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Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro IV. DNA damage and repair kinetics assessed by single cell gel electrophoresis (SCGE) assay on Chinese hamster ovary (CHO) cells

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Abstract

Single cell gel electrophoresis (SCGE) was used to analyse dithiocarbamate zineb- and the zineb-containing technical formulation azzurro-induced DNA damage and repair in CHO cells. Cells were treated with zineb (50.0 μg/ml) or azzurro (100.0 μg/ml) for 80 min, washed and reincubated in pesticide-free medium for 0–12 h until SCGE. Viability of treated cells (0 h) did not differ from control remaining unchanged up to 6 h of incubation. After 12 h, viability decreased up to 70 and 54% in zineb- and azzurro-treated cultures, respectively. SCGE revealed at 0 h the absence of undamaged cells and an increase of slightly damaged and damaged cells in zineb-treated cultures or by an increase in damaged cells in azzurro-treated cultures. For both chemicals, a time-dependent repair of pesticide-induced DNA damage within a 0–12 h post-treatment incubation period was observed. Overall, damaged cells decreased as a function of the repair time for both pesticides while the slightly damaged cells decreased as a function of the repair time of zineb-induced DNA damage. Concomitantly, a time-dependent increase of undamaged cells was observed within the 0.5–12 h repair time for both pesticides. At 12 h after treatment, no differences in the frequencies of undamaged, slightly damaged and damaged cells were found between both zineb- or azzurro-treated cultures and control values as well as between zineb- and azzurro-treated cells. Immediately after exposure, nuclear DNA from zineb and azzurro-treated cells were larger and wider than nuclear DNA from untreated cells. When damaged cells were allowed to repair, a time-dependent decrease of the amount of free DNA migrating fragments was observed committed only to damaged cells but not in slightly or undamaged cells. On the other hand, no time-dependent alteration on nuclear DNA width within the 0–12 h repair period was observed.

Keywords: Comet assay; Chinese hamster ovary (CHO) cells; Zineb; Azzurro; Dithiocarbamates; Repair kinetics

1. Introduction

Among ethylene bis(dithiocarbamate) pesticides, the ethylene bis(dithiocarbamate)zinc (zineb) is a widely used foliar fungicide and is registered for

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use on a large number of fruits, vegetable and field crops, as well as on a large number of ornamental plants and for the treatment of several seeds. Zineb is also registered for use as fungicide in paints and for mould control on fabrics, leather, linen, painted surfaces, surfaces to be painted and paper, plastic and wood surfaces [1]. Though carcinogenic and teratogenic properties were reported for ethylenethiourea, the main metabolic and degradation product of the ethylene bis(dithiocarbamate) pesticides is well documented [2], very little is known about the deleterious effect of zineb [1,2].

Available reported data of the deleterious effect of the zineb is scarce and even contradictory. It has been demonstrated that zineb can be considered as a non-mutagenic agent when using plate incorporation assay with *Salmonella typhymurium* whereas gene conversion and point mutation assays with *Saccharomyces cerevisae* and *Bacillus subtillis* gave positive results [3–5]. Zineb exerted a high dose-related cytotoxicity in BALB/c 3T3 mouse cells in vitro but only in the absence of an exogenous metabolising system [6]. Trigathy et al. [7] reported zineb as positive genotoxic agent to somatic and germ cells in *Drosophila*. While Chernov and Khitsenko [8] observed an increased incidence of lung tumours after its oral administration to C57BL mice, negative results have been also reported in other mouse strains [8,9] and in rats [10,11]. Moreover, after its subcutaneous administration, systematic reticulum-cell carcinoma and a variety of sarcomas were observed in mice [12] and rats [10], respectively. In humans, development of sulphaemoglobinaemia, haemolytic anaemia and Heinz body formation has been reported in one person suffering hypocatalasemia after contact with zineb [13]. Even though zineb has been considered a non-mutagenic agent in bacterial systems by the International Agency for Research on Cancer [1], this does not necessarily imply that the dithiocarbamate cannot directly damage genetic material. In favour with this assumption, are reports of the induction of point mutations [3–5], chromosomal aberrations in lymphocytes from crop workers occupationally exposed to zineb [14], and our recent report of its ability to induce chromosomal aberrations, in human lymphocytes in vitro [15]. 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 is limited to B $CD20^+$ and T suppressor/cytotoxic $CD8^+$ cells among the different lymphocyte subsets [16].

