SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 31, No. 5, pp. 968–972, 2012
© 2012 SETAC
Printed in the USA
DOI: 10.1002/etc.1799

Environmental Toxicology and Chemistry in Latin America

OXIDATIVE DAMAGE AND ANTIOXIDANT RESPONSE OF *ALLIUM CEPA* MERISTEMATIC AND ELONGATION CELLS EXPOSED TO METRONIDAZOLE

NANCY ANDRIOLI,† SEBASTIÁN E. SABATINI,‡§ MARTA D. MUDRY,†§ and MARÍA DEL CARMEN RÍOS DE MOLINA*‡§
†Department of Ecology, Genetics, and Evolution, School of Exact and Natural Sciences, University of Buenos Aires, Argentina
‡Department of Biological Chemistry, College of Exact and Natural Sciences, University of Buenos Aires, Argentina
§National Council of Scientific and Technological Research (CONICET), Buenos Aires, Argentina

(*Submitted* 27 *April* 2011; *Returned for Revision* 22 *August* 2011; *Accepted* 12 *September* 2011)

Abstract—The toxicity of metronidazole (MTZ) in meristematic and elongation zones of *Allium cepa* roots was analyzed for 30 h of exposition. Toxic effects were evaluated by lipid peroxidation (content of thiobarbituric-reactive substances [TBARS]), reduced glutathione (GSH) levels, ascorbate acid and dehydroascorbate acid content, and enzymatic activities of superoxide dismutase and catalase. The root zones showed differentiated susceptibility to MTZ. In the elongation zone, MTZ induced an increase of TBARS content and a significant rise in GSH levels, whereas in the meristematic zone, lipid peroxidation was not observed and all antioxidant defense parameters analyzed were significantly increased. These results indicate that MTZ exposure induced oxidative stress in *A. cepa* roots, and that the antioxidant defenses in the meristematic zone are more efficient compared with the elongation zone, which is probably related to higher oxidative metabolism of meristematic tissue. Environ. Toxicol. Chem. 2012;31:968–972. © 2012 SETAC

Keywords—Metronidazole *Allium cepa* Oxidative stress Antioxidant defenses

INTRODUCTION

Metronidazole (MTZ), 1-hydroxyethyl-2-methyl-5-nitroimidazole, is a potent drug used in human and veterinary medicine to treat infections caused by anaerobic bacteria or microaerophilic protozoa as well as to increase the effectiveness of ionizing radiation or alkylating agents in antitumor treatment [1,2]. The selective toxicity of MTZ to anaerobic microorganisms and hypoxic cells such as those found in solid tumors is attributed to the bioreduction of nitroimidazole, which is enhanced by the presence of electron transport systems with a low redox potential that are able to mediate reduction of the drug [3,4]. The reductive pathway of metabolism is associated with the therapeutic effect of nitroimidazoles and other compounds that have a nitro substituent, which causes a short-lived hydroxylamine and then a primary amine [5]. Because hydroxylamine metabolites react with nucleophilic biopolymers, compounds produced by the reductive pathway in the biotransformation of MTZ are particularly important in genetic toxicology, because they can bind covalently to various macromolecules including the DNA molecule [6,7].

In aerobic cells, the biotransformation of MTZ may take oxidative or reductive pathways mediated by the cytochrome P 450 system. Reduction under aerobic conditions could take place, with the addition of a single electron to the nitro radical anion, which is oxidized in the presence of oxygen, reconstituting the original molecule and the formation of superoxide anion. This futile cycle generates reactive oxygen species (ROS) without loss of the original molecule [8]. Several authors agree that the radical nitro anion generated by the bioreduction of MTZ is hardly detectable in aerobic cells, either because the nitro anion radical reacts with oxygen in this futile cycle or because MTZ competes with oxygen for electrons [9]. This mechanism leads to the formation of free radicals, which damage DNA and other cell components [10].

