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# Vitamin E prevents ethylene bis(dithiocarbamate) pesticide zineb-induced sister chromatid exchange in Chinese hamster ovary cells

**Sonia Soloneski[1], Miguel A.Reigosa and Marcelo L.Larramendy**

Laboratorio de Citogenética, Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina

**The *in vitro* effect of the antioxidant α-tocopherol, vitamin E, on deleterious effects induced by the dithiocarbamate fungicide zineb and its commercial formulation azzurro on Chinese hamster ovary (CHO) cells was studied by using frequency of sister chromatid exchanges (SCEs), cell cycle progression and mitotic index (MI) as genetic end points. Both zineb and azzurro activities were tested within the range 0.1–100.0 μg/ml on exponentially growing CHO cells preincubated for 24 h in the presence or absence of 50.0 μg/ml vitamin E. SCE frequencies increased significantly over control values in a concentration-dependent manner in zineb- and azzurro-treated cultures at concentrations of 0.1–10.0 and 0.1–25.0 μg/ml, respectively. When target cells were preincubated with vitamin E, the number of SCEs was significantly lower than that observed in cells exposed only to 1.0–10.0 μg/ml zineb or 1.0–25.0 μg/ml azzurro, but higher than control values. Cytotoxicity was observed at concentrations higher than 25.0 and 50.0 μg/ml zineb and azzurro, respectively, regardless of the absence or presence of vitamin E. Regression analysis showed that the proliferative rate index decreased as a function of the concentration of zineb (0.1–10.0 μg/ml concentration range) and azzurro (0.1–25.0 μg/ml concentration range) titrated into cultures. For both chemicals, progressive concentration-related inhibition of the mitotic activity from cultures was observed when 10.0 μg/ml zineb or 1.0–25.0 μg/ml azzurro was employed. However, no significant alteration in cell cycle progression or MI was observed between vitamin E-preincubated cultures and those treated only with zineb and azzurro.**

## Introduction

Dithiocarbamates are chemicals used in agriculture with a variety of applications. Furthermore, dithiocarbamate derivatives are currently used for eradication of fungal infection on fruit plants and vegetables such as tomato, rice, potato and sugar beet, among others. Most of these compounds have produced conflicting results in mutagenicity tests, since conclusions vary according to both the test and the compound employed (IARC, 1976, 1987a, 1991).

According the International Agency for Research on Cancer (IARC, 1976, 1983, 1986, 1991) zineb has been ranked as category 3 (i.e. insufficient available data to evaluate carcinogenicity to humans), since the evidence for its deleterious properties has been determined as not adequate and/or inadequate in humans and other animal systems, respectively (IARC, 1987b). Based on the similarities of the chemical structure of zineb with other ethylene bis(dithiocarbamate) pesticides and the comparable toxicological profiles of this type of pesticide as well as on the toxic effect of their main metabolic and degradation product, ethylenethiourea, the acceptable human daily intake for zineb has been estimated to be 0–0.03 mg/kg body wt/day (Vettorazzi *et al*., 1995), while its residue tolerance for most raw agricultural crop products has been established at 7 mg/kg (Environmental Protection Agency, 1974).

So far, zineb has been considered a non-mutagenic agent in bacterial systems (IARC, 1976), although this does not necessarily imply that dithiocarbamate cannot directly damage genetic material. In agreement with this assumption are findings that show induction of point mutations (Shiau *et al*., 1980; Franekic *et al*., 1994; Della Croce *et al*., 1996), chromosomal aberrations in lymphocytes from occupationally exposed crop workers (Pilinskaya, 1974) and our findings demonstrating an ability to induce chromosomal aberrations in human lymphocytes *in vitro* (Soloneski *et al*., 2001), sister chromatid exchanges (SCEs) and a delay in cell cycle progression of human lymphocytes and Chinese hamster ovary (CHO) cells (Soloneski *et al*., 2001, 2002a). We have also demonstrated that zineb, as well as the zineb-containing formulation azzurro, are not only able to induce micronuclei in human lymphocytes *in vitro*, but also that such induction was restricted to B $CD20^+$ and T suppressor/cytotoxic $CD8^+$ cell subsets (Soloneski *et al*., 2002b). So far, the exact mechanism(s) of ethylene bis(dithiocarbamate)-induced DNA damage is not known. The possibility of zineb-induced lesions in DNA via free radical reactions and production of active oxygen species has been previously suggested by us (Soloneski *et al*., 2002a). On the other hand, when assessing DNA damage and repair kinetics analyzed using the single cell gel electrophoresis assay on zineb- and azzurro-exposed CHO cells, we observed that single strand breaks introduced into the DNA likely reflect those induced by alkylating agents rather than those produced by active oxygen species (González *et al*., 2003).

