fiskesjö, 1985; Grant, 1999). In the anaphase-telophase aberration test, two groups of end points should be taken into account (Feretti, 2007), namely:

1) Lagging chromosomes, multipolar anaphases, disorganized anaphases, C- mitosis and polyploidy, all of which indicate spindle disturbance.

2) Fragments, rings and bridges, which are indicative of clastogenic effects.

The presence of condensed chromosomes randomly distributed in the cell or C-mitosis suggests inhibition of spindle formation, which affects the metaphase–anaphase transition, similar to the colchicine effect.

Micronuclei frequency is another biomarker of genotoxic damage. Micronuclei are composed of either acentric chromosome fragments or whole chromosomes and they increase by exposure to clastogenic and aneugenic agents (Fenech, 2000; Lindberg *et al.*, 2007). Micronuclei are formed at the end of telophase and are more frequently observed during interphase. Furthermore, binucleate cell induction has been taken into account in genotoxicity analysis on account of the fact that microtubular disruption affects the consolidation of the phragmoplast in telophase (Soliman, 2001).

In view of the above, and taking into account the wide use of TBZ and the contradictory findings reported to date on the use of this chemical

compound, root meristem cells of *A. cepa* were exposed to the commercial formulation Foldan® at different TBZ concentrations in order to characterize its genotoxic effects.

MATERIALS AND METHODS

Chemicals
Foldan® (TBZ 500 mg, Excipients c.s.) was obtained from Andrómaco (Buenos Aires, Republic of Argentina). Dimethyl sulfoxide (DMSO, CAS 67-68-5) was purchased from Baker (Buenos Aires, Republic of Argentina) and Orcein (CAS Nº 1400-62-0) was obtained from BioPack (Buenos Aires, Republic of Argentina).

Control and thiabendazole-treated cells
Bulbs of *A. cepa*, variety *valcatorce* (INTA), were placed into glass jars containing filtered tap water (SPA® Filter) at 24ºC in darkness. Oxygen was supplied by constant bubbling. Roots between 1 and 2 mm in length were exposed to a series of TBZ concentrations. A stock solution containing 500 mg of Foldan® in 20 ml of DMSO was prepared and all test concentrations were obtained by appropriate dilution of the stock solution in filtered tap water. Four experimental concentrations were chosen following the criterion according to which all should be lower than those inhibiting root growth in a percentage higher than 50% with respect to the control (Fiskesjö, 1985). Test concentrations were 10, 50, 100 and 250 µg/ml TBZ. DMSO 1% maximum concentration was used as solvent control. Independent experiments were performed in triplicate. Exposure was maintained for 30 h to guarantee two complete mitotic cycles (Grant, 1999). At the end of exposure time, roots were cut and root tips fixed in ethanol and glacial acetic acid (3:1) for 24 h. Squash preparations of the meristem-tip cells were made and stained using 2% acetic orcein. The slides were examined under a Leica DMLB light microscope (1000 x).

End points analysis
The following endpoints were scored for genotoxicity analysis:
1) Mitotic index was analyzed by counting 1000 cells in all stages of the mitotic cycle.
2) Chromosome aberrations were determined by the examination of 300 anaphases-telophases per concentration, including: a) lagging chromosomes, multipolar telophases, disorganized anaphases, polyploid cells and C-mitosis; b) bridges and acentric fragments.
3) Index phases as percentage of each mitotic phase over total mitotic cells.
4) Micronuclei frequency was characterized as the percentage of micronucleated cells per 1000 interphase cells.
5) Frequency of binucleate cells as percentage of the total cells observed.

Statistical analysis
Student's t test was used to compare the average values of anaphase-telophase aberrations, mitotic index, binucleate cells and index phases between treated and untreated cells. The X^2 test was used to detect statistical differences in micronucleus frequencies. Analyses were carried out with STATISTICA (StatSoft Inc. 1999).

RESULTS

Mitotic and phase index
The IM inhibition decreased as the TBZ concentration increased (Fig. 1). Results showed both a significant decrease in mitotic index frequency at 100 and 250 µg/ml of TBZ and an increase in metaphase and anaphase frequency at 50, 100 and 250 µg/ml of TBZ (Table 1).

Anaphase-telophase aberrations, binucleate cells and micronuclei
Significant differences in the frequency of anaphase-telophase aberrations were observed at 10, 50, 100 and 250 µg/ml TBZ ($p < 0.01$) whereas no increase was observed after the 50 µg/ml concentration reached a plateau (Fig. 2). The types observed were: polyploid, C-mitosis, lagged chromosomes and disorganized anaphases. No bridges or fragments as indicative of clastogenic effect were observed. The frequency of micronuclei and binucleate cells was significantly increased in all treatments in comparison to the control ($p < 0.05$). Binucleate cells were found in interphase or early prophase and were considered to be perturbers of cytokinesis (Table 2).

