Short Communication

DNA Damage in Wistar Rats Exposed to Dithiocarbamate Pesticide Mancozeb

(comet assay / DNA damage / mancozeb / micronucleus test / pesticides)

A. GOLDONI[1], C. R. KLAUCK[1], S. T. DA SILVA[2], M. D. DA SILVA[2], P. G. ARDENGHI[2,3], L. B. DA SILVA[1,2,3]

[1]Research Group on Environmental Quality Indicators, [2]Research Group on Human Health and Environment, [3]Graduate Program in Environmental Quality, Universidade Feevale, Novo Hamburgo, RS, Brazil.

Abstract. Pesticides are used in large amounts in agriculture and the evaluation of their toxic effects is of major concern to public and environmental health. The aim of the present study was to investigate the genotoxic potential of a commercial formulation of the fungicide mancozeb by the micronucleus test in bone marrow and the comet assay in total blood of Wistar rats. Adult male Wistar rats were treated with a solution of mancozeb at a concentration of 40 mg/kg/day, administered intraperitoneally for 18 consecutive days, and compared to a control group. The results indicate that mancozeb induced significantly higher DNA damage as detected by the comet assay and increased the frequency of micronuclei. The results show that mancozeb is genotoxic and may adversely affect the DNA integrity of exposed organisms.

Introduction

Pesticides represent a heterogeneous category of chemicals specifically designed to control pests, weeds, and plant diseases. Their application is the most effective means of protecting plants, contributing to enhanced agricultural productivity and crop yields throughout the world (Bolognesi, 2003). However, many of these compounds are environmentally persistent and may not be entirely specific for their target organisms, endangering other living species, including humans (Aprea et al., 2002).

Pesticides are characterized by varying degrees of toxicity. The genotoxic potential is a primary risk factor for long-term effects, such as carcinogenic and reproductive toxicology and degenerative diseases (Bolognesi et al., 2011). Among the methods currently available for the detection of DNA damage, the micronucleus (MN) test and the comet assay have been widely employed due to their simplicity, sensitivity and relatively low cost. The MN test detects acentric chromosome fragments, acentric chromatid fragments, or whole chromosomes that fail to be included in the daughter nuclei during cell division (Fenech et al., 2011), while the comet assay is able to detect single-strand breaks or other lesions, such as alkali-labile sites and DNA cross-links (Jha, 2008).

Mancozeb is a broad-spectrum fungicide of the ethylene-bis-dithiocarbamate (EBDC) family, commonly used for foliar application and seed treatment in agriculture (Marrs and Ballantyne, 2004). Mancozeb has low acute toxicity with an oral LD_{50} value of 8 g/kg/day in Wistar rats (Edwards et al., 1997). However, mancozeb is suspected to be carcinogenic for mammals (Belpoggi et al., 2002) and has induced DNA damage in cells exposed *in vitro* through oxidative mechanisms (Calviello et al., 2006; Srivastava et al., 2012). The genotoxic potential of mancozeb has been previously studied using *in vivo* assays, but conflicting results have been reported (Georgian et al., 1983; Gautam and Kapoor, 1991; Vasudev and Krishnamurthy, 1994). Therefore, the aim of this study was to evaluate the genotoxic effects in Wistar rats exposed to a mancozeb-based commercial formulation using the comet assay and the MN test.

Material and Methods

A commercial formulation of mancozeb was obtained from a local supplier as a wettable powder containing 80 % (w/w) mancozeb as the active ingredient.

Healthy adult male Wistar rats, weighing 250–350 g, were used in the experiment. Animals were purchased from the animal house of the Feevale University, Novo Hamburgo, Brazil. They were housed in groups of five in plexiglass cages and provided with food and water *ad libitum*. All animals were maintained in a 12-h light/

Received August 30, 2013. Accepted March 28, 2014.

Corresponding author: Luciano Basso da Silva, Universidade Feevale, RS 239, 2755. CEP 93352-000, Novo Hamburgo, RS, Brazil. Phone: (+55) 51 35868800; Fax: (+55) 51 35868836; e-mail: lucianosilva@feevale.br

Abbreviations: bw – body weight, EBDC – ethylene-bis-dithiocarbamate, MN – micronucleus, PCE – polychromatic erythrocyte, ROS – reactive oxygen species.

dark cycle at a temperature of 22 °C ± 1 °C. The study protocol was approved by the Research Ethics Committee of Universidade Feevale, Brazil. All experiments and procedures were carried out in accordance with the ethical principles involving animals in research defined by the Brazilian College for Animal Experimentation (COBEA).

The animals were divided into two groups of 10 individuals each, as follows: Group 1: rats received a single dose of mancozeb (40 mg/kg body weight [bw]) dissolved in saline solution (0.9% NaCl) at a volume of 1 ml/kg, administered intraperitoneally once a day for 18 consecutive days; Group 2 (control): control rats received injections of saline solution (0.9% NaCl) during the same period. At the end of the exposure period, all rats were sacrificed by decapitation. Femurs were dissected from each animal for the MN test, and blood samples were collected for the comet assay.

