Original Article

The Effect of Ascorbic Acid on Mancozeb-Induced Toxicity in Rat Thymocytes

(mancozeb / ascorbic acid / thymocytes / toxicity / ROS)

V. PAVLOVIC[1], S. CEKIC[1], B. KAMENOV[2], M. CIRIC[1], D. KRTINIC[3]

[1]Institute of Physiology, Medical Faculty University of Nis, Serbia
[2]Pediatric Clinic, [3]Clinic for Gynaecology and Obstetrics, University Clinical Centre, Nis, Serbia

Abstract. Mancozeb, as a dithiocarbamate fungicide, has been found to exhibit toxicological manifestations in different cells, mainly by generation of free radicals which may alter antioxidant defence systems in cells. The effect of mancozeb on the cells of a primary lymphoid organ has not been studied. In the present study, the effects of mancozeb (0.2, 2 and 5 µg/ml) or mancozeb+ascorbic acid (100 µg/ml), or ascorbic acid alone or control medium alone on the levels of cell viability, apoptosis, intracellular reactive oxygen species production (ROS), mitochondrial membrane potential (MMP) and ATP levels in rat thymocytes were examined *in vitro*. Cells treated with mancozeb displayed a concentration-dependent increase of hypodiploid cells and ROS production followed by markedly decreased viability of the cells, MMP and ATP levels. Application of ascorbic acid significantly reduced cytotoxicity in cell cultures treated with 0.2 and 2 µg/ml of mancozeb, together with significantly decreased ROS levels and increased MMP and ATP levels. In cells treated with 5 µg/ml of mancozeb, ascorbic acid failed to reduce toxicity while simultaneously increasing the apoptosis rate of thymocytes. These results suggest that ROS plays a significant role in mancozeb-induced toxicity, through alteration of mitochondrial function. Ascorbic acid administration reduced the toxicity rate in cells treated with lower mancozeb concentrations, while it may have the ability to shift cells from necrosis to apoptosis in the presence of highest mancozeb concentrations.

Received February 15, 2015. Accepted March 30, 2015.

Corresponding author: Voja Pavlovic, Institute of Physiology, Medical Faculty University of Nis, Bulevar dr Zorana Djindjica 81, 18000 Nis, Serbia. Phone: (+381) 66 276736; e-mail: vojapav@yahoo.com

Abbreviations: CCK-8 – cell counting kit, CM – culture medium, EBDC – bis-dithiocarbamate, ETU – ethylene thiourea, FCS – foetal calf serum, H2DCF-DA – 2',7'-dichlorofluorescin diacetate, MFI – mean fluorescence intensity, MMP – mitochondrial membrane potential, PBMC – peripheral blood mononuclear cells, PI – propidium iodide, ROS – reactive oxygen species.

Introduction

Pesticides are chemical compounds that are widely used throughout the world against plant diseases, which results in increased agricultural production. In the past years, due to the increasing demand for food safety, great concern was raised about the pesticide residues in food and food products and their potential risk to the consumer. Mancozeb is a contact fungicide of the ethylene bis-dithiocarbamate (EBDC) family, widely used as a dithiocarbamate fungicide to protect field crops, fruits and vegetables against different fungal diseases (Paro et al., 2012). These fungicides, due to low acute toxicity in humans and short environmental persistence, are used globally. However, even though agricultural workers are the main population with high risk of EBDC intoxication, the general population can also be affected by residues present in the food (Rossi et al., 2006). Further, EBDCs are very unstable and rapidly degraded into ethylene thiourea (ETU), a major metabolite of EBDCs, which shows long persistence in the soil (Houeto et al., 1995) or is decomposed in water, forming products with high toxicological potential (Corsini et al., 2006).

It has been shown that mancozeb causes adverse effects in humans and experimental animals. Various studies documented the toxic effect of mancozeb in non-immune cells (Calviello et al., 2006; Domico et al., 2006; Tsang and Trombetta, 2007), as well as in different cells of the immune system, including rat peripheral blood mononuclear cells (PBMC) (Calviello et al., 2006), human lymphocytes (Srivastava et al., 2012) and human PBMCs (Calviello et al., 2006). Formation of reactive oxygen species (ROS) has been implicated as the main contributor of toxicity induced by mancozeb (Williams et al., 2013). This might be a consequence of its chemical structure, since it contains transitional metals known to induce ROS formation through the Fenton reaction (Calviello et al., 2006). Ascorbic acid (vitamin C) represents the most important antioxidant in extracellular fluids (Dwivedi et al., 2013). This water soluble vitamin has the ability to trap radicals in the aqueous phase and protect biomembranes from peroxidative damage (Eroglu et al., 2013). The ameliorating effects

of ascorbic acid have been shown in sperm toxicity induced by mancozeb (Khan and Sinha, 1996). Also, the anti-mutagenic effect of ascorbic acid has been well documented in various *in vivo* and *in vitro* systems exposed to pesticides (Durak et al., 2009). However, despite numerous studies regarding the toxic effect of mancozeb on different cells, its effect on the cells of the primary lymphoid organ has not been studied. Therefore, the current study was undertaken to determine a possible toxic effect of mancozeb in thymocytes and to investigate whether there is any preventive effect of ascorbic acid, along with potential mechanisms involved.