In a previous study, Soloneski et al. [17] reported that both zineb and azzurro induced a significant increase in SCEs and a delay in cell-cycle progression of Chinese hamster ovary (CHO) cells. Furthermore, we analysed the capacity of the single cell gel electrophoresis (SCGE) methodology in detecting lesions introduced in the DNA of CHO cells exposed to a pulse treatment of either zineb and the zineb-containing technical formulation azzurro within a 1.0–100.0 μg/ml dose range. However, no attempt to analyse the repair kinetics of the induced lesions have been assessed [17]. In the present study we analysed whether CHO cells are able to repair the zineb-induced DNA strand breaks and to provide further knowledge of the real risk of the genetic damage of the dithiocarbamate pesticide.

2. Materials and methods

2.1. Chemicals

Ethylene bis(dithiocarbamate)zinc (zineb; CAS no. 12122-67-7) was obtained from Riedel-de Haën (Pestanal®, Hanover, Germany). Azzurro (zineb 70%) was kindly provided by Chemiplant (Buenos Aires, Argentina). Dimethyl sulphoxide (DMSO) was purchased from Sigma.

2.2. Cell cultures and pesticide treatment

CHO cells were grown in Ham's F10 medium (Gibco, Grand Island, NY) supplemented with 10% foetal calf serum (Gibco), 100 units/ml of penicillin (Gibco) and 10 μg/ml of streptomycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Prior to drug treatment, exponentially growing CHO cells were detached with a rubber-policeman, collected by centrifugation and resuspended in complete culture medium and then counted. Afterwards, aliquots containing 3.5×10^5 cells/ml were incubated during 80 min at 37 °C in a 5% CO_2 atmosphere in culture medium containing the test compounds. Both zineb and azzurro were dissolved in DMSO prior to use and then were diluted in culture medium such the addition of

100 μl to cultures allowed them to reach the required concentration. Zineb and azzurro were used at the final concentration of 50.0 and 100.0 μg/ml, respectively. The final solvent concentration was lower than 1% for all the treatments. Negative control (untreated cell and solvent-vehicle-treated cells) were performed and run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium even the highest concentration of zineb and azzurro. Thereafter, the cells were washed twice with pesticide-free complete culture medium, collected by centrifugation, resuspended in 1.0 ml of pesticide-free complete culture medium. The SCGE and cell viability assays were performed immediately after the 80 min treatment or after a post-treatment incubation period of 0–12 h at 37 °C in a 5% CO_2 atmosphere with harvesting times at 0, 0.5, 1, 2, 3, 6 and 12 h. Cultures were duplicated for each experimental point, during at least three independent experiments. The same batches of culture medium, sera and reagents were used throughout the study.

2.3. Cell viability

Cell viability was determined using the ethidium bromide/acridine orange assay described elsewhere [18]. Briefly, one aliquot of 5 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml, Sigma) and acridine orange (100 μg/ml, Sigma) was mixture with 50 μl of the cell suspension. Afterwards, cells were analysed using an Olympus BX50 fluorescence photomicroscope equipped with appropriated filter combination. Viable cells appear green-fluorescent whereas orange-stained nuclei indicate dead cells. At least, 500 cells were counted per experimental point, and results expressed as percentage of viable cells among all cells. Cell viability was expressed as proportion of living cells.