For the MN test, bone marrow cells from one femur were prepared as described by Schmid (1975). After dissection, the proximal and distal ends of the femurs of each animal were cut off and bone marrow cells were flushed out with foetal calf serum. The suspension was centrifuged and the supernatant was discarded. The pellet was resuspended in ethanol-acetic acid (3 : 1) and dropped onto a clean slide. The slides were air dried for 12 h and stained with Giemsa (Newprov, Pinhais, Paraná). All slides were coded and the cells were scored blindly by light microscopy at ×1000 magnification. A total of 2000 polychromatic erythrocytes (PCEs) were scored per animal for the presence of micronuclei. All slides were scored by the same observer.

The alkaline comet assay was performed according to Tice et al. (2000). After the animals were killed, a 5-µl blood sample was collected and mixed with 95 µl of low melting point agarose. After homogenization, the mixture was layered onto slides pre-coated with normal melting point agarose, covered with a coverslip, and then allowed to solidify for 7 min in the refrigerator. The coverslips were gently removed and the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO) for at least 6 h. After lysis, the slides were placed in a horizontal electrophoresis box filled with freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min. Electrophoresis was performed at 25 V and 300 mA for 20 min. The slides were then neutralized with 0.4 M Tris buffer at pH 7.5, stained with silver nitrate (Nadin et al., 2001), and examined under a light microscope Nikon Eclipse E200 (Nikon, Tokyo, Japan). From each individual, 100 cells were scored at ×400 magnification. DNA damage was quantified by visual classification of cells into five categories corresponding to the tail length: 0 – undamaged; I – low-level damage; II – medium-level damage; III – high-level damage; IV – complete damage (Anderson et al., 1994). Based on the arbitrary levels assigned to the different categories, a genetic damage index was calculated for each rat (Pitarque et al., 1999), as follows: [(No. of Type 0 cells × 0) + (No. of Type I cells × 1) + (No. of Type II cells × 2) + (No. of Type III cells × 3) + (No. of Type IV cells × 4)]. Therefore, the total score per individual ranged from 0 (all undamaged) to 400 (all maximally damaged).

Differences between treated and control groups were compared using the Mann-Whitney test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 15.0 for Windows, at a significance level of $P \leq 0.05$.

Results and Discussion

The present study was undertaken to evaluate the genotoxic potential of mancozeb in Wistar rats as assessed by the comet assay and MN test. The results of MN test in rat bone marrow are shown in Table 1. The mean number of micronucleated cells/per 1000 cells in rats treated with mancozeb (7.2 ± 1.1) was significantly higher than that in the control group (3.2 ± 0.7) ($P = 0.0004$). The results obtained by the comet assay in blood lymphocytes of rats are also shown in Table 1. The damage index showed a statistically significant difference between treated and control groups ($P = 0.001$), with increased DNA damage in mancozeb-treated rats. Accordingly, the results obtained by both methods for the detection of DNA damage indicate that mancozeb is genotoxic at the dose tested (40 mg/kg bw/day).

Genotoxicity studies with mancozeb have produced controversial results in rodent bone marrow cells. Vasudev and Krishnamurthy (1994) found no genotoxic effects for mancozeb at doses of 1687.5, 3375, and 5962.5 mg/kg bw using the MN test in Swiss albino male mice. Gautam and Kapoor (1991), however, reported induction of chromosomal aberrations in the bone marrow cells of male mice injected intraperitoneally with mancozeb at doses of 30, 40, and 300 mg/kg bw. In another study with mice, mancozeb significantly induced micronuclei in bone marrow cells only at the dose of 1000 mg/kg bw (Hemavathi and Rahiman, 1996). In the study by Georgian et al. (1983), Wistar rats received mancozeb at doses of 2.5, 5, and 10 mg/kg bw as a single injection for each dose, and chromosomal aberration frequency in bone marrow cells was then evaluated. Almost all rats died within 2–4 h after the highest dose, and dose-related clastogenic effects were found for the 2.5 and 5 mg/kg bw doses of mancozeb.

Table 1. Number of micronucleated erythrocytes (per 1000 cells) as assessed by the micronucleus (MN) test in bone marrow and damage index in lymphocytes as estimated by the comet assay (mean ± standard deviation) in mancozeb-treated and control male Wistar rats

Treatment	N	MN (‰)	Damage index
Mancozeb	8*	7.2 ± 1.1	23.8 ± 4.4
Control	10	3.2 ± 0.7	7.8 ± 2.3
P		0.0004	0.001

* Two rats died during the treatment with mancozeb.