Material and Methods

Animals

Experiments were performed on 11 adult male Wistar rats (180–200 g), 9–11 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions and in accordance with national animal protection guidelines.

Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. CM contained 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% foetal calf serum (FCS).

2',7'-dichlorofluorescin diacetate (H2DCF-DA), Cell Counting Kit (CCK-8), Rhodamine 123 and ATP Bioluminiscent Assay Kit were purchased from Sigma-Aldrich. Propidium iodide (PI) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Vitamin C (L-ascorbic acid) was purchased from Galenika a.d., Belgrade, Serbia. Mancozeb was purchased from Galenika-Fitofarmacija a.d., Belgrade, Serbia.

Preparation of thymocytes and mancozeb solution

Rat thymocytes were isolated as described earlier (Pavlovic et al., 2007). Briefly, thymus was extirpated using sterile technique and placed in CM with 10% FCS. Thymocytes were released by teasing the thymus through a steel mesh. Cell suspensions were filtered through sterile nylon filter to remove the stroma and then the cells were washed twice with CM/10% FCS. Thymocytes were counted and adjusted to a density of 1×10^6 cells/ml. The viability of the isolated cells, as determined by trypan blue dye exclusion test, was always over 93 %. Mancozeb solutions were prepared immediately before use in dimethyl sulphoxide and diluted in appropriate amount of CM. Control cells were treated with the same amount of vehicle alone. The final dimethyl sulphoxide concentration never exceeded 0.5 % (v/v).

Cell culture

Isolated cells were cultivated in 96-well round-bottom plates (NUNC, Aarhus, Denmark), containing 100 μl of cell suspension (5×10^5 cells) in each well. Thymocytes were treated with increasing concentrations of mancozeb (0.2, 2 and 5 μg/ml) and vitamin C (100 μg/ml), only mancozeb (0.2, 2 and 5 μg/ml), only vitamin C (100 μg/ml) or left in CM alone. All cell cultures were done in triplicates and incubated in an incubator (Galaxy, Wolf Laboratories, Edison, NJ) at 37 °C for 24 h in an atmosphere of 95% air and 5% carbon dioxide.

The dose response study was conducted by exposing the rat thymocytes to different concentrations of mancozeb (0.2–10 μg/ml) for 24 h. The results revealed a dose-dependent decrease in cell viability, with an IC_{50} value of 2.75 μg/ml, and therefore we selected 0.5, 2 and 5 μg/ml doses of mancozeb for 24 h exposure for further studies. The concentration of ascorbic acid used in the current study was based on a previous report (Turkez and Aydin, 2012).

Analysis of cell viability

Cell viability of rat thymocytes after the cultivation period was estimated by CCK-8 assay as was previously described (Hori et al., 2002). Briefly, 100 μl of cell suspension (5×10^5 cells) was plated per well in 96-well plates and cultured in CM containing 10% FCS. After the cells underwent exposure to the compounds described above, they were washed with phosphate-buffered saline, resuspended in CM and plated in a 96-well plate. Ten μl of reaction mixture, containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, was added into each well. After 2 h of incubation, the solubilized formazan product was quantified spectrophotometrically, by using a Perkin-Elmer microplate reader (Wallac Victor^{2}V, Turku, Finland). Absorbance was measured at 450 nm. For each sample, basal intensity values were subtracted from those obtained after different treatments. Absorbances were presented as a ratio of control for further comparison.

Apoptotic DNA analysis

Thymocytes undergoing apoptosis were identified by their reduced relative nuclear DNA content as previously described (Nicoletti et al., 1991). Briefly, at the end of the cultivation period, cells were collected, centrifuged ($200 \times g$) and gently resuspended in 1.5 ml of hypotonic fluorochrome solution (PI 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100) in polypropylene tubes. The tubes were placed at 4 °C in the dark overnight before the flow-cytometric analysis. Single apoptotic cells were detected using an Epics®XL flow cytometer (Coulter, Krefeld, Germany) as a reduction in fluorescence of the DNA-binding dye PI in apoptotic nuclei. The percentage of apoptotic cells (subdiploid DNA) was determined and presented as a ratio of control for further comparison.

Measurement of intracellular reactive oxygen species (ROS) production

A redox-sensitive probe (H2DCF-DA) was used to determine changes in overall cellular ROS levels, as described previously (Boldogh et al., 2003; Das et al., 2005). Briefly, after the cultivation period ended, cell suspensions were loaded with H2DCF-DA at 5 µM for 15 minutes at 37 °C. The change in fluorescence (excitation 485 nm; emission 530 nm) was measured using a Perkin-Elmer fluorometer (Wallac Victor^{2}V). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and the results were presented as the mean fluorescence intensity (MFI) ratio of control for further comparison.