TBZ (µg/ml)	MN ± SD	BN ± SD	Anaphase-telophase aberration				AC± SD (%)
			Lag	C-met	Des an	Polyp	
0	1.00±0.50	–	–	1	1	–	1.92±0.93
0 (DMSO 1%)	1.00±0.40	–	–	1	1	–	1.87±1.32
10	5.9±2.50**	2.30±1.4*	1	1	3	1	5.22±1.83**
50	10.50±0.16**	2.70±0.9*	5	12	17	14	46.28±14.38**
100	22.8±3.33**	7.7±2.5**	4	9	19	17	48.82±7.39**
250	38,6±6,00**	13,2±7,4**	3	10	18	14	47.48±18.86*

Table I. Micronuclei frequency (MN), binucleated cells (BN) and anaphase - telophase aberrations (AC) in meristematic cells of *A. cepa* exposed to TBZ and negative controls and solvent control (DMSO).

*Student t-test * p <0.05, ** p <0.01. MN: micronucleous; BN: binucleated cells; Lag: lagging chromosome, C-met: C-metaphase, Des. An.: disorganized anaphases, Polyp: polyploid cells, CA: chromosome aberrations.*
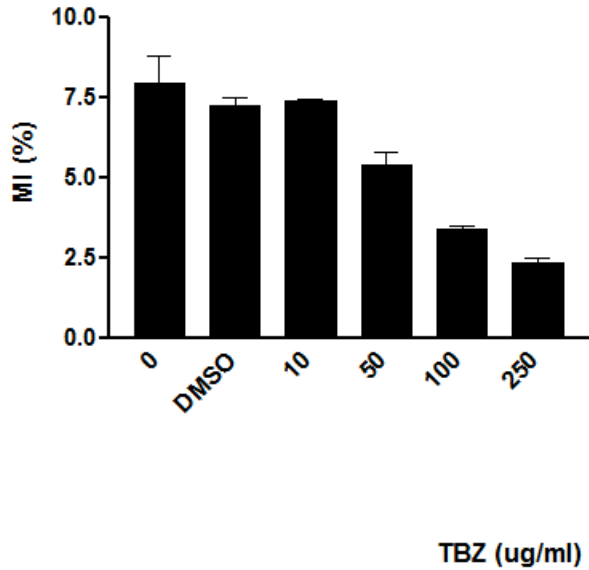

Figure 1.

TBZ(µg/ml)	MI ± SD (%)	Phase Index			
		P	M	A	T
0	7.82±1.29	0.58±0.09	0.13±0.02	0.10±0.04	0.19±0.06
0 (DMSO 1%)	7.28±0.41	0.52±0.03	0.11±0.02	0.16±0.04	0.21±0.05
10	7.42±0.09	0.49±0.07	0.21±0.08	0.10±0.04	0.20±0.06
50	5.40±0.69	0.35±0.01	0.23±0.02*	0.21±0.05*	0.20±0.04
100	3.40±0.18**	0.36±0.10	0.30±0.02*	0.12±0.06	0.22±0.08
250	2.36±0.27**	0.31±0.12	0.27±0.08*	0.20±0.11	0.22±0.06

Table II. Phase Index in *A. cepa* meristematic root cells exposed to TBZ (µg/ml) and negative controls and solvent control (DMSO).

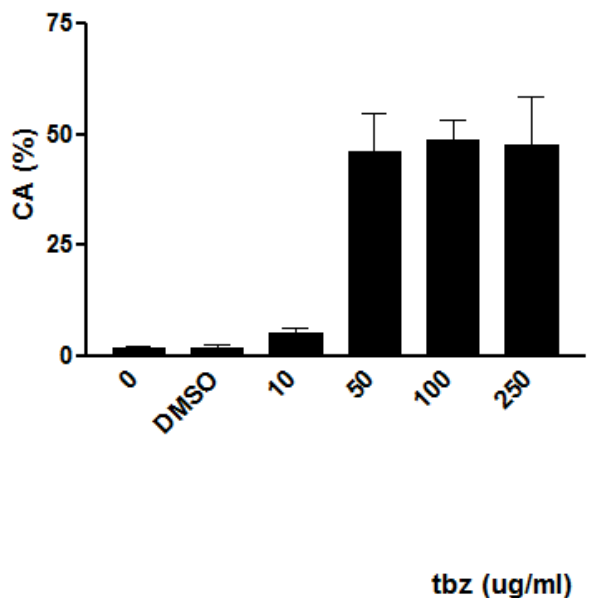*Student t-test. * p < 0,05. TBZ: thiabendazole, P: prophase, M: metaphase, A: anaphase, T: telophase.*

Figure 2.