To analyze whether oxidative damage is involved in the deleterious effects exerted by zineb and azzurro, we set out to investigate the possible protective role of the antioxidant vitamin E using SCE frequency and cell cycle progression analyses as *in vitro* cytogenetic end-points in CHO cells.

## Materials and methods

### *Chemicals*

Zineb [ethylene bis(dithiocarbamate) zinc; CAS no. 12122-67-7] (Pestanal®) was obtained from Riedel-de Haën (Hannover, Germany). Azzurro (70% zineb) and vitamin E (*dl*-α-tocopherol) were kindly provided by Chemiplant (Buenos Aires, Argentina) and Roche (Basel, Switzerland), respectively.

[1]To whom correspondence should be addressed. Tel: +54 221 14 4741840; Fax: +54 221 14 4741840; Email: ssoloneski@yahoo.com.ar

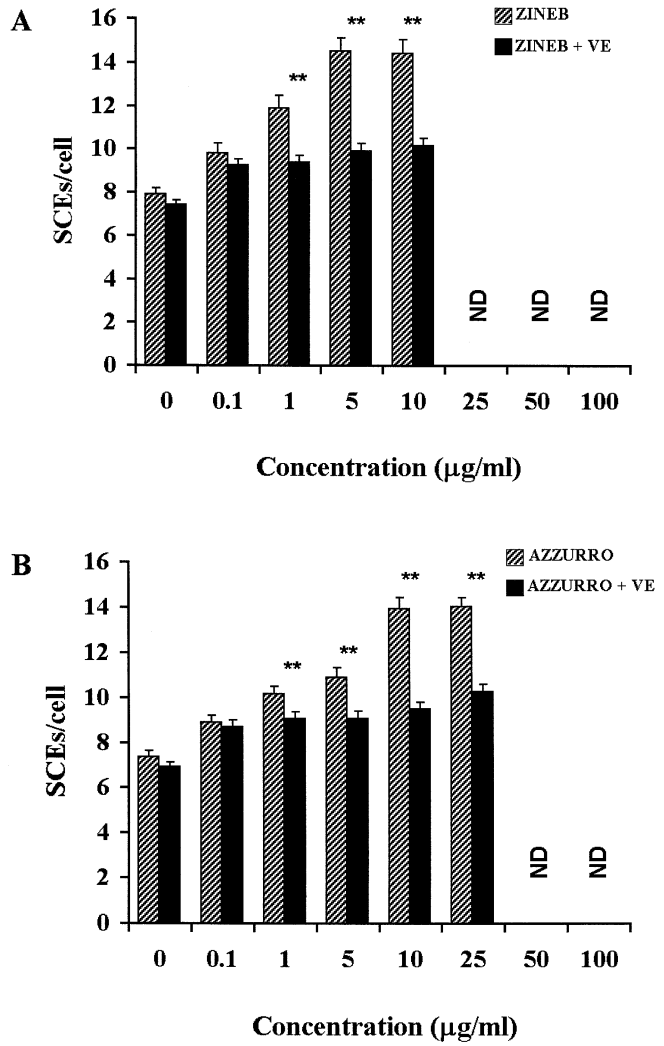


**Fig. 1.** Effect of vitamin E on SCEs induced by zineb and its commercial formulation azzurro in CHO cells. Cultures were set up with culture medium supplemented with vitamin E, treated with pesticides 24 h later and harvested 48 h after seeding. The proportion of SCEs was determined in 50 $M_2$ mitoses at each experimental point. For both fungicides, pooled data from three independent experiments are reported as mean SCE values ± SE (*y*-axis) and plotted against the fungicide concentration (0–100.0 μg/ml concentration range, *x*-axis). ND, not determined since no cells were present in cultures. $^{**}P < 0.01$.