2.4. Single cell gel electrophoresis (SCGE) assay

The remaining cell culture (950 μl) was used for microgel electrophoresis. The comet assay was performed following the alkaline procedure described by Singh [19] with minor modifications. Slides were cleaned with 100% ethanol and air-dried. Two solutions containing 0.5% normal melting agarose (NMA), and 0.5% low melting agarose (LMA) solution in Ca^{2+}–Mg^{2+}-free PBS were performed. Briefly, 75 μl of 0.5% NMA was transferred onto a pre-cleaned slide, spread evenly, and placed at 37 °C to solidify the agarose. Afterwards, 95 μl of 0.5% LMA together with 7×10^3 cells (20 μl cell suspension + 75 μl of 0.5% LMA) was applied, covered with a coverslip, and placed at 4 °C for 15 min. After this layer had solidified, a third layer of 75 μl of 0.5% LMA was added, and slides placed at 4 °C for 15 min. Immediately after, slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, 10% DMSO) and then lysed in the dark at 4 °C for an overnight period. After the overnight period, slides were placed in a horizontal electrophoresis device filled with freshly prepared electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 20 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer at 4 °C for 20 min at 25 V and 250 mA. Afterwards, slides were neutralised with a solution comprising 0.4 M Tris–HCl (pH 7.5) and stained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were coded and scored blind by one cytogeneticist. Analysis of the slides was performed in a Olympus BX50 fluorescence photomicroscope equipped with appropriated filter combination with a X63 fluorescence objective. Cellular images were acquired with the Leica IM50 Image Manager (Imagic Bildverarbeitung AG), based on an integrated high-sensitivity monochrome charge-coupled device (CCD) camera and automated capture image software. The cellular nucleus diameter and the comet length, determined as the diameter of the nucleus plus migrated DNA, were measured by CarioFISH 1.2 software. Width of the nucleus and comet length (expressed in μm) were determined from 50 randomly captured cells per experimental point of each experiment. Two parallel slides were performed for each experimental point. Cells were visually graded into four categories as previously suggested by us [17]. Depending on DNA damage level the categories were as follows: undamaged (no tail comet, diameter $\leq$30 μm), slightly damaged (diameter 31–45 μm), damaged (tail comet length

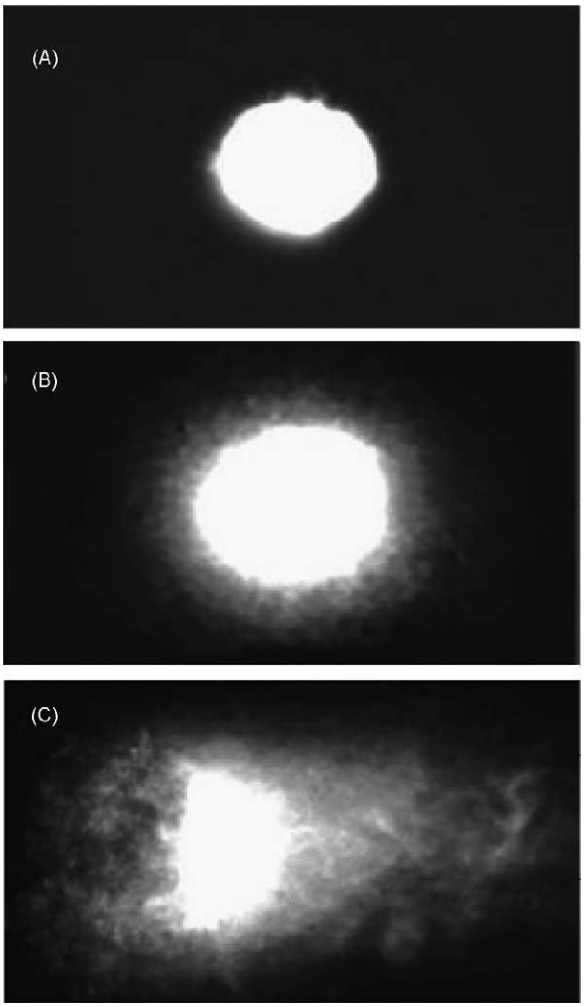

Fig. 1. Microphotographs of CHO cells treated with zineb showing various degrees of DNA damage: (A) undamaged cell (diameter ≤30 μm); (B) slightly damaged cell (no tail comet, diameter 31–45 μm); (C) damaged cell (tail comet length >45 μm).

>45 μm) and highly damaged (dying or dead cells; Fig. 1).

2.5. Statistical analysis

The non-parametric Kruskal–Wallis test was used to compare the global effect of a pesticide over control cells while individual comparisons between pairs of data were performed using the Mann–Whitney test employing the SPSS 9.0 software. The level of significance chosen was 0.05 unless indicate otherwise.

3. Results

Since no differences of cell viability were observed between negative and positive controls (untreated and DMSO-treated cells, respectively), pooled data are presented for control cultures. Cell viability after 80 min treatment with 50.0 μg/ml of zineb or 100.0 μg/ml of azzurro showed no significant changes until 12 h of incubation when a significant decrease reaching values of 70 and 54% in zineb- and azzurro-treated cultures, respectively, was observed ($P < 0.05$; Fig. 2). Statistical analysis revealed that at this harvesting time, cell viability was lower in azzurro-treated cultures than in those cells treated with zineb ($P < 0.05$; Fig. 2).