Determination of mitochondrial membrane potential

Changes in mitochondrial membrane potential (MMP) of thymocytes were evaluated by uptake of lipophilic cation Rhodamine 123 into mitochondria, as previously described (Pathak and Khandelwal, 2006; Wang et al., 2007). Briefly, treated cells were incubated with Rhodamine 123 (5 µg/ml as final concentration) for 60 min in the dark at 37 °C. Thymocytes were harvested and resuspended in phosphate-buffered saline. The fluorescence of intracellular Rhodamine 123 (excitation 485 nm; emission 530 nm) was measured by a Perkin-Elmer fluorometer, as described earlier (Yang et al., 2008). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and the results were expressed as the MFI ratio of control for further comparison.

ATP measurements

ATP levels in cultured thymocytes after the end of the incubation period were measured by using a Sigma ATP Bioluminiscent Assay Kit and chemiluminescence was determined by a luminometer (Wallac Victor^{2}V) according to the manufacturers' guidelines. The results were presented as a ratio of control for further comparison.

Statistical analysis

All values are expressed as mean ± SD. The comparisons among groups were carried out using the analysis of variance (ANOVA) coupled to the Dunnett's post hoc test. A P value < 0.05 was considered significant.

Results

The cytotoxic effects of mancozeb on rat thymocytes were evaluated after 24 h of incubation with increasing concentrations (0.2, 2, 5 µg/ml) of the tested compound. Exposure to increasing concentrations of mancozeb resulted in a dose-dependent decrease in cell viability (Fig. 1), indicating a significant cytotoxic potential of mancozeb in rat thymocytes. As shown in Fig. 1, the most prominent cytotoxicity was observed in the cells exposed to the highest mancozeb concentration (P < 0.01).

To determine whether mancozeb-induced cytotoxicity is mediated by increased apoptosis, we evaluated the

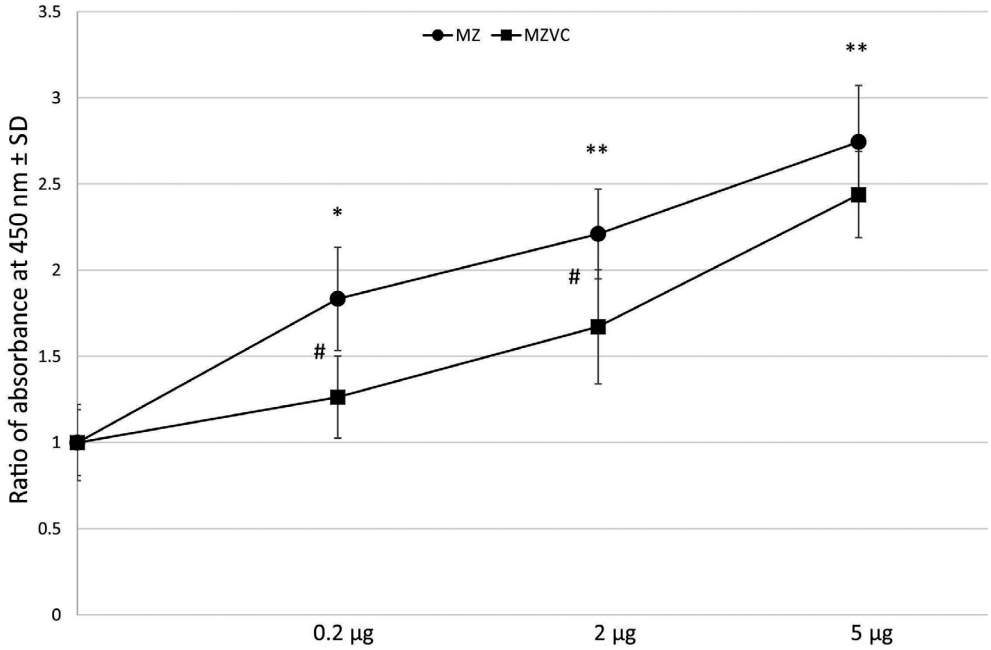

Fig. 1. The effect of mancozeb and ascorbic acid on cytotoxicity. Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of mancozeb (0.2, 2, 5 µg/well) and/or vitamin C for 24 h and cell toxicity was determined. Results are presented as mean percentage ± SD. Results are presented as the absorbance ratio of control for further comparison.

MZ – cells treated with mancozeb, MZVC – cells treated with mancozeb and vitamin C, SD – standard deviation, *P < 0.05, **P < 0.01 compared to control cells, #P < 0.05 compared to mancozeb-treated cells