Dimethylsulfoxide (DMSO) was purchased from Sigma Chemical Co. (St Louis, MO).

*Cell cultures and pesticide treatment for cytogenetic assays*

CHO cells were grown in Ham's F10 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin (Gibco) and 10 μg/ml streptomycin (Gibco) at 37°C in a 5% $CO_2$ atmosphere. Experiments were set up with cultures in the log phase of growth. The cells were seeded in T75 flasks at a density of $10^6$ cells/flask. A stock solution of vitamin E was made in 96% ethanol prior to use and incorporated into flasks immediately after seeding to a final concentration of 50 μg/ml. Treatments with test compounds were performed 24 h after plating without removal of vitamin E-supplemented culture medium. Both zineb and azzurro were dissolved in DMSO prior to use and were then diluted in culture medium such that addition of 100 μl to cultures produced the required concentration. Immediately after seeding zineb and azzurro were used within the concentration range 0.1–100.0 μg/ml. The final solvent concentration was <1% for all the treatments. Negative controls (untreated cells and solvent vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Afterwards, 10 μg/ml bromodeoxyuridine (BrdUrd) (Sigma Chemical Co.) was incorporated into cultures and then the cells were incubated at 37°C in a 5% $CO_2$ atmosphere under a safety light for 36 h until harvesting. Cultures were duplicated for each experimental point, in at least three independent experiments. The same batches of culture medium, sera and reagents were used throughout the study.

*Chromosome preparations*

During the last 3 h of culture, the cells were treated with 0.2 μg/ml colchicine (Sigma Chemical Co.). Cells were detached by trypsinization, collected by centrifugation, hypotonically shocked (0.075 M KCl, 37°C, 15 min) and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air drying technique.

*Fluorescence plus Giemsa (FPG) method for sister chromatid differentiation*

Chromosome spreads were stained using the FPG technique for sister chromatid differentiation as described previously (Larramendy and Knuutila, 1990). Slides were coded and scored blind by one researcher.

*Cell cycle kinetics and mitotic index*

A minimum of 200 metaphase cells per sample were scored to determine the percentage of cells that had undergone one ($M_1$), two ($M_2$) and three or subsequent mitoses ($M_{3+}$). The proliferative rate index (PRI) was calculated for each experimental point according to the formula $PRI = [(\% M_1) + 2(\% M_2) + 3(\% M_{3+})]/100$, which indicated the average number of times the cells had divided in the medium from the time of addition of BrdUrd until harvesting (Lamberti *et al*., 1983). The mitotic index (MI) was determined by scoring 2000 cells from each experimental point and expressed as the number of mitoses among 1000 nuclei. Changes in MI were expressed as a factor (*f*) of the mean MI from treated cultures ($MI_t$) over the mean MI from controls ($MI_c$) ($f = MI_t/MI_c$) (Miller and Adler, 1989).

*Sister chromatid exchange analysis*

For the SCE assay a total of 50 well-spread diploid metaphases were scored per experimental point from each triplicate experiment in $M_2$ cells. The data were expressed as the mean number of SCE per cell ± SE from 150 pooled cells scored per fungicide concentration.

*Statistical analysis*

The two-tailed Student's *t*-test was used to compare SCE frequencies. The $\chi^2$ test was used for cell cycle progression and MI data. The chosen level of significance was 0.05 unless indicated otherwise.

## Results

Since no differences in cell cycle progression, SCE and PRI values were observed between negative controls (untreated and DMSO-treated cells), pooled data are presented for control cultures.