DISCUSSION

The concentrations of TBZ assayed in the present study were in accordance with those used in previous studies conducted to analyze its genotoxic effects. In *Aspergillus nidulans*, the 50-200 µg/ml TBZ range was observed to have the capacity of disrupting microtubules (Kiso *et al.*, 2004). In *Hordeum vulgare,* the 25-250 µg/ml TBZ range was reported to have no effect on microtubule arrays (Voutsinas *et al.*, 1997). In addition, results from CHO cells exposed to 10-25 µg/ml TBZ show that TBZ promotes the inhibition of tubulin synthesis and defects in the spatial organization of microtubules in mitosis, thus disturbing the normal organization of microtubule arrays and inducing formation of aberrant spindles (Pisano *et al.*, 2000). In CHO cells, a significant dose-dependent increase in sister chromatid exchange frequency (SCE) using 50-100 µg/ml TBZ dose-range also showed a significant increase in mitotic spindle anomalies (Carballo *et al.,* 2006). Furthermore, while in *A. cepa* MI inhibition evidenced a concentration-response relation (Fig. 1), the frequency of anaphase-telophase aberrations did not evidence such relation on account of the fact that in the second concentration assayed frequency values reached a plateau (Fig. 2).

An increased damage derived from an increased exposure concentration may lead cells either to arrest in interphase or to die. As these cells do not enter mitosis, they are not detected and the frequency of anaphase telophase aberrations may therefore begin to decrease when IM values are very low as a result of mitotic toxicity. The 100-250 TBZ concentration range was observed to inhibit IM, with very toxic effects observed. Still, anaphase telophase aberrations were not found to decrease but remained. This could support the hypothesis of TBZ binding to free tubulin. This occurs in cells throughout the whole cell cycle, thus being indicative of the fact that cells are affected even when they enter mitosis. The binding of TBZ to tubulin could affect the self-regulatory control of tubulin synthesis resulting in a change in the global dynamics of microtubule formation. An increase in metaphase frequency is considered a consequence of spindle disturbance. The C-mitotic effect was originally observed after treatment with colchicines and was interpreted as a difficulty to enter anaphase, thus preventing microtubule formation. In *A.*

cepa meristem root cells disorganized anaphases, polyploidy and laggard chromosomes were also induced and could be related to defects in microtubule polymerization dynamics. Failure in the exchange of tubulin subunits, intact microtubules or actin linkage probably leads to a global effect producing a deficient spatial organization of every microtubule array.

On the other hand, according to previous research the absence of a full phragmoplast in telophase is likely to lead to the formation of binucleate cells and polyploidization (Giménez-Abián *et al.*, 2004). This is in agreement with the presence of binucleate and polyploid cells observed in the present study. Within this framework, micronuclei frequency could be considered to be another biomarker of alterations in the mitotic spindle. The substantial increase in micronuclei frequency indicates nuclear envelope entrapment of whole chromosomes or DNA fragments or DNA in excess that were extruded from the nucleus (Fernandes *et al.*, 2007). Our observations regarding the absence of TBZ clastogenic effect are in agreement with previous results obtained from a screening for clastogenicity with benomyl, carbendazim, thiophanate-methyl, fuberidazole and thiabendazole by other authors in *A. cepa* and other species such as *Allium sativum* and *Vicia fava*. Only benomyl and carbendazim were found to evidence clastogenic effects (Sahu *et al.*, 1983) in agreement with findings from our study. In addition, in spite of findings collected from animal models, no multipolar cells were observed in *A. cepa* meristem root cells (Carballo *et al.*, 2006). This could be due to differences between microtubular center organizations on account of the fact that centrosomes are not present in plants, thus suggesting that different molecular mechanisms are involved in multipolar cells induction in plants and animals (Shimamura *et al.*, 2004).

Taken together, findings from the present study lead us to conclude that, under the conditions of the present experimental design, Foldan® evidences ability to promote both genotoxicity and cytotoxicity in *A. cepa* meristem root tips cells. The formation of aberrant spindles and phragmoplast disruption could be consistent with the interaction between tubulins and TBZ action.

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REFERENCES

Albertini S. (1990). Analysis of nine known or suspected spindle poisons for mitotic chromosome malsegregation using saccharomyces cervisiae D61.M. Mutagenesis 5:453-459.

Carballo M.A., Hick A.S., Solonesky S., Larramendy M.L., Mudry M.D. (2006). Genotoxic and aneugenic properties of an imidazole derivative. J. Appl. Toxicol. 26:293-300.

Cassimeris L., Pryor N.K., Salmon E.D. (1988). Real-time observations of microtubules dynamic instability in living cells. J. Cell Biol. 107:2223-2231.

De la Torre C. (2005). Aceleración y frenado de la proliferación celular. An. R. Acad. Nac. Farm. 71:535-569.