Equivalent frequencies of undamaged/damaged cells revealed by SCGE assay from both negative and positive controls (untreated and DMSO-treated cells) were observed, then pooled data are presented

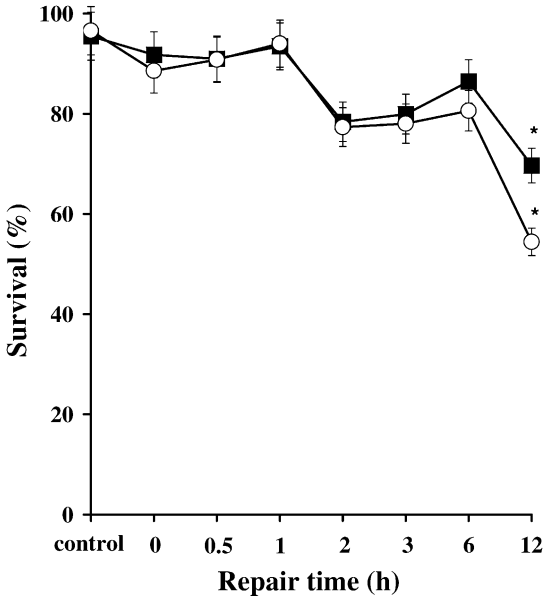

Fig. 2. Effect of in vitro treatment with zineb (50.0 μg/ml; black squares) and the zineb-containing technical formulation azzurro (100.0 μg/ml; empty circles) on Chinese hamster ovary (CHO) cells viability. Cell viability was determined using the ethidium bromide/acridine orange assay and expressed as the proportion of living cells at different harvesting times after a 80 min pulse treatment. For each harvesting time, pooled data from three independent experiments are reported as mean values ± S.E. (*y*-axis) and plotted against incubation time (*x*-axis). $^{*}P < 0.05$.

for control cultures. Moreover, a clear increase in DNA damage after fungicide 80 min pulse treatment (0 h) was revealed by the total absence of undamaged cells and by either an increase over control values of the proportion of slightly damaged ($P < 0.01$) and damaged cells ($P < 0.01$) in zineb-treated cultures (Figs. 1 and 3A) or by an increase in the frequency of damaged cells ($P < 0.01$) in azzurro-treated cultures (Fig. 3B). No highly damaged cells were observed either in zineb- (Fig. 3A) or azzurro-treated cells