Damage to eukaryote cells is widely induced by free radicals and involves several macromolecules including DNA [11]. One way to assess whether tissues have been exposed to ROS is to analyze enzymatic and nonenzymatic antioxidant responses and oxidative damage [12–14]. Enzymes more frequently used to characterize the oxidative stress response produced by a variety of xenobiotics are superoxide dismutase (SOD), glutathione reductase, and catalase (CAT); nonenzymatic biomarkers are ascorbate (ASC), dihydroascorbate (ADH), and reduced glutathione (GSH) [14,15]. Malonedialdehyde (MDA) levels measured as reactive with thiobarbituric acid (TBARS) indicate lipid peroxidation [16].

In previous work from our laboratory, *Allium cepa* roots exposed to 10, 50, 100, 250, and 500 mg/L MTZ concentrations showed length inhibition and nonadverse effects in meristematic cells [17]. The use of this organism in toxic and genotoxic evaluations of some chemical compounds and complex mixtures has increased notably since its implementation by Levan in 1938. In view of its sensitivity, an *A. cepa* root length inhibition bioassay has been recommended for routine monitoring [18]. The root is a polar organ that has a differential metabolism from apical meristematic to onion base. Physiological studies during the first decades of the 20th century confirmed the differential electrical polarity and oxygen demand of the meristematic elongation and differentiation regions [19]. More recently, along the axis of onion roots, a differential localization was reported of antioxidant enzymes, such as CAT, ASC oxidase, ADH reductase, and GSH reductase, [20].

The aim of the present study was to increase our knowledge of the mechanisms involved in the toxicity of MTZ on *A. cepa* roots during 30 h of exposure. Oxidative effects are evaluated through different oxidative stress biomarkers, such as lipid peroxidation, GSH levels, ascorbate acid (AA) and ADH content, and activity of the enzymes SOD and CAT.

* To whom correspondence may be addressed
(mcrios@qb.fcen.uba.ar).
Published online 12 March 2012 in Wiley Online Library
(wileyonlinelibrary.com).

MATERIALS AND METHODS

Organisms

Bulbs of *A. cepa*, Valcatorce variety, were used for the bioassay. The bulbs were purchased in the local market and stored at 4°C until use.

Chemicals

Tablets containing 500 mg of MTZ (CAS 443-48-1, Rhone-Poulenc Rorer Laboratories) were dissolved in 20 ml of dimethylsulfoxide (CAS 67-68-5, Backer Laboratories), and the solution was diluted with filtered water to a concentration of 250 μg/ml MTZ.

Experimental design

Bulbs in filtered tap water with dimethylsulfoxide 1% were used as controls. For each treatment, four bulbs of the same size were placed in glass boxes. Previously, the exterior cataphylls were removed and the inferior disc was cleaned of rootlets without destroying the primordium, which would be exposed to experimental MTZ solutions. The *A. cepa* bulbs were then immersed up to the fourth part in filtered tap water for 24 h. Those with a root growth of less than 2 cm were discarded. Roots obtained from this exposition were detached from bulbs and cut into two zones, each 2 mm long. Zone I comprised the apical region of the root, rich in meristematic cells, whereas the subjacent zone II was rich in elongation cells. Conditions were kept constant throughout the test. The temperature was 22°C, aeration was kept constant, and bulbs were kept in a culture stove protected from light and air currents. The assay lasted for 30 h. After exposure of roots to each solution, an average was calculated and used to determine the percentage of MTZ-exposed root growth compared with controls [18].

A 250 μg/ml metronidazole concentration was chosen because toxicity was significant but lower than median effective concentration (EC50) parameters indicating moderate toxicity [17].

Protein content

Total soluble protein content was measured by the Bradford method [21] using bovine serum albumin as the standard. Results were expressed as μg protein per ml.

Lipid peroxidation

The level of lipid peroxidation was measured by dosage of TBARS according to Vavilin et al. [22]. Results were expressed as umol TBARs per mg protein.

Ascorbate acid and dehydroascorbate acid (DHA) content

Ascorbate content was estimated using the bipyridyl method as described by Okamura [23]. Results were expressed as nmol AA per mg protein, nmol DHA per mg protein, and the AA:DHA ratio.

Reduced glutathione content

The GSH levels were measured following the Anderson procedure [24] in the presence of 5,5-dithiobis nitrobenzoic acid. A freshly prepared solution of glutathione was used to generate a standard curve. Results were expressed as nmol of GSH per mg protein.