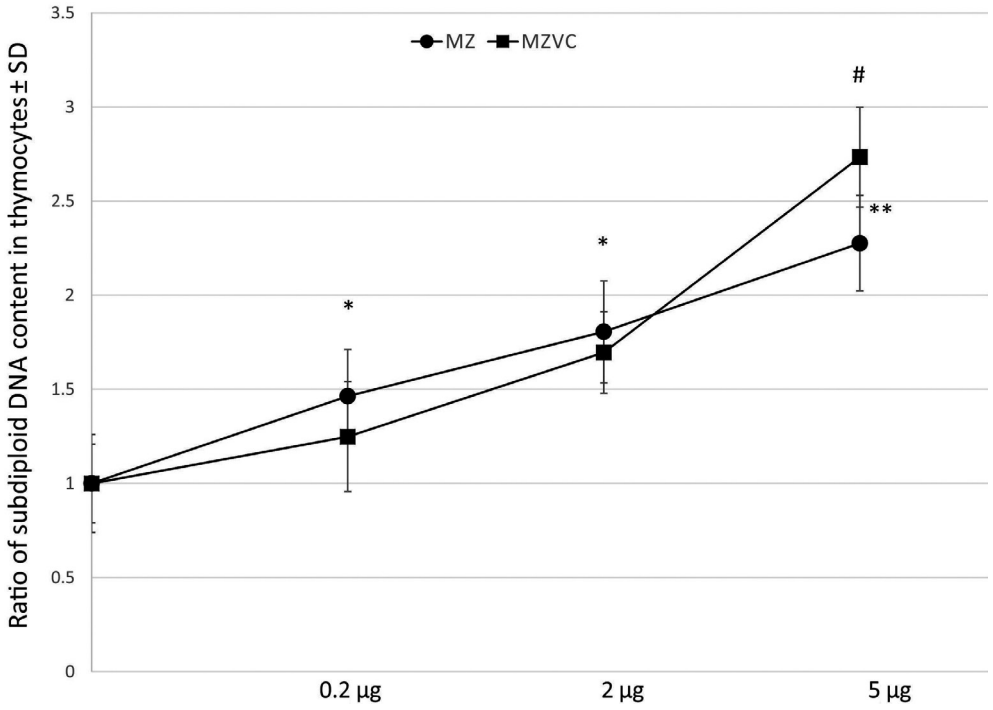

Fig. 2. The effect of mancozeb and ascorbic acid on rat thymocyte apoptosis. Cells (5×10^5 cells/well) were treated with increasing concentrations of mancozeb (0.2, 2, 5 µg/well) and/or vitamin C for 24 h and apoptosis was evaluated by flow cytometry. The percentage of apoptotic cells (subdiploid DNA) was determined and presented as a ratio ± SD of control for further comparison.
MZ – cells treated with mancozeb, MZVC – cells treated with mancozeb and vitamin C, SD – standard deviation, *P < 0.05, **P < 0.01 compared to control cells, #P < 0.05 compared to mancozeb-treated cells

effect of increased mancozeb concentrations on rat thymocyte apoptosis. Mancozeb exposure (0.2, 2, 5 µg/ml) led to a significant increase of sub-G1 peak in all tested concentrations (Fig. 2). The results showed that the percentage of apoptotic cells in sub-G1 phase of the cell cycle increased in a dose-dependent manner after mancozeb exposure, as determined by flow-cytometric analysis, confirming the pro-apoptotic potential of mancozeb.

By using fluorometric analysis, intracellular ROS levels were determined in terms of MFI. As shown in Fig. 3, exposure to increased mancozeb concentrations resulted in a significant dose-dependent increase of ROS production in rat thymocytes, suggesting its pro-oxidant potential. Simultaneously, to evaluate alterations of mitochondrial parameters during mancozeb-induced cytotoxicity, MMP and ATP levels were examined. Fluorometric analysis showed a significant decrease of both MMP and ATP levels in rat thymocytes following increased mancozeb exposure (Fig. 4, Fig. 5).

The potential capacity of ascorbic acid to reduce mancozeb-induced toxicity in rat thymocytes was evaluated in *in vitro* experiments where cells were simultaneously cultured with both compounds. The results shown in Fig. 1 demonstrated that application of ascorbic acid significantly reduced cytotoxicity in cell cultures treated with 0.2 and 2 µg/ml of mancozeb, while in cultures treated with 5 µg/ml of mancozeb, no significant reduction was observed. On the other hand, ascorbic acid exposure was found out to significantly increase the apoptosis rate in cultures with 5 µg/ml of mancozeb, compared to the thymocytes treated only with mancozeb (Fig. 2). Further, application of ascorbic acid in cell cultures treated with 0.2 and 2 µg/ml of mancozeb resulted in significantly decreased ROS production in thymocytes (Fig. 3), as evaluated by H2DCF-DA fluorescence intensity. Simultaneously, decreased ROS levels were followed by significantly increased MMP and ATP levels in thymocytes, compared to their respective controls (Fig. 4 and Fig. 5).

Discussion

The concern about the environmental impact of pesticides has been growing in the last years. However, thanks to its short environmental persistence, mancozeb is intensely used, even though it has been reported that many pesticides enhance oxidative stress with resulting teratogenic and/or carcinogenic effects (Paskova et al., 2011). In the present study, we investigated the cytotoxic effects of mancozeb on rat thymocytes and the mechanisms involved, as well as a potential beneficial role of ascorbic acid in these processes. Rat thymocytes were chosen to determine the effect of mancozeb on cells of a primary lymphoid organ and the potential development of secondary immunological consequences (including adaptive immune system failure with resulting immunodeficiency and high susceptibility to infec-