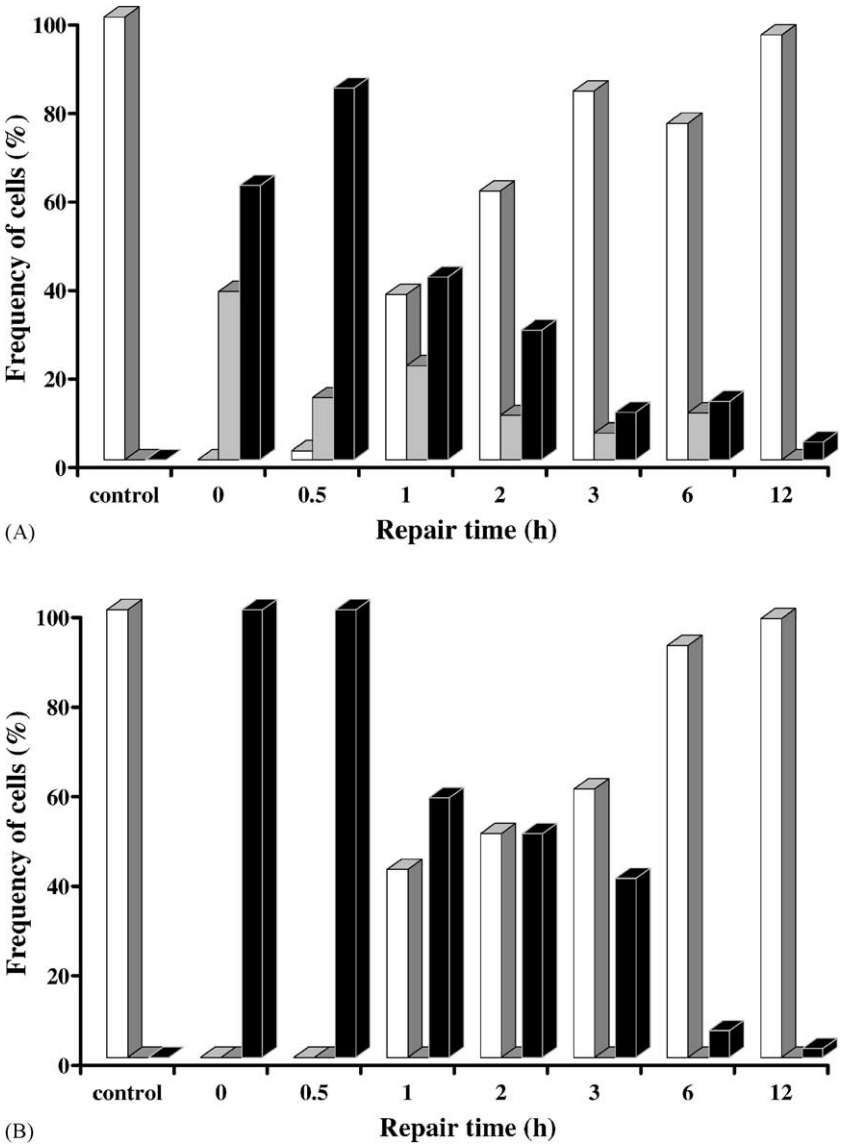

Fig. 3. Time course of the repair of in vitro DNA damage induced in Chinese hamster ovary (CHO) cells by a 80 min pulse treatment (0 h) treatment with zineb (50.0 μg/ml; A) and the zineb-containing technical formulation azzurro (100.0 μg/ml; B) detected by SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells were stained with DAPI. For each harvesting time, pooled data from three independent experiments are reported, a total 150 randomly selected cells were visualised for DNA damage. Cells classified as undamaged (empty bars), slightly damaged (grey bars) and damaged (black bars) and their frequencies plotted against harvesting times.

(Fig. 3B). For both chemicals, a time-dependent repair of zineb- and azzurro-induced DNA damage was observed by a progressive decrease of slightly damaged and damaged cells simultaneously with an increase in the frequency of undamaged cells within the 12 h repair period. A regression test showed that the number of damaged cells decreased as a function of the repair time of both zineb- ($r = -0.73$, $P < 0.01$; Fig. 3A) or azzurro-induced DNA damage ($r = -0.86$, $P < 0.01$; Fig. 3B) as well as the number of slightly damaged cells of zineb-induced DNA damage ($r = -0.69$, $P < 0.01$; Fig. 3A). Concomitantly, a time-dependent increase in the number of undamaged cells was observed within the 0.5–12 h repair time either in zineb- ($r = 0.78$, $P < 0.01$) or in azzurro-treated cultures ($r = 0.85$, $P < 0.01$; Fig. 3). At 12 h after treatment, no differences in the frequencies of undamaged, slightly damaged and damaged cells were found between both zineb- or azzurro-treated cultures and control values ($P > 0.05$) as well as between zineb- and azzurro-treated cells ($P > 0.05$; Fig. 3).

Mann–Whitney test demonstrates that both the length and width of nuclear DNA from zineb-treated