Antioxidant enzyme activities

Each group of *A. cepa* roots was homogenized with chilled sodium phosphate buffer 50 mM (pH 7.0) containing ethylenediamine tetraacetic acid 1 mM and supplemented with protease inhibitors (0.2 mM benzamidine and 0.5 mM phenyl methyl sulfonyl fluoride). The homogenate was centrifuged at 11,000 *g* at 4°C for 30 min, and the supernatant was used as enzyme extract.

Superoxide dismutase (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium [25]. Results were expressed as SOD units per mg of proteins. One SOD unit was defined as the amount of enzyme necessary to inhibit 50% nitroblue tetrazolium reduction rate.

Catalase (EC 1.11.1.6) activity was measured as the decrease in H_2O_2 concentration by recording absorbance at 240 nm [26]. Results were expressed as CAT units per mg of protein. One CAT unit was defined as the amount of enzyme that transforms 1 mmol of H_2O_2 per min.

Statistical analyses

Results from different treatments were compared statistically by *t* test analysis. Suppositions of normality and homogeneity of variances were tested by Lillieford and Bartlett tests, respectively [27]. GraphPad Prism 3 software was used for statistical analysis.

RESULTS

Lipid peroxidation

Lipid peroxidation (TBARS content) was statistically significant ($p < 0.01$) in elongation tissue exposed to MTZ compared with controls, whereas no differences ($p > 0.05$) were observed in meristematic tissue (Fig. 1).

Ascorbate acid and DHA content

Although AA and DHA showed no significant differences ($p > 0.05$) in tissues exposed to MTZ (Fig. 2), the AA:DHA ratio was modified ($p < 0.05$) because of MTZ effects on meristematic tissue (Fig. 3).

Reduced glutathione content

Changes in GSH levels after exposure to MTZ are shown in Figure 4. Both tissues showed a significant increase in GSH levels ($p < 0.05$), with values 100% higher than controls in meristematic and elongation tissue.

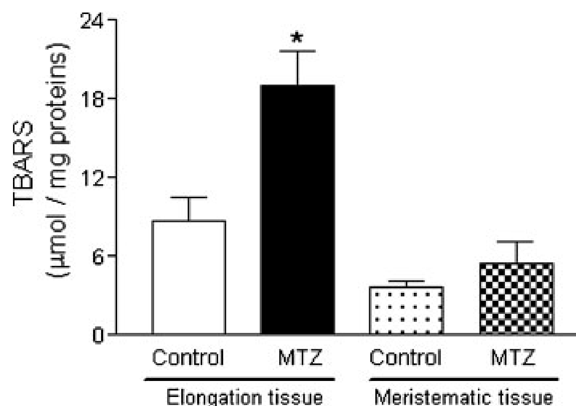

Fig. 1. Thiobarbituric acid-reactive substances (TBARS) content (μmol/mg proteins) in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). Significant differences between control and 250 μg/ml metronidazole (MTZ) exposure for elongation and meristematic tissues are indicated by asterisks: $^{*}p < 0.01$.

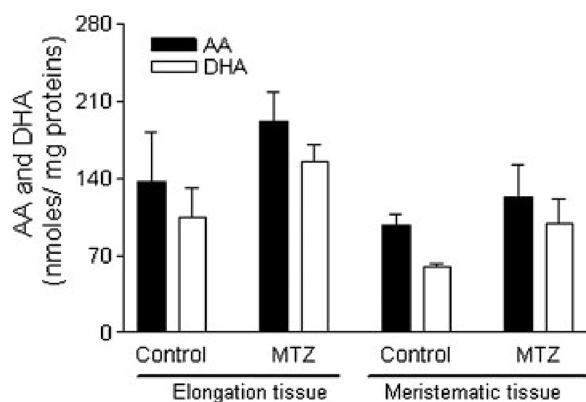

Fig. 2. Ascorbate acid (AA) and dehydroascorbate acid (DHA) content (nmol/mg proteins) in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). MTZ = metronidazole.