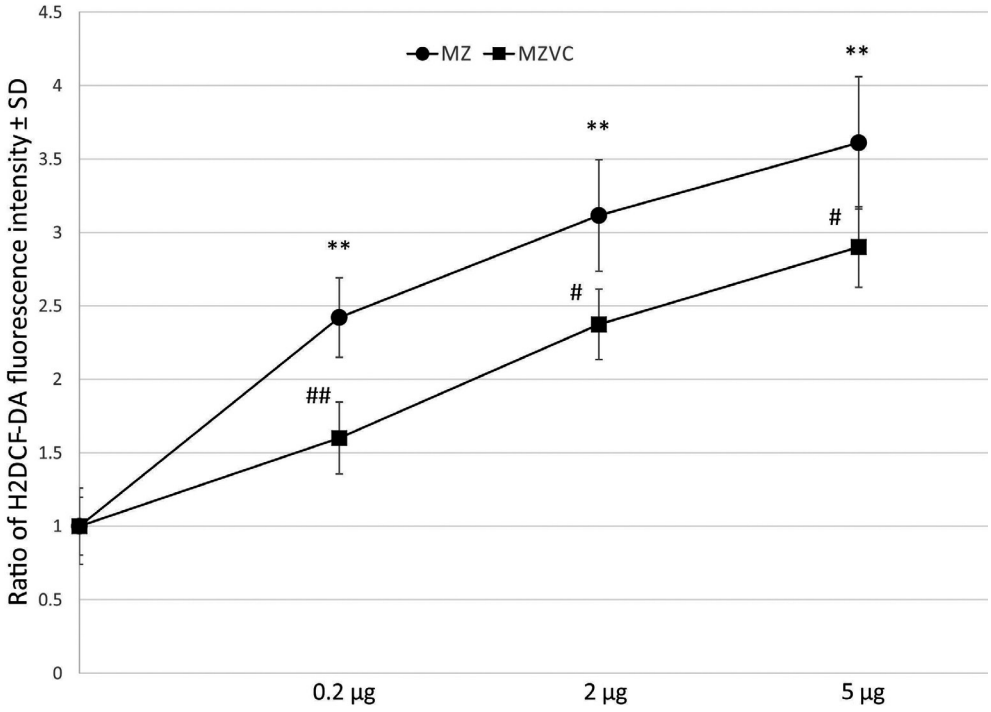

Fig. 3. The effect of mancozeb and ascorbic acid on ROS production in rat thymocytes. Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of mancozeb (0.2, 2, 5 µg/well) and/or vitamin C for 24 h and intracellular ROS production was evaluated by using a redox-sensitive probe (H2DCF-DA). Results are presented as a ratio of mean fluorescence intensity ± SD of control for further comparison.
MZ – cells treated with mancozeb, MZVC – cells treated with mancozeb and vitamin C, SD – standard deviation, **P < 0.01 compared to control cells, #P < 0.05, ##P < 0.01, compared to mancozeb-treated cells

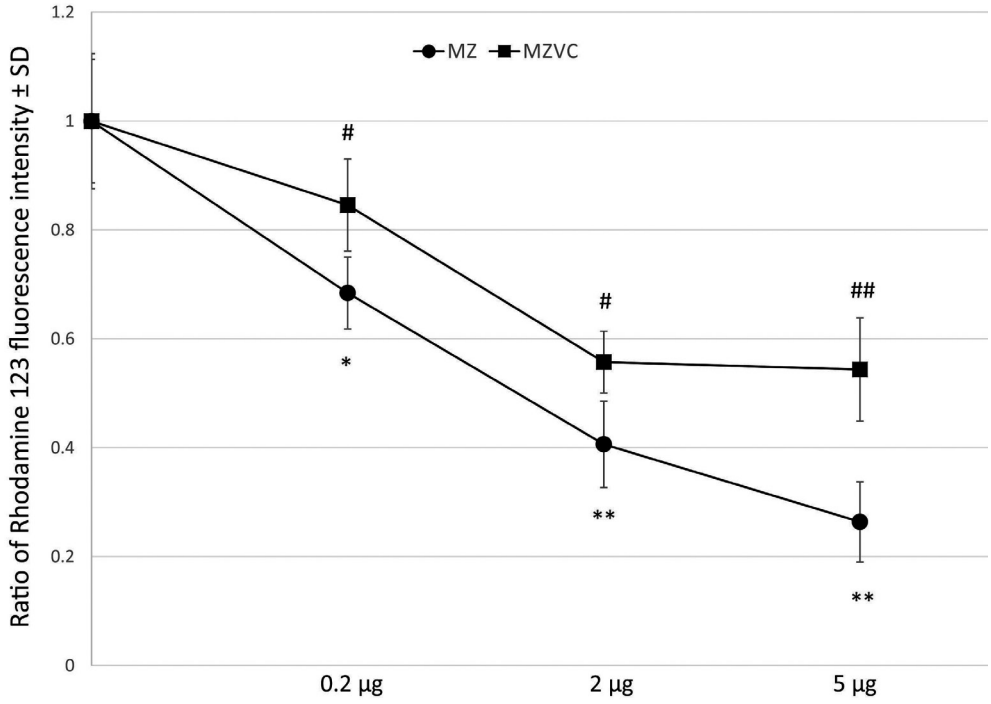

Fig. 4. The effect of mancozeb and ascorbic acid on mitochondrial membrane potential of rat thymocytes. Cells (5×10^5 cells/well) were treated with increasing concentrations of mancozeb (0.2, 2, 5 µg/well) and/or vitamin C for 24 h and mitochondrial membrane potential was evaluated by using Rhodamine 123. Results are presented as a ratio of mean fluorescence intensity ± SD of control for further comparison.
MZ – cells treated with mancozeb, MZVC – cells treated with mancozeb and vitamin C, SD – standard deviation, *P < 0.05, **P < 0.01 compared to control cells, #P < 0.05, ##P < 0.01, compared to mancozeb-treated cells