Figure 1 and Table I show the results of SCE analyzed in CHO cells treated for 24 h with different concentrations of zineb or the zineb-containing commercial fungicide azzurro in the presence or absence of vitamin E. The SCE frequencies observed in cultures treated with either zineb or azzurro at concentrations of 0.1–10.0 and 0.1–25.0 μg/ml, respectively, were significantly higher than control values (Figure 1, $P < 0.01$). SCEs increased in a concentration-dependent manner. Regression tests showed that SCE frequencies increased as a function of the concentration of zineb titrated into cultures (Figure 1, $r = 0.70$, $P < 0.05$) and the concentration of azzurro (Figure 1, $r = 0.83$, $P < 0.01$). When target cells were preincubated with vitamin E, the number of SCEs was significantly lower than that observed when exposed to only 1.0–10.0 μg/ml zineb (Figure 1, $P < 0.01$) or 1.0–25.0 μg/ml azzurro (Figure 1, $P < 0.01$), but higher than control values ($P < 0.05$). Regression tests showed that SCE frequencies increased as a function of the concentration of the zineb titrated into cultures (Figure 1, $r = 0.70$, $P < 0.05$) and the concentration of azzurro (Figure 1, $r = 0.74$, $P < 0.01$). Furthermore, for zineb and azzurro, no mitoses were observed at concentrations higher than 25.0 and 50.0 μg/ml, respectively, regardless of the absence or presence of vitamin E in the culture medium (Figure 1).

**Table 1.** SCE frequencies in control and zineb- and azzurro-treated CHO cells with and without vitamin E (50 μg/ml)[a]

| Concentration (μg/ml) | SCE frequencies[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Zineb | | | | Azzurro | | | |
| | Exp. 1 | Exp. 2 | Exp. 3 | Pooled data[c] | Exp. 1 | Exp. 2 | Exp. 3 | Pooled data[c] |
| 0 | 7.84 ± 0.32 | 7.88 ± 0.40 | 7.60 ± 0.33 | 7.92 ± 0.20 | 7.48 ± 0.37 | 7.29 ± 0.37 | 7.24 ± 0.37 | 7.32 ± 0.21 |
| 0 + VE | 6.64 ± 0.47 | 7.63 ± 0.51 | 7.88 ± 0.51 | 7.43 ± 0.28 | 6.83 ± 0.23 | 7.00 ± 0.36 | 7.08 ± 0.44 | 6.93 ± 0.20 |
| 0.1 | 9.24 ± 0.37 | 10.12 ± 0.75 | 10.00 ± 0.76 | 9.79 ± 0.38 | 8.92 ± 0.54 | 8.88 ± 0.52 | 8.68 ± 0.53 | 8.83 ± 0.30 |
| 0.1 + VE | 8.20 ± 0.46 | 7.92 ± 0.60 | 8.04 ± 0.51 | 9.33 ± 0.34 | 9.00 ± 0.53 | 8.46 ± 0.49 | 8.92 ± 0.49 | 8.68 ± 0.29 |
| 1.0 | 10.96 ± 0.59 | 12.72 ± 0.89 | 12.60 ± 0.89 | 12.09 ± 0.47 | 10.16 ± 0.60 | 10.16 ± 0.59 | 10.32 ± 0.56 | 10.21 ± 0.33 |
| 1.0 + VE | 11.00 ± 0.59 | 8.16 ± 0.60 | 9.48 ± 0.46 | 9.55 ± 0.34 | 9.71 ± 0.50 | 8.88 ± 0.58 | 8.54 ± 0.46 | 9.07 ± 0.30 |
| 5.0 | 13.96 ± 0.59 | 15.08 ± 0.98 | 14.96 ± 0.95 | 14.67 ± 0.49 | 10.00 ± 0.60 | 11.80 ± 0.71 | 11.96 ± 0.75 | 11.25 ± 0.41 |
| 5.0 + VE | 10.13 ± 0.46 | 9.36 ± 0.55 | 9.32 ± 0.42 | 9.61 ± 0.28 | 9.67 ± 0.56 | 8.54 ± 0.56 | 8.88 ± 0.58 | 9.01 ± 0.33 |
| 10.0 | 14.28 ± 0.85 | 14.52 ± 0.97 | 14.00 ± 0.94 | 14.27 ± 0.53 | 9.92 ± 0.61 | 13.64 ± 0.91 | 13.49 ± 0.93 | 13.79 ± 0.50 |
| 10.0 + VE | 9.24 ± 0.74 | 10.44 ± 0.89 | 8.96 ± 0.44 | 9.55 ± 0.41 | 10.00 ± 0.66 | 9.25 ± 0.52 | 9.17 ± 0.51 | 9.48 ± 0.32 |
| 25.0 | ND | ND | ND | ND | 14.24 ± 0.76 | 13.36 ± 0.83 | 12.84 ± 0.78 | 13.64 ± 0.47 |
| 25.0 + VE | ND | ND | ND | ND | 9.75 ± 0.57 | 10.38 ± 0.54 | 10.46 ± 0.64 | 10.24 ± 0.33 |
| 50.0 | ND | ND | ND | ND | ND | ND | ND | ND |
| 50.0 + VE | ND | ND | ND | ND | ND | ND | ND | ND |
| 100.0 | ND | ND | ND | ND | ND | ND | ND | ND |
| 100.0 + VE | ND | ND | ND | ND | ND | ND | ND | ND |