Although the dose of mancozeb used in the present study (40 mg/kg bw/day) was higher than that used by Georgian et al. (1983), we were not able to confirm the results reported by the authors with respect to the high lethality of mancozeb observed in Wistar rats at a dose of 10 mg/kg bw. Toxicity studies with mancozeb, and with pesticides in general, have yielded controversial results, depending on the compound or assay used. Moreover, in order to explain inconsistent results with respect to the induction of toxic effects by pesticides, additional factors, such as different sources, purity levels, strains and sub-strains of animals, doses and treatment schedules, and manufacturing processes, should be taken into consideration (Surrallés et al., 1995).

Several studies have shown the involvement of reactive oxygen species (ROS) and oxidative stress in pesticide toxicity, and it is generally accepted that ROS eventually cause DNA damage (Bertram and Hass, 2008). Tissue culture studies have shown that mancozeb may induce oxidative stress and that ROS may play a critical role in mancozeb-induced genotoxicity *in vitro* (Calviello et al., 2006; Srivastava et al., 2012).

The data reported here demonstrate that mancozeb exerts toxic effects on rat cells, inducing DNA damage. Moreover, the results indicate that mancozeb may adversely affect the DNA integrity of exposed organisms, including humans.

References

Anderson, D., Yu, T. W., Phillips, B. J., Schmezer, P. (1994) The effect of various antioxidants and other modifying agents on oxygen-radical generated DNA damage in human lymphocytes in the comet assay. *Mutat. Res.* **307**, 261-271.

Aprea, C., Colosio, C., Mammone, T., Minoia, C., Maroni, M. (2002) Biological monitoring of pesticide exposure: a review of analytical methods. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **769**, 191-219.

Belpoggi, F., Soffritti, M., Guarino, M., Lambertini, L., Cevolani, D., Maltoni, C. (2002) Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. *Ann. N. Y. Acad. Sci.* **982**, 123-136.

Bertram, C., Hass, R. (2008) Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biol. Chem.* **389**, 211-220.

Bolognesi, C. (2003) Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat. Res.* **543**, 251-272.

Bolognesi, C., Creus, A., Ostrosky-Wegman, P., Marcos, R. (2011) Micronuclei and pesticide exposure. *Mutagenesis* **26**, 19-26.

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F. I., Palozza, P. (2006) DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. *Toxicol. Appl. Pharmacol.* **211**, 87-96.

Edwards, I. R., Ferry, D. G., Temple, W. A. (1997) Thiodithiocarbamates. In: *Pesticide Profiles (Toxicity, Environmental Impact and Fate)*, ed. Kamrin, M. A., pp 91-132. CRC Lewis Publishers, New York.

Fenech, M., Kirsch-Volders, M., Natarajan, A. T., Surralles, J., Crott, J. W., Parry, J., Norppa, H., Eastmond, D. A., Tucker, J. D., Thomas, P. (2011) Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* **26**, 125-32.

Gautam, D. C., Kapoor, L. (1991) Genotoxic effects of dithane M-45 on the bone marrow cells of mice in vivo. *Experientia* **47**, 280-282.

Georgian, L., Moraru, I., Draghicescu, T., Dinu, I., Ghizelea, G. (1983) Cytogenetic effects of alachlor and mancozeb. *Mutat. Res.* **116**, 341-348.

Hemavathi, E., Rahiman, M. A. (1996) Effect of ziram, thiram, and dithane M-45 on bone marrow cells of mice assessed by micronucleus test. *Bull. Environ. Contam. Toxicol.* **56**, 190-196.

Jha, A. N. (2008) Ecotoxicological applications and significance of the comet assay. *Mutagenesis* **23**, 207-221.

Marrs, T. C Ballantyne, B. (2004) *Pesticide Toxicology and International Regulation*. John Wiley and Sons, New Jersey.

Nadin, S. B., Vargas-Roig, L. M., Ciocca, D. R. (2001) A silver staining method for single-cell gel assay. *J. Histochem. Cytochem.* **49**, 1183-1186.

Pitarque, M., Creus, A., Marcos, R., Hughes, J. A., Anderson, D. (1999) Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. *Mutat. Res.* **440**, 195-204.

Schmid, W. (1975) The micronucleus test. *Mutat. Res.* **31**, 9-15.

Srivastava, A. K., Ali, W., Singh, R., Bhui, K., Tyagi, S., Al-Khedhairy, A. A., Srivastava, P. K., Musarrat, J., Shukla, Y. (2012) Mancozeb-induced genotoxicity and apoptosis in cultured human lymphocytes. *Life Sci.* **90**, 815-824.

Surrallés, J., Xamena, N., Creus, A., Catalán, J., Norppa, H., Marcos, R. (1995) Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. *Mutat. Res.* **341**, 169-184.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C., Sasaki, Y. F. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **35**, 206-221.

Vasudev, V., Krishnamurthy, N. B. (1994) In vivo cytogenetic analyses of the carbamate pesticides Dithane M-45 and Baygon in mice. *Mutat. Res.* **323**, 133-135.