Elhajouji A., Cunha M., Kirsch-Volders M. (1998) Spindle poisons can induce polyploidy by mitotic slippage and micronucleate mononucleates in the cytokinesis-block assay. Mutagenesis 13:193-198.

Fenech M. (2000). The *in vitro* micronucleus technique. Mutat. Res. 455:81-95.

Feretti D., Zerbini I., Zani C., Ceretti E., Moretti M., Monarca S. (2007). *Allium cepa* chromosome aberrations and micronucleus tests applied to study genotoxicity of extracts from pesticide-treated vegetables and grapes. Food Addit. Contam. 24(6):561-572.

Fernandes T.C.C., Mazzeo D.E., Marin-Morales M. (2007). Mechanism of micronuclei formation in poliploidized cells of *Allium cepa* exposed to trifluralin herbicida. Pestic. Biochem. Phys. 88:252-259.

Fiskesjö G.F. (1985). The *Allium* test as a standard in environmental monitoring. Hereditas 102:99-112.

Giménez-Abián M.I., Giménez-Abián J.F., Utrilla L., De la Torre C. (2004). Nuclear ploidy is contingent on the microtubular cycle responsible for plant cytokinesis. Protoplasma 224:41-47.

Grant W.F. (1999). Chromosome aberration assays in *Allium*. A report of the US Environmental Agency Gene–Tox. Program. Mutat. Res. 99:273-291.

Grover J.K., Vats V., Uppal G., Yadav S. (2001). Anthelmintic: A review. Trop. Gastroenterol. 22(4):180-189.

Hepler P.K., Hush J.M. (1996). Behavior of microtubules in living plant cells. Plant Physiol. 112:455-461.

Kiso T., Fujita K., Ping X., Tanaka T., Taniguchi M. (2004). Screening for microtubule-disrupting antifungal agents by using a mitotic-arrest mutant of *Aspergillus nidulans* and novel action of phenylalanine derivatives accompanying tubulin loss. Antimicrob. Agents Chemother. 48(5):1739-1748.

Lankas G.R., Nakatsuka T., Ban Y., Komatsu T., Matsumoto H. (2001). Developmental toxicity of orally administered thiabendazole in ICR mice. Food Chem. Toxicol. 39:367-374.

Lindberg H.K., Wang X., Jarventaus H., Falck G.C.M., Norppa H., Fenech M. (2007). Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat. Res. 617(1-2):33-45.

Mudry de Pargament M.D., Labal de Vinuesa M., Larripa I. (1987). Mutagenic bioassay of certain pharmacological drugs: I. Thiabendazole (TBZ). Mutat. Res. 188:1-6.

Pisano C., Battistoni A., Dagrasi F., Tanzarella C. (2000). Changes in microtubule organization after exposure to benzimidazole derivative in Chinese hamster cells. Mutagenesis 15:507-515.

Rank J., Nielsen M.H. (1997). *Allium cepa* anaphase-telophase root tip chromosome aberration assay on N-methyl-N-nitrosourea, maleic hydrazide, sodium azide and ethyl methanesulfonate. Mutat. Res. 390:121-127.

Riffell J.L., Zimmerman C., Khong A., Mc Hardy L.M., Roberge M. (2009). Effects of chemical manipulation of mitotic arrest and slippage on cancer cell survival and proliferation. Cell Cycle 8 (18):3029-3042.

Sahu R.K., Behera B.N., Sharma C.B.S.R. (1983). Cytogenetic effects from agricultural chemicals. Environ. Exp. Bot. 23(1):79-83.

Sammak P.J., Borisy G.G. (1988). Detection of single fluorescent microtubules and methods for determining their dynamics in living cells. Cell Motil. & the Cytoskel.10(1-2):237-245.

Shimamura M., Brown R.C., Lemmon B.E., Akashi T., Naohisa K., Mizuno N., Tomizawa K., Yoshimoto K., Deguchi H., Hosoya H., Horio T., Mineyuki Y. (2004). Tubulin in basal land plants: Characterization, localization and implication in the evolution of acentriolar microtubule, organizing centers. Plant Cell. 16:45–59.

Soliman M.I. (2001). Genotoxicity testing of neem plant (*Azadirachta indica* A. Juss) using the *Allium cepa* chromosome aberration assay. Online J. Biol. Sci. 1(11):1021-1027.

StatSoft Inc. 1999. www.statsoft.com

Tsuiki H., Nitta M., Tada M., Inagaki M., Ushio Y., Saya H. (2001). Mechanism of hyperploid cell formation induced by microtubule inhibiting drug in glioma cell lines. Oncogene 20(4):420-429.

Voutsinas G., Zarani F, Kappas A. (1997). The effect of environmental aneuploidy-inducing agents on the microtubule architecture of meristematic root cells in *Hordeum vulgare*. Cell Biol. Int. 21(7):411-418.