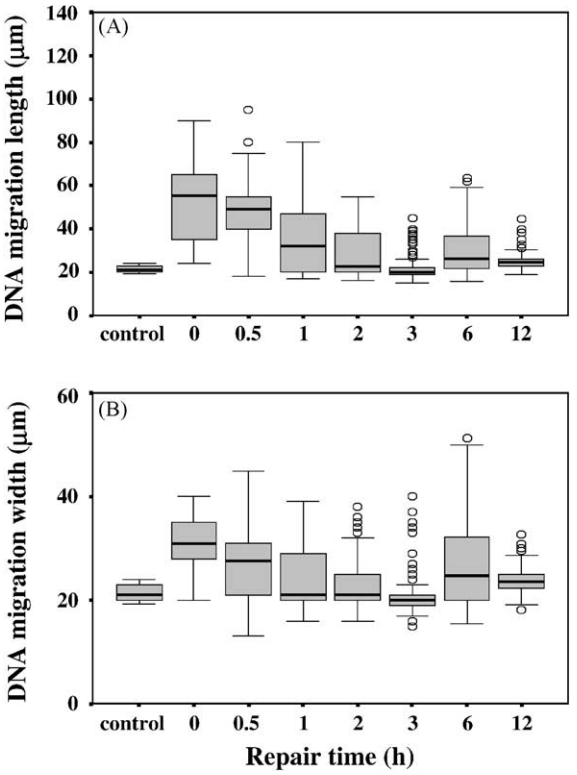

Fig. 4. Zineb-induced DNA damage and repair in CHO cells measured by SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells were stained with DAPI. For each harvesting time, pooled DNA damage relative to 150 zineb-treated individual cells from three independent experiments are reported. The nuclear DNA length (A) and the nuclear DNA width (B) expressed in μm are plotted against the different repair times. The data are displayed as box plots, where the *y*-axis shows the range data. Each box encloses 50% of the data, with the median value of the variable displayed as a line. The top and the bottom of the box mark the limits ±25% of the variable population. The lines extending from the top and the bottom of each box mark the minimum and maximum values that fall within an acceptable range. Any value outside this range is displayed as an individual point (empty circles).

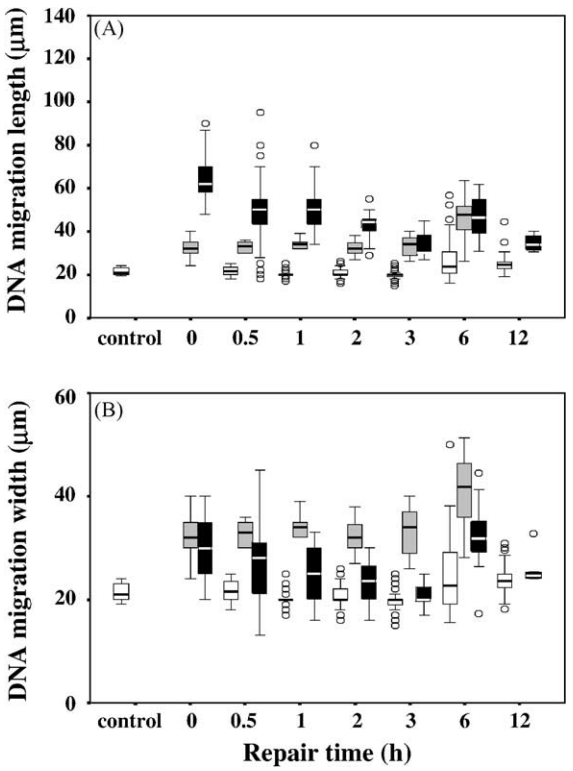

Fig. 5. Zineb-induced DNA damage and repair in CHO cells measured by SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells were stained with DAPI. For each harvesting time, pooled DNA damage relative to 150 zineb-treated individual cells from three independent experiments are reported. The nuclear DNA length (A) and the nuclear DNA width (B) expressed in μm for undamaged (empty bars), slightly damaged (grey bars) and damaged cells (black bars) are plotted against are plotted against the different repair times. The data are displayed as box plots (for details see the caption of Fig. 4).

cells immediately after exposure (0 h) are higher and wider than untreated cells, respectively ($P < 0.01$; Fig. 4). When damage cells were allowed to repair, a time-dependent decrease of the amount of free DNA fragments that could migrate (i.e. a strong reduction in the average of nuclear DNA length) was observed ($r = -0.60$, $P < 0.05$; Fig. 4A). Statistical analysis reveals that such strong reduction of nuclear DNA length was committed to damaged cells ($P < 0.01$) among the treated cell population (Fig. 5A). On the other hand, no time-dependent alteration on nuclear DNA width within the 0–12 h repair period was observed ($r = -0.2$, $P > 0.05$; Fig. 4B), though an increase in nuclear DNA width in regard to control values was observed either for damaged cells during the 12 h repair time ($P < 0.01$) or for slightly damaged cells found at 6 h ($P < 0.01$; Fig. 5B). Overall, the zineb-damaged cell population did not recover completely the features of the control cells even after 12 h repair being the nuclear DNA from treated cells longer (Figs. 4A and 5A) and wider (Figs. 4B and 5B) than control cells, respectively ($P < 0.01$).