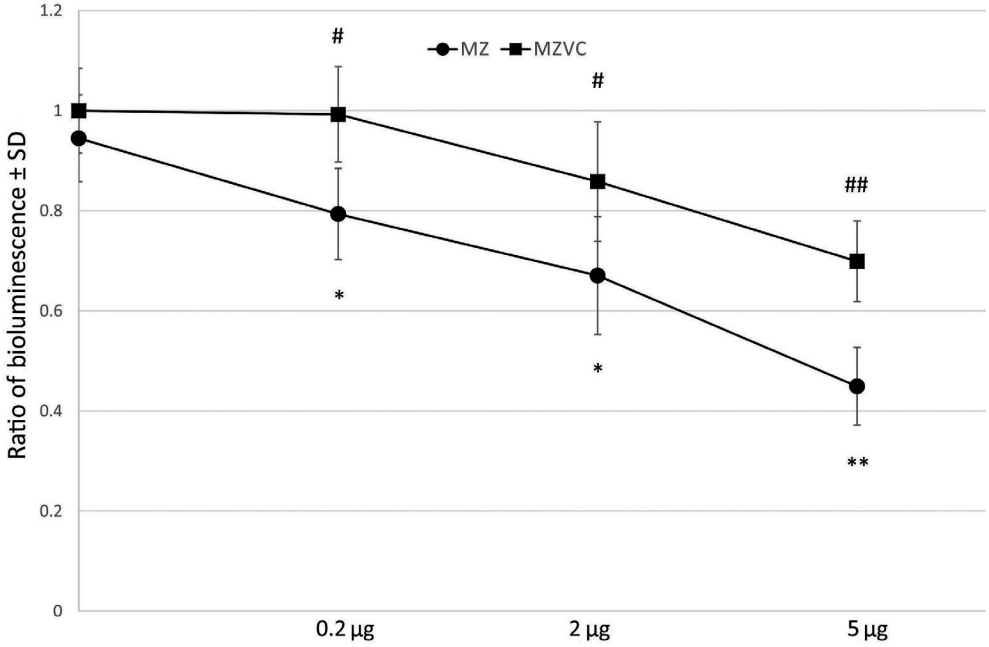

Fig. 5. The effect of mancozeb and ascorbic acid on ATP levels in rat thymocytes. Cells (5×10^5 cells/well) were treated with increasing concentrations of mancozeb (0.2, 2, 5 µg/well) and/or vitamin C for 24 h and ATP levels in rat thymocytes were evaluated by using ATP Bioluminiscent Kit. The results were presented as a ratio of bioluminescence of control for further comparison.
MZ – cells treated with mancozeb, MZVC – cells treated with mancozeb and vitamin C, SD – standard deviation, *P < 0.05, **P < 0.01 compared to control cells, #P < 0.05, ##P < 0.01, compared to mancozeb-treated cells

tion). The results of the current study demonstrate that exposure of rat thymocytes to mancozeb was able to induce cytotoxicity in a dose-dependent manner. Flow-cytometric analysis revealed that some of the cells die via the apoptotic mechanism in the presence of increased mancozeb concentrations. These findings are in line with previous reports showing cytotoxic effects of mancozeb in rat PBMCs (Calviello et al., 2006), human lymphocytes (Srivastava et al., 2012), rat astrocytes (Tsang and Trombetta, 2007), rat mesencephalic cells (Domico et al., 2006) and rat fibroblasts (Calviello et al., 2006), along with pro-apoptotic mancozeb potential in lymphocytes (Srivastava et al., 2012), PBMCs (Calviello et al., 2006) and mammary adenocarcinoma cells (Lin and Garry, 2000).

Since it has been well documented that ROS generation may cause damage to DNA, lipids and other biomolecules, with resulting cytotoxic effect (Pelicano et al., 2004), we next evaluated the potential mechanisms involved in these processes. Exposure to increased mancozeb concentrations resulted in a dose-dependent increase of ROS production, together with depletion of MMP and ATP levels, which corresponds with decreased viability of the rat thymocytes. Due to the high content of polyunsaturated fatty acids in their plasma membranes, cells of the immune system are expressly sensitive to oxidative stress (Victor et al., 2002). Intensive ROS production may induce apoptosis by oxidative stress or direct ROS damage to cellular components (Hildeman et al., 2003). Cell death depends, in part, upon mitochondrial dysfunction, which is often characterized by increased production of ROS (Exline and Crouser, 2008). Functional alterations of mitochondria are usually manifested in changes of MMP, which is essential for mitochondrial functions such as oxidative phosphorylation and ATP synthesis (Zhang et al., 2008). Taken together with our results, it seems that elevated ROS production and mitochondrial dysfunction may represent a key factor in mancozeb-induced toxicity, with potential secondary immunological consequences.