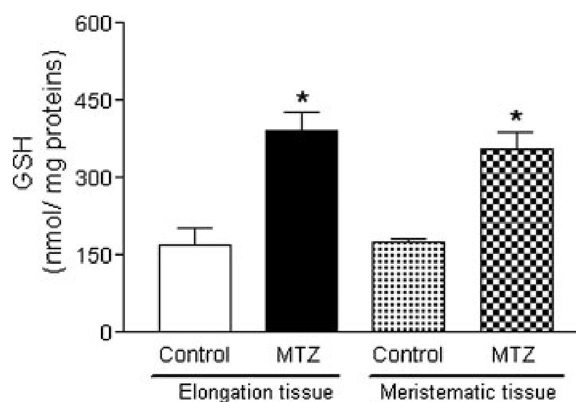

Fig. 4. Reduced glutathione (GSH) content (nmol/mg proteins) in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). Significant differences between control and 250 μg/ml metronidazole (MTZ) exposure for elongation and meristematic tissues are indicated by asterisks: * $p < 0.05$.

Antioxidant enzyme activities

Analysis of CAT and SOD levels showed a significant increase ($p < 0.01$) in activity of both enzymes in the meristematic tissue of *A. cepa* roots exposed to MTZ. No significant differences ($p > 0.05$) were found in levels of either enzyme in elongation tissue exposed to MTZ compared with controls (Figs. 5 and 6).

DISCUSSION

The present study shows oxidative stress effects of MTZ in *A. cepa* roots during chronic exposure. In in vitro studies, MTZ genotoxicity was evaluated using concentrations from 0.1 to 500 μg/ml in peripheral blood lymphocytes and in Chinese hamster ovary line cells [28,29]. As we previous reported, 250 μg/ml of MTZ produced toxic effects inhibiting root elongation in *A. cepa* roots [17]; therefore, this concentration was selected for the study of oxidative stress effects.

The formation of free radicals is involved in the toxicity of a wide range of xenobiotics. Many chemicals exert toxic action once they are metabolically activated to reactive intermediates, which may be free radical species [30]. Oxygen affects the full bioreduction of MTZ [9,31]. If MTZ was bioreducted by one electron in the futile cycle, an increase in ROS would be expected. Reactive oxygen species are a natural product of the normal metabolism of oxygen (photosynthetic electron transport processes and respiration) and are commonly produced in cells through the oxidative metabolism in mitochondria [32]. During the process of energy production by the mitochondrial respiratory chain, part of the oxygen that enters the cell is the source of superoxide anion, hydroxyl radical, or hydrogen peroxide and reacts with other nonradical species. Under normal physiological conditions ROS usually do not have harmful effects, because they are unstable and are rapidly changed to nonradical products. However, during several stressful conditions ROS levels increase dramatically, resulting in oxidative damage to proteins, DNA, and lipids [33].

The results of this investigation suggest that ROS generation was induced in meristematic and elongation fractions of *A. cepa* roots. Elongation tissue analysis showed membrane damage resulting from peroxidative reactions of polyunsaturated fatty acids (lipid peroxidation). Reactive oxygen species can seriously injure plants by increasing oxidative damage to lipids. Lipid peroxidation is usually used as an indicator of the effect of ROS generated by oxidative stress [34]. Although the system reacts by increasing its antioxidant defenses, they would not be enough to counteract oxidative stress, which is evidenced by the increased lipid peroxidation.

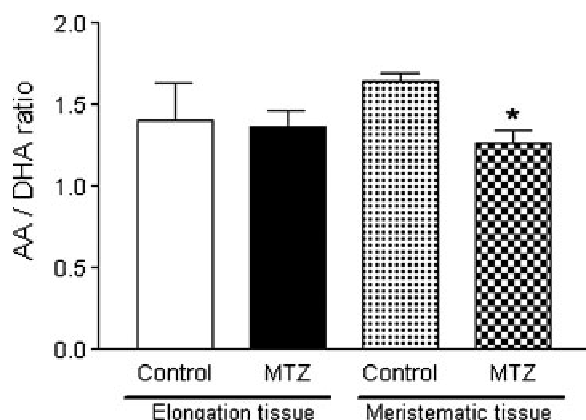

Fig. 3. Ascorbate acid (AA) and dehydroascorbate acid (DHA) ratio in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). Significant differences between control and 250 μg/ml metronidazole (MTZ) exposure for elongation and meristematic tissues are indicated by asterisks: * $p < 0.05$.