The length and width of nuclear DNA from azzurro-treated cells are larger and wider than untreated cells immediately after exposure (0 h, $P < 0.01$; Fig. 6). During the 0–12 h repair time, a time-dependent decrease in the average of nuclear

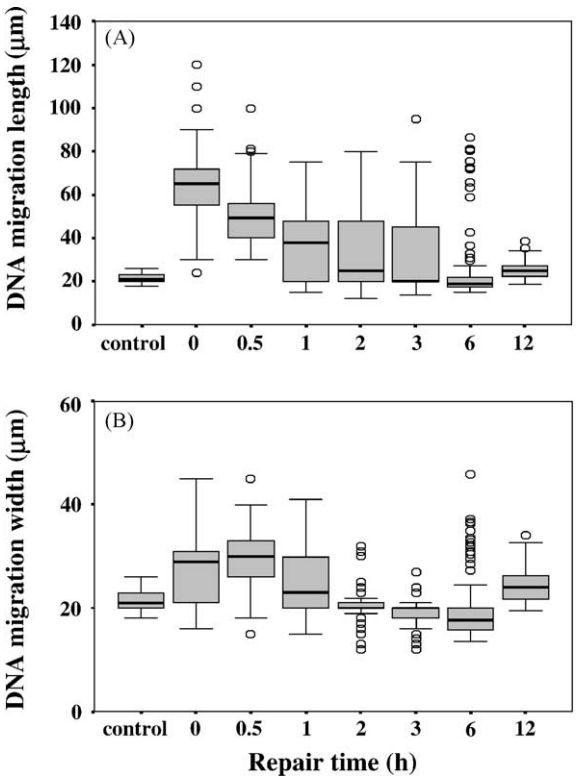

Fig. 6. Azzurro-induced DNA damage and repair in CHO cells measured by SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells were stained with DAPI. For each harvesting time, pooled DNA damage relative to 150 azzurro-treated individual cells from three independent experiments are reported. The nuclear DNA length (A) and the nuclear DNA width (B) expressed in μm are plotted against the different repair times. The data are displayed as box plots. The data are displayed as box plots (for details see the caption of Fig. 4).

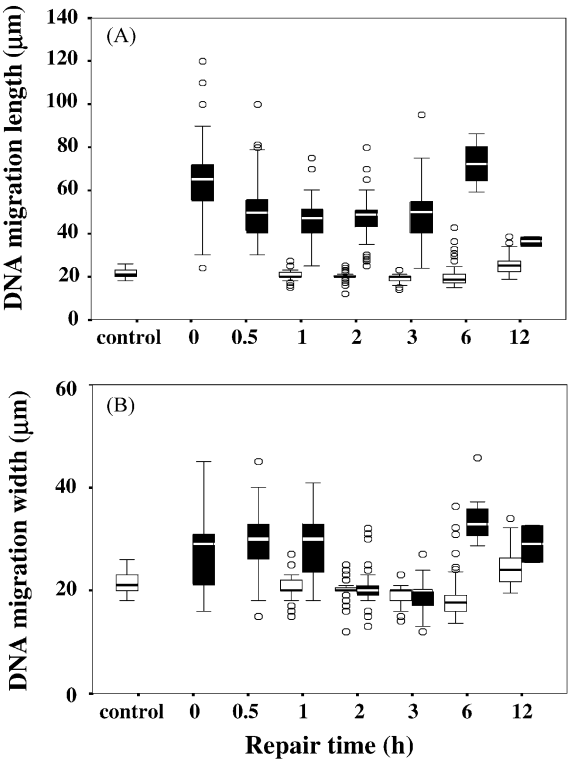

Fig. 7. Azzurro-induced DNA damage and repair in CHO cells measured by SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells were stained with DAPI. For each harvesting time, pooled DNA damage relative to 150 azzurro-treated individual cells from three independent experiments are reported. The nuclear DNA length (A) and the nuclear DNA width (B) expressed in μm for undamaged (empty bars) and damaged cells (black bars) are plotted against are plotted against the different repair times. The data are displayed as box plots (for details see the caption of Fig. 4).