Based on the previous findings, we hypothesized that ascorbic acid may modulate the mancozeb-induced toxicity. Application of ascorbic acid significantly decreased ROS production in mancozeb-exposed cells, suggesting its protective role in mancozeb-induced ROS generation. In addition, our results showed that ascorbic acid significantly decreased toxicity in cells treated with 0.2 and 2 µg/ml of mancozeb. The protective role of ascorbic acid in mancozeb-induced toxicity has been shown earlier in sperm toxicity (Khan and Sinha, 1996) and here we demonstrate the protective role in rat thymocytes. Ascorbic acid is a non-enzymatic antioxidant that defends the cellular compartment against water-soluble oxygen and nitrogen radicals. On the other hand, different reports demonstrate that increased ROS production induces mitochondrial dysfunction, which sensitizes T cells to apoptosis (Hildeman et al., 2003; Pavlovic et al., 2007).

Our results support previous studies indicating that ascorbic acid enhances antioxidant defences of T cells (Wu et al., 2000) and inhibits various forms of T-cell death (Campbell et al., 1999). Further, in rat thymocytes

exposed to the highest (5 μg/ml) mancozeb concentrations, ascorbic acid failed to reduce the toxicity but, simultaneously, markedly increased the apoptosis rate. These findings correlate with mitochondrial analysis, which showed the most significant increase, after ascorbic acid application, of MMP and ATP levels in the cells treated with highest mancozeb concentrations. Besides MMP, cellular ATP levels are another characteristic of mitochondrial dysfunction. ATP is necessary for apoptosome development, and ATP depletion can cause block of apoptosis steps (Nicotera et al., 1998). Based on the obtained study results we can speculate that ascorbic acid may have the ability to shift cells from necrosis to apoptosis, by increasing MMP and ATP levels in the cells exposed to the highest (5 μg/ml) mancozeb concentrations. In line with this, it has been postulated that cells triggered to undergo apoptosis will die by necrosis when the intracellular ATP levels are depleted (Leist et al., 1997). T-cell necrosis is always associated with an inflammatory response compared to apoptosis (Jin et al., 2007). Since high levels are required to sustain apoptosis, it seems that ascorbic acid enables maintaining the bioenergetics status of immune cells and prevents further inflammatory response that results in impaired immune function.

In summary, we have shown that exposure of rat thymocytes to mancozeb resulted in increased cytotoxicity mainly through induction of oxidative stress and resulting mitochondrial dysfunction. Ascorbic acid administration reduced the toxicity rate in cells treated with lower mancozeb concentrations while increasing the apoptosis rate of cells in the presence of highest mancozeb concentrations. Understanding the mechanism of the protective role of ascorbic acid at the cellular level may provide a rationale for antioxidant therapy in humans who are exposed to different pesticides and prevent possible secondary immunological consequences.

References

Boldogh, I., Roy, G., Lee, M. S., Bacsi, A., Hazra, T. K., Bhakat, K. K., Das, G. C., Mitra, S. (2003) Reduced DNA double strand breaks in chlorambucil resistant cells are related to high DNAPKCs activity and low oxidative stress. *Toxicology* **193**, 137-152.

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.

Campbell, J. D., Cole, M., Bunditrutavorn, B., Vella, A. T. (1999) Ascorbic acid is a potent inhibitor of various forms of T cell apoptosis. *Cell. Immunol.* **194**, 1-5.

Corsini, E., Viviani, B., Birindelli, S., Gilardi, F., Torri, A., Codecà, I., Lucchi, L., Bartesaghi, S., Galli, C. L., Marinovich, M., Colosio, C. (2006) Molecular mechanisms underlying mancozeb-induced inhibition of TNF-α production. *Toxicol. Appl. Pharmacol.* **212**, 89-98.

Das, A., Hazra, T. K., Boldogh, I., Mitra, S., Bhakat, K. K. (2005) Induction of the human oxidized base-specific DNA glycosylase Neil1 by reactive oxygen species. *J. Biol. Chem.* **280**, 35272-35280.

Domico, L. M., Zeevalk, G. D., Bernard, L. P., Cooper, K. R. (2006) Acute neurotoxic effects of mancozeb and maneb in mesencephalic neuronal cultures are associated with mitochondrial dysfunction. *Neurotoxicology* **27**, 816-825.

Durak, D., Uzun, F. G., Kalender, S., Ogutcu, A., Uzunhisarcikli, M., Kalender, Y. (2009) Malathion-induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E in vitro. *Environ. Toxicol.* **24**, 235-242.

Dwivedi, N., Kumar, S., Ansari, K. M., Khanna, S. K., Das, M. (2013) Skin tumorigenic potential of benzanthrone: prevention by ascorbic acid. *Food Chem. Toxicol.* **59**, 687-695.

Eroglu, S., Pandir, D., Uzun, F. G., Bas, H. (2013) Protective role of vitamins C and E in dichlorvos-induced oxidative stress in human erythrocytes in vitro. *Biol. Res.* **46**, 33-38.