[a]CHO cells were treated 24 h after seeding with different test compounds and harvested 24 h later.
[b]Results are expressed as mean SCEs/cell ± SE.
[c]Data from three independent experiments (Exp. 1–3).
ND, not determined.

Both chemicals were cytotoxic, since both a significant delay in cell cycle progression and a significant reduction in PRI were observed in zineb- and azzurro-treated cultures (Figure 2). Pesticide concentrations ranging from 0.1–1.0 μg/ml did not alter the rate of cell proliferation ($P > 0.05$). For both test compounds, a significant decrease in the frequency of $M_2$ ($P < 0.01$) and a significant increase in the frequency of $M_1$ ($P < 0.01$) were observed in cultures treated with either 5.0–10.0 μg/ml zineb (Figure 2A) or 5.0–25.0 μg/ml azzurro (Figure 2C) with respect to control values. Regression tests showed that the PRI decreased as a function of the concentration of zineb (0.1–10.0 μg/ml concentration range) or azzurro (0.1–25.0 μg/ml concentration range) titrated into cultures ($r = –0.97$, $P < 0.01$). However, no significant alteration in cell cycle progression was observed between these vitamin E-preincubated cultures and those treated only with zineb (Figure 2B, $P > 0.05$) or azzurro (Figure 2D, $P > 0.05$). In those vitamin E-preincubated cultures, regression tests showed that the PRI decreased as a function of the concentration of zineb (0.1–25.0 μg/ml concentration range, $r = –0.98$, $P < 0.01$) and azzurro (0.1–10.0 μg/ml concentration range, $r = –0.89$, $P < 0.01$) titrated into cultures.

The MI data from both zineb- and azzurro-treated cultures are presented in Figure 3. For both chemicals, a progressive concentration-related inhibition of mitotic activity in cultures was observed when 10.0 μg/ml zineb ($P < 0.01$) or 1.0–25.0 μg/ml azzurro ($P < 0.01$) was used. Overall, regression tests showed that mitotic activity decreased as a function of the concentration of zineb (Figure 3A, $r = –0.90$, $P < 0.01$) and azzurro (Figure 3B, $r = –0.77$, $P < 0.01$) titrated into cultures. When either 10.0 μg/ml zineb or 25.0 μg/ml azzurro was used, the mitotic activity of cultures decreased with respect to the control values ($f = 1.00$) by a mean $f$ of 0.34 ± 0.12 and 0.42 ± 0.02, respectively. No significant alteration in MI was observed between these vitamin E-preincubated cultures and those treated only with zineb (Figure 3A, $P > 0.05$) or azzurro (Figure 3B, $P > 0.05$). In these vitamin E-preincubated cultures, regression test showed that the MI decreased as a function of the concentration of zineb ($r = –0.96$, $P < 0.01$) and azzurro ($r = –0.90$, $P < 0.01$) titrated into cultures. When either 10.0 μg/ml zineb or 25.0 μg/ml azzurro was used, the mitotic activity of these vitamin E-preincubated cultures decreased with respect to the control values ($f = 1.00$) by a mean $f$ of 0.47 ± 0.08 and 0.50 ± 0.03, respectively.