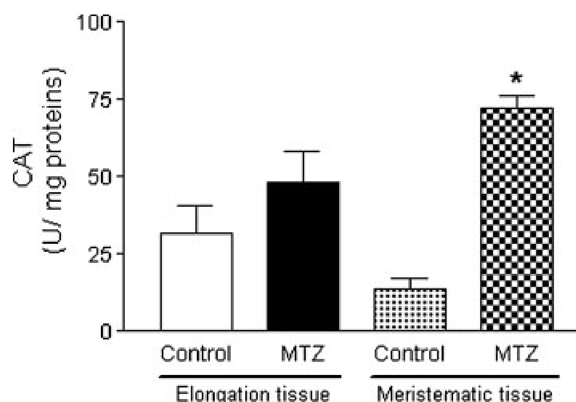

Fig. 5. Catalase (CAT) activity (U/mg proteins) in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). Significant differences between control and 250 μg/ml metronidazole (MTZ) exposure for elongation and meristematic tissues are indicated by asterisks: * $p < 0.01$.

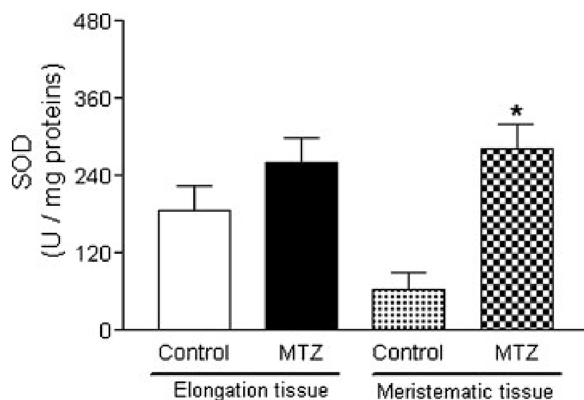

Fig. 6. Superoxide dismutase (SOD) activity (U/mg proteins) in *Allium cepa* roots. Data are expressed as means ± SD ($n = 6$). Significant differences between control and 250 μg/ml metronidazole (MTZ) exposure for elongation and meristematic tissues are indicated by asterisks: $^{*}p < 0.01$.

Plant cells respond defensively to oxidative stress by removing the concentration of toxic intracellular ROS levels and guaranteeing normal cellular function [35]. Plants have developed an enzymatic (SOD, CAT, ascorbate peroxidase) and nonenzymatic (glutathione, ascorbate, carotenoids) antioxidant system to keep the concentrations of harmful ROS lower [33]. Plant damage occurs when the capacity of antioxidant processes and detoxification mechanisms are lower than the amount of ROS production. In the present study, antioxidant defenses responded to MTZ toxicity by increasing SOD and CAT activity, increasing GSH levels, and changing the AA:DHA ratio. The lower AA:DHA ratio observed in the meristematic tissue of *A. cepa* root exposed to MTZ can be related to scavenging of lipid peroxides. The AA:DHA ratio is considered an important indicator of the redox state of the cell and the degree of oxidative stress experienced [36]. Nonenzymatic scavengers such as AA, glutathione, and thiols are essential in protecting biomolecules from most ROS, but they cannot cope with reducing radicals such as superoxide or hydroperoxides [37]. Antioxidant enzymes such as SOD and CAT constitute the major defensive system against ROS [38]. The increase in this mechanism of antioxidant defense in the meristematic tissue of *A. cepa* root exposed to MTZ is enough to prevent oxidative damage in lipids. The increase in SOD activity could be related to augmentation of superoxide anion production caused by the reaction of the nitro anion with oxygen in the futile cycle. These findings are consistent with the inhibition of root elongation without damage to meristematic cells previously reported by our group [17]. The observed differences between tissues may be the result of differences in oxygen consumption. The controversial results of MTZ genotoxic effects cited in the literature could be partially because of metabolic differences and the redox status of the tissue used in each experimental model [28,29,39,40].