Exline, M. C., Crouser, E. D. (2008) Mitochondrial mechanisms of sepsis induced organ failure. *Front. Biosci.* **13**, 5030-5041.

Hildeman, D. A., Mitchell, T., Aronow, B., Wojciechowski, S., Kappler, J., Marrack, P. (2003) Control of Bcl-2 expression by reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **100**, 15035-15040.

Hori, R., Kashiba, M., Toma, T., Yachie, A., Goda, N., Makino, N., Soejima, A., Nagasawa, T., Nakabayashi, K., Suematsu, M. (2002) Gene transfection of H25A mutant heme oxygenase-1 protects cells against hydroperoxide-induced cytotoxicity. *J. Biol. Chem.* **277**, 10712-10718.

Houeto, P., Bindoula, G., Hoffman, J. R. (1995) Ethylenebisdithiocarbamates and ethylenethiourea: possible human health hazards. *Environ. Health Perspect.* **103**, 568-573.

Jin, S., DiPaola, R. S., Mathew, R., White, E. (2007) Metabolic catastrophe as a means to cancer cell death. *J. Cell Sci.* **120**, 379-383.

Khan, P. K., Sinha, S. P. (1996) Ameliorating effect of vitamin C on murine sperm toxicity induced by three pesticides (endosulfan, phosphamidon and mancozeb). *Mutagenesis* **11**, 33-36.

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.* **185**, 1481-1486.

Lin, N., Garry, V. F. (2000) In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. *J. Toxicol. Environ. Health* **60**, 423-439.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271-279.

Nicotera, P., Leist, M., Ferrando-May, E. (1998) Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicol. Lett.* **102-103**, 139-142.

Paro, R., Tiboni, G. M., Buccione, R., Rossi, G., Cellini, V., Canipari, R., Cecconi, S. (2012) The fungicide mancozeb induces toxic effects on mammalian granulosa cells. *Toxicol. Appl. Pharmacol.* **260**, 155-161.

Paskova, V., Hilscherova, K., Blaha, L. (2011) Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress. *Rev. Environ. Contam. Toxicol.* **211**, 25-61.

Pathak, N., Khandelwal, S. (2006) Modulation of cadmium induced alterations in murine thymocytes by piperine: oxidative stress, apoptosis, phenotyping and blastogenesis. *Biochem. Pharmacol.* **72**, 486-497.

Pavlovic, V., Pavlovic, D., Kocic, G., Sokolovic, D., Jevtovic-Stoimenov, T., Cekic, S., Velickovic, D. (2007) Effect of monosodium glutamate on oxidative stress and apoptosis in rat thymus. *Mol. Cell. Biochem.* **303**, 161-166.

Pelicano, H., Carney, D., Huang, P. (2004) ROS stress in cancer cells and therapeutic implications. *Drug Resist. Updat.* **7**, 97-110.

Rossi, G., Buccione, R., Baldassarre, M., Macchiarelly, G., Palmerini, M. G., Cecconi, S. (2006) Mancozeb exposure in vivo impairs mouse oocyte fertilizability. *Reprod. Toxicol.* **21**, 216-219.

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.

Tsang, M. M., Trombetta, L. D. (2007) The protective role of chelators and antioxidants on mancozeb-induced toxicity in rat hippocampal astrocytes. *Toxicol. Ind. Health.* **23**, 459-470.

Turkez, H., Aydin, E. (2012) The protective role of ascorbic acid on imazalil-induced genetic damage assessed by the cytogenetic tests. *Toxicol. Ind. Health.* **28**, 648-654.

Victor, V. M., Guayerbas, N., De la Fuente, M. (2002) Changes in the antioxidant content of mononuclear leukocytes from mice with endotoxin-induced oxidative stress. *Mol. Cell. Biochem.* **229**, 107-111.

Wang, Z. B., Liu, Y. Q., Zhang, Y., Li, Y., An, X. X., Xu, H., Guo, Y., Jin, W., Jiang, Z. J., Cui, Y. F. (2007) Reactive oxygen species, but not mitochondrial membrane potential, is associated with radiation-induced apoptosis of AHH-1 human lymphoblastoid cells. *Cell Biol. Int.* **31**, 1353-1358.

Williams, C. A., Lin, Y., Maynard, A., Cheng, S. Y. (2013) Involvement of NFκB in potentiated effect of Mn-containing dithiocarbamates on MPP(+) induced cell death. *Cell. Mol. Neurobiol.* **33**, 815-823.

Wu, C. C., Doriarajan, T., Lin, T. L. (2000) Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. *Vet. Immunol. Immunopathol.* **74**, 145-152.

Yang, Z., Wu, D., Bui, T., Ho, R. J. (2008) A novel human multidrug resistance gene MDR1 variant G571A (G191R) modulates cancer drug resistance and efflux transport. *J. Pharmacol. Exp. Ther.* **327**, 474-481.

Zhang, H., Zhang, J., Chen, Y., Zhu Y. (2008) Microcystin-RR induces apoptosis in fish lymphocytes by generating reactive oxygen species and causing mitochondrial damage. *Fish Physiol. Biochem.* **34**, 307-312.