## Discussion

The present study was carried out to test the protective effect of the antioxidant vitamin E on damage induced in mammalian cells *in vitro* by zineb and the zineb-containing commercial formulation azzurro. CHO cells were exposed to increasing concentrations of both pesticides after a pretreatment with vitamin E and the induced damage was evaluated using cytogenetic tests: analysis of frequency of SCEs, follow-up of cell cycle progression for 24 h after treatment and assessment of mitotic activity in the cultures. The results demonstrate that vitamin E prevents the induction of SCEs by either zineb or azzurro, but show the ineffectiveness of vitamin E in modulating both the delay in cell cycle kinetics and decrease in mitotic activity induced by the pesticide.

High concentration-related cytotoxicity revealed by the absence of cells was observed in cultures harvested 24 h after treatment with concentrations >25.0 μg/ml zineb or 50.0 μg/ml azzurro, respectively. We have previously reported that complete cell death is achieved in CHO cells exposed to 50.0–100.0 μg/ml of both compounds, when the culture period is extended up to 36 h after treatment (Soloneski *et al*., 2002a). A similar cytotoxic effect was observed in human lymphocytes cultured in an erythrocyte-free environment, such as plasma leukocyte cultures (Soloneski *et al*., 2002b), but not in lymphocytes exposed to 50.0 μg/ml of either pesticide in the presence of red blood cells (Soloneski *et al*., 2001).