Our findings demonstrated that as a result of exposure to 250 μg/ml of MTZ, lipid peroxidation levels were enhanced in elongation fractions of *A. cepa* roots. This may indicate that oxidative stress probably occurred because the defense system was overwhelmed and was thus unable to maintain correct concentrations of harmful active oxygen species, whereas in the meristematic fraction roots this response was not observed. Our results contribute to the understanding of the toxicity caused by MTZ exposure in *A. cepa* roots during 30 h in a laboratory study, through analysis of oxidative damage and antioxidant defense determinations, thereby expanding our knowledge concerning the toxic mechanisms involved in nitroimidazolic compounds. This is important because the risk of their application is still insufficiently characterized. However, more detailed studies are needed to elucidate the mechanisms behind the stimulatory effect of MTZ on ROS production by plants.

Acknowledgement—The present work was supported by grants: MCRM UBACYT X310 and CONICET PIP 5590, and MDMX 154.

REFERENCES

1. Stratford I, Adams GE. 1982. Nitroimidazoles as hypoxia-mediated drugs in cancer chemotherapy. In Breccia A, Cavalleri B, Adams GE, eds, *Nitroimidazoles: Chemistry, Pharmacology, and Clinical Application*. Plenum, London, UK, pp 91–104.
2. Freeman CD, Klutman NE, Lamp KC. 1997. Metronidazole. A therapeutic review and update. *Drugs* 54:679–708.
3. Adams GE, Stratford LJ, Bremmer JCM, Cole S, Edwards HS, Fielden EM. 1991. Nitroheterocyclic compounds as radiation sensitizers and bioreductive drugs. *Radiother Oncol Suppl* 20:85–91.
4. Rasoloson D, Vanacova S, Tomkova E, Razga J, Hrdy I, Tachezy J, Kulda J. 2002. Mechanisms of in vitro development of resistance to metronidazole in *Trichomonas vaginalis*. *Microbiology* 148:2467–2477.
5. Dobías L, Cerná M, Rossner P, Srám R. 1994. Genotoxicity and carcinogenicity of metronidazole. *Mutat Res* 317:177–194.
6. Edwards DI. 1986. Reduction of nitroimidazoles in vitro and DNA damage. *Biochem Pharmacol* 35:53–58.
7. Kedderis GL, Miwa GT. 1988. The metabolic activation of nitroheterocyclics therapeutic agents. *Drug Metab Rev* 19:33–62.
8. Martínez Cayuela M. 1998. Toxicidad de xenobióticos mediada por radicales libres de oxígeno. *Ars Pharm* 39:5–18.
9. Moreno SNJ, Mason RP, Muniz RPA, Cruz FS, Docampo R. 1983. Generation of free radicals from metronidazole and other nitroimidazoles by *Tritichmonas foetus*. *J Biol Chem* 258:4051–4054.
10. Abreu FC, Ferraz PA, Goulart MOF. 2002. Some applications of electrochemistry in biomedical chemistry. Emphasis on the correlation of electrochemical and bioactive properties. *J Braz Chem Soc* 13:19–35.
11. Barzilai A, Yamamotob K. 2004. DNA damage responses to oxidative stress. *DNA Repair* 3:1109–1115.
12. Offord E, van Poppel G, Tyrrell R. 2000. Markers of oxidative damage and antioxidant protection: Current status and relevance to disease. *Free Radic Res* 33:5–19.
13. Sabatini SE, Juárez AB, Eppis MR, Bianchi L, Luquet CM, Ríos de Molina MC. 2009. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. *Ecotox Environm Saf* 72:1200–1206.
14. Sabatini SE, Leonardi PI, Rodríguez MC. 2011. Responses to sublethal copper exposure in two strains of *Chlamydomonas reinhardtii* in autotrophic and mixotrophic conditions. *Phycologia* 50:78–88.
15. Schutzendubel A, Polle A. 2002. Plant responses to abiotic stress: Heavy metal induced oxidative stress and protection by mycorrhization. *J Exp Botany* 53:1351–1365.
16. Shalata A, Mittova V, Guy M, Tal M. 2001. Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicum pennellii* to salt dependent oxidative stress: The root antioxidant system. *Physiol Plant* 112:487–494.
17. Andrioli NB, Wulff AF, Mudry MD. 2006. *Allium cepa* como biomonitor de toxicidad y genotoxicidad del metronidazol. *Teoría* 15:9–16.
18. Fiskejo G. 1985. The *Allium* test as an alternative in environmental studies: The relative toxicity of metal ions. *Mutat Res* 197:243–260.
19. Berry LJ, Brock MJ. 1946. Polar distribution of respiratory rate in the onion root tip. *Plant Physiol* 21:542–549.
20. Córdoba Pedregosa MC, Córdoba F, Villalba JM, González Reyes JA. 2003. Zonal changes in ascorbate and hydrogen peroxide contents, peroxidase, and ascorbate-related enzyme activities in onion roots. *Plant Physiol* 131:697–706.
21. Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248–254.
22. Vavilin DV, Ducruet JM, Matorin DN, Venediktov PS, Rubin AB. 1998. Membrane lipid peroxidation, cell viability and photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. *Photochem Photobiol* 42:233–239.
23. Okamura M. 1980. An improved method for determination of ascorbic acid and dehydroascorbic acid in blood plasma. *Clin Chim Acta* 103: 259–268.