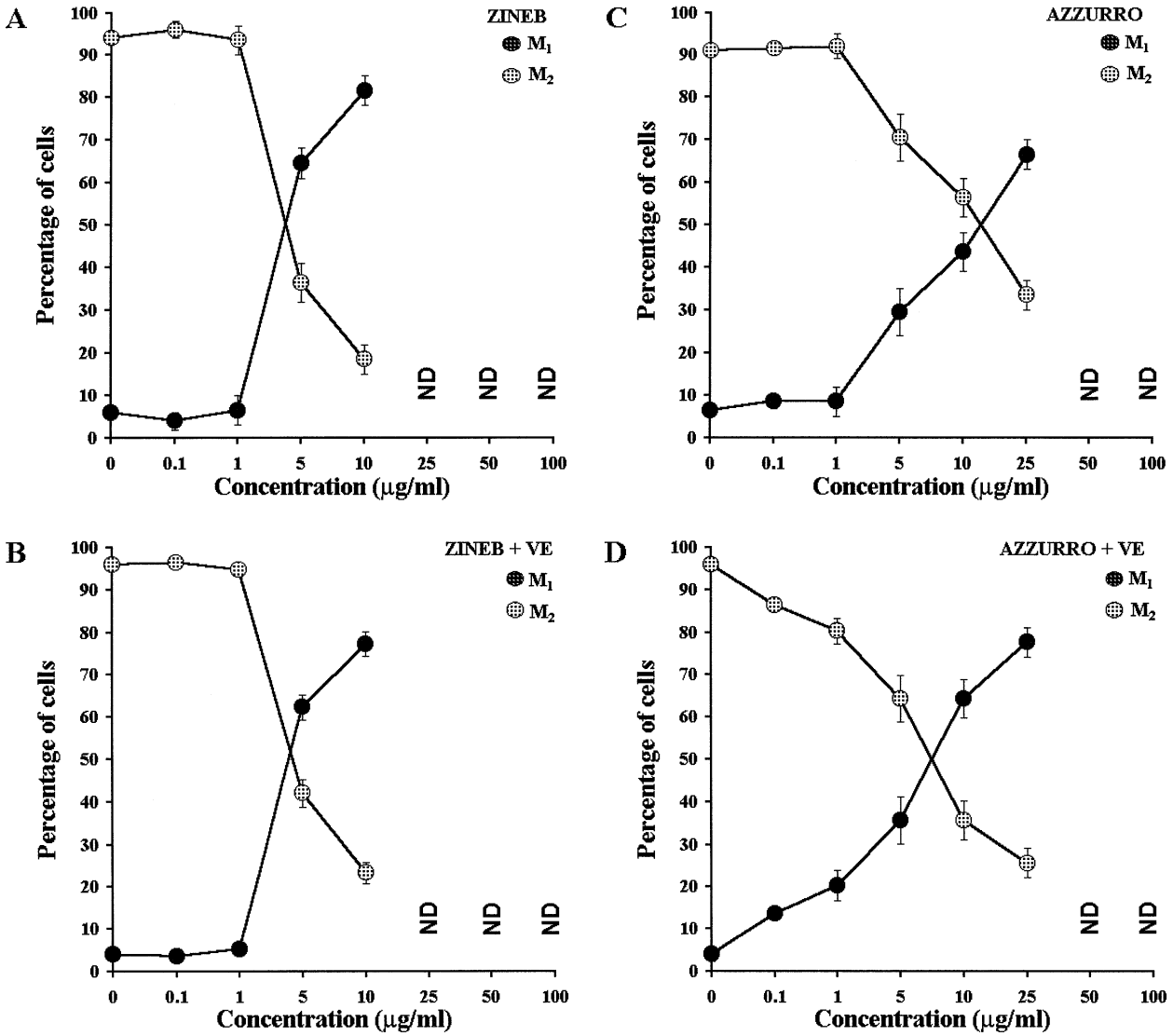


**Fig. 2.** Effects of vitamin E (**B** and **D**) on zineb (**A**) and its commercial formulation azzurro (**C**) cytotoxicity measured as cell cycle progression of CHO cells. Cultures were set up with culture medium supplemented with vitamin E, treated with pesticides 24 h later and harvested 48 h after seeding. For both fungicides, pooled data from three independent experiments are reported as mean frequencies ± SE (*y*-axis) and plotted against the fungicide concentration (0–100.0 μg/ml concentration-range, *x*-axis). ND, not determined since no cells were present in cultures.

Accordingly, we suggested a detoxification role for the erythrocytes present in whole blood cultures (Soloneski *et al*., 2001, 2002a), being most probably related to the antioxidant enzymes present in red blood cells but absent from erythrocyte-free human leukocyte cultures as well as CHO cell cultures. In agreement with this suggestion are the observations of Perocco *et al*. (1995), who found a drastic reduction in clonal efficiency of zineb-treated BALB/c 3T3 cells in the absence of an exogenous metabolizing system, S9 mix. Thus, they suggested that the endogenous metabolic system of BALB/c 3T3 cells can lead to the formation of a very toxic product(s) which abolishes the transforming properties of the mouse system (Perocco *et al*., 1995). These findings could indicate an indirect mode of action of this dithiocarbamate pesticide, most probably inducing DNA damage via free radical reactions and production of active oxygen species, as previously suggested (Soloneski *et al*., 2001, 2002a,b). Moreover, reports from the early 1980s indicate that tumor promoters like 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can induce chromosomal aberrations and DNA damage by an indirect action, i.e. via free radical reactions and production of active oxygen species (Emerit and Cerutti, 1981, 1982). Although TPA is not mutagenic (Thompson *et al*., 1980) or carcinogenic in any *in vivo* or *in vitro* system (Diamond *et al*., 1980), it was found to be capable of enhancing the *in vitro* transformation initiated by relatively low concentrations of chemical carcinogens (Popescu *et al*., 1980) or by low concentrations of UV- and X-irradiation (DiPaolo *et al*., 1981). The genotoxic profile of zineb that emerges from our results together with the scarce literature available on its effects (Pilinskaya, 1974; IARC, 1976; Soloneski *et al*., 2001, 2002b) is reminiscent of the effects assigned to TPA. In brief, zineb was not mutagenic in bacterial systems but exerted cytotoxicity in *in vitro* cultured rodent cells (IARC, 1976). It induced chromosomal aberrations both *in vivo* and *in vitro* and should be considered a weak inducer of SCEs (Pilinskaya, 1974; IARC, 1976; Soloneski

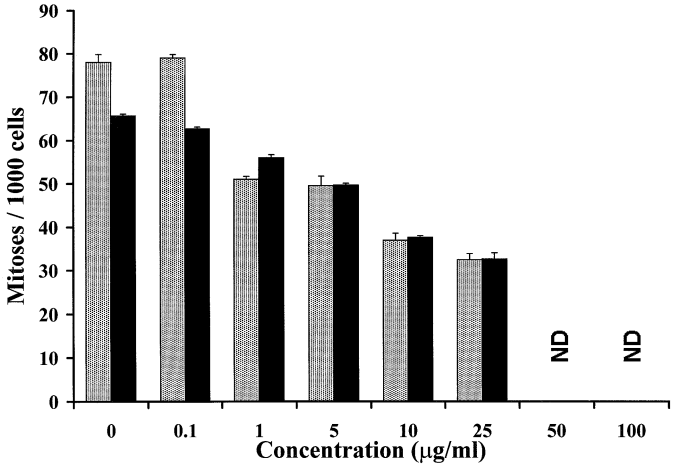


**Fig. 3.** Effect of vitamin E on zineb and its commercial formulation azzurro mitotic indices in CHO cells. Cultures were set up with culture medium supplemented with vitamin E, treated with pesticides 24 h later and harvested 48 h after seeding. The proportion of mitoses were determined in 2000 cells for each experimental point and expressed as number of mitoses/1000 cells. For each fungicide, pooled data from three independent experiments are reported as mean values ± SE, and the mitotic indices (*y*-axis) are plotted against the fungicide concentration (0–100.0 μg/ml concentration-range, *x*-axis). ND, not determined since no cells were present in cultures.

*et al*., 2001, 2002b). However, in order to match the ways in which TPA and zineb operate it should be demonstrated that the pesticide is able to induce aneugenic processes (Soloneski *et al*., 2003).

Active oxygen species arise during mono- or bivalent reduction of molecular oxygen. Although their steady-state concentrations in the cell are normally kept low by antioxidant enzymes and low molecular weight antioxidants, the extent to which these species might be involved in numerous degenerative processes has been investigated (Fridovich, 1975). It is well documented that when mammalian cells are exposed to active oxygen species, lesions appear in the DNA (Meneghini and Hoffmann, 1980; Birboin, 1982). So far, chromosomal aberrations (Emerit *et al*., 1982; Phillips *et al*., 1984; Estervig and Wang, 1984; Nicotera *et al*., 1985), SCEs (Emerit *et al*., 1982; Speit and Vogel, 1982; Estervig and Wang, 1984; Nicotera *et al*., 1985; Larramendy *et al*., 1987, 1989; Weitberg *et al*., 1987) and delays in cell cycle progression (Larramendy *et al*., 1987, 1989) have been determined as a consequence of the damage inflicted on DNA by active oxygen species.

Vitamin E is a hydrophobic antioxidant whose principal function is to protect the lipid components of an organism from uncontrolled and/or spontaneous autoxidation (Witting, 1980; Bieri *et al*., 1983). Our results show that vitamin E is protective against SCE formation but not against a delay in cell cycle progression and diminished mitotic activity induced by the fungicide zineb. The protection afforded against the induction of SCEs can be explained by scavenging of free radicals before they cause damage to cellular macromolecules. Because vitamin E protected the CHO target cells against fatty acid oxidation, the possibility that a membrane-derived secondary product(s) behaves as an intermediate agent responsible for the cellular damage can be suggested. In good agreement with this observation, only chronic (Sarma and Kesavan, 1993; Umegaki and Ichikawa, 1995) and not acute (Morales-Ramírez *et al*., 1998) vitamin E administration was found to protect against chromosomal damage induced by different clastogens when they inflict DNA lesions via production of free radical species.

In conclusion, the finding that vitamin E reduces genetic damage in CHO cells suggests that the ethylene bis(dithiocarbamate) pesticide zineb is a possible promoter of active oxygen species.

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