24. Anderson ME. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 113:548–553.
25. Beauchamp C, Fridovich I. 1971. Superoxide dismutase: Improved assays and an assay applicable to polyacrylamide gels. *Anal Biochem* 44:276–286.
26. Aebi H. 1984. Catalase in vitro. *Methods Enzymol* 105:121–126.
27. Sokal RR, Rohlf FJ. 1999. *Introducción a la Bioestadística*. Reverté, Barcelona, Spain.
28. Rodríguez Ferreiro G, Cancino Badías L, López-Nigro M, Palermo A, Mudry MD, Prieto González E, Carballo MA. 2002. DNA single strand breaks in peripheral blood lymphocytes induced by three nitroimidazole derivatives. *Toxicol Lett* 132:109–115.
29. López Nigro MM, Palermo AM, Mudry MD, Carballo MA. 2003. Cytogenetic evaluation of two nitroimidazole derivatives. *Toxicol In Vitro* 17:35–40.
30. Halliwel B, Gutteridge JMC. 1989. *Free Radicals in Biology and Medicine*, 2nd ed. Oxford University Press, Clarendon, Oxford, UK.
31. Raheter W, Hanel H. 2003. Nitroheterocyclic drugs with broad spectrum activity. *Parasit Res* 90:19–39.
32. Gutteridge JMC, Halliwell B. 1999. Antioxidant protection and oxygen radical signaling. In Gilbert DL, Colton CA, eds, *Reactive Oxygen Species in Biological Systems*. Kluwer Academic/Plenum, New York, NY, USA, pp 189–218.
33. Apel K, Hirt H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399.
34. Janero DR. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med* 9:515–540.
35. Horváth E, Pál M, Szalai G, Páldi E, Janda T. 2007. Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. *Biol Plant* 51:480–487.
36. Hatata MM, Adel Abdel-Aal E. 2008. Oxidative stress and antioxidant defense mechanisms in response to cadmium treatments. *Am Eurasian J Agric Environ Sci* 4:655–669.
37. Diere J, Ferrari-lliou R. 1999. Intracellular antioxidants: From chemical to biochemical mechanisms. *Food Chem Toxicol* 37:949–962.
38. Sies H. 1993. Strategies of antioxidant defense. *Eur J Biochem* 215:213–219.
39. Palermo AM, Reynoso AS, López Nigro M, Carballo MA, Mudry MD. 2004. Teratogenic evaluation of metronidazole and ornidazole using *Drosophila melanogaster* as an experimental model. *Birth Def Res (Part A)* 70:157–162.
40. Mudry MD, Palermo AM, Merani MS, Carballo MA. 2007. Metronidazole-induced alterations in murine spermatozoa morphology. *Reprod Toxicol* 23:246–252.