DNA length was achieved ($r = -0.68$, $P < 0.01$; Fig. 6A). On the other hand, no time-dependent alteration on nuclear DNA width within the 0–12 h repair time was observed ($r = -0.31$, $P > 0.05$; Fig. 6B), though an increase in its width from damaged cells was observed in regard to control values during the whole repair time period ($P < 0.01$) but not in the undamaged cells (Fig. 7B). Overall, the azzurro-damaged cell population did not completely achieve the nuclear DNA length (Figs. 6A and 7A) and the width of the control cells (Figs. 6B and 7B) even after a 12 h post-treatment repair time appearing the DNA migration longer and wider than the untreated cell population ($P < 0.01$).

4. Discussion

We employed the alkaline SCGE assay to analyse the repair kinetics of lesions introduced in the DNA of CHO cells after an 80 min pulse treatment of 50.0 μg/ml zineb and 100.0 μg/ml azzurro within the first 12 h after exposure. We have recently reported equivalent levels of DNA damage in CHO cells induced by these different pesticide doses [17]. The present results are in total accord with our previous observations [17]. Accordingly, we could assume that the effect induced by azzurro can be accounted for the zineb component of the mixture alone, and suggest that a non-clastogenic agent(s) other than zineb might be present in the technical formulation containing 70% zineb. However, the identity of the components of the commercial product was not made available to us.

The reports concerning the use of the SCGE assay for assessing damage and/or repair of pesticides are scarce, specially among carbamates. Quantification of DNA single-strand breaks induction has been reported in mouse splenocytes and peripheral lymphocytes after oral administration of thiram as well as in human lymphocytes in vitro [20]. Garaj-Vrhovac and Zeljezic [21,22] have evaluated the risk assessment of workers occupationally exposed to a mixture of different agrochemicals. They found that after pesticide exposure, increased DNA damage in circulating lymphocytes, namely tail length and tail moment were observed which decreased significantly compared with the first sampling point after workers spend 6–8 months out of the pesticide exposure zone [21,22]. Blasiak et al. [23] analysed the DNA damaging effect of malathion and both its major metabolites malaoxon and isomalathion in human lymphocytes in vitro. They observed that while malathion did not cause any significant changes in the comet length of the lymphocytes, its metabolites introduced damage to DNA in a dose-dependent manner, and that the effect of malaoxon was more pronounced than that caused by isomalathion [23]. Saleha et al. [24] studied damage and repair of DNA strand breaks induced by monocrotophos in the nucleated erythrocytes of *Tilapia mossambica* in vivo. Their results revealed that the DNA damage induced within a 0.313–4.375 ppm dose range of monocrotophos returned to control levels after a 96 h repair period [24].

Our SCGE results demonstrated that zineb and azzurro induced a significant increase not only in the number of CHO damaged cells but also in the comet tail length. In the genotoxicity, measured by the SCGE, the effects on cell viability could be ruled out since cell viability after 80 min treatment with both test compounds showed non-significant changes until 12 h of repair time when it significantly decreased reaching values of 70 and 54% in zineb- and azzurro-treated cultures. It should be pointed out that at the concentrations employed, no evidence of a relationship between cytotoxic effect and comet length was observed, though equivalent doses of 50.0 μg/ml of zineb and 100.0 μg/ml of azzurro, respectively, induced a complete inhibition of cellular growth of CHO cells after a continuous 36 h treatment [17].

The exact mechanism(s) of zineb-induced DNA damage is not known. It is well documented that the repair kinetic profile of the single-strand breaks induced by active oxygen species when analysed by SCGE is totally different from the repair profile of those zineb-induced lesions. In fact, the in vitro repair of the active oxygen species in mammalian cells is very rapid, being the majority of the breaks resealed within 10 min [25,26]. Accordingly, the possibility that zineb-introduced lesions into the DNA through the formation of active oxygen species, previously suggested by us when using other cytogenetic end-points [15–17], could be ruled out since the present results clearly show the necessity of a 12 h repair period for a complete resealing of the lesions analysed with the SCGE under our experimental conditions. Another possible explanation could be

alkylation of the DNA molecule by this ethylene bis(dithiocarbamate) pesticide. It has been previously demonstrated that single-strand breaks introduced in the DNA molecule of CHO cells after treatments with different ethylating agents, e.g. *N*-ethyl-*N*-nitrosurea and *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine, are long lasting, being repaired in a period of up to 24 h after pulse treatment [26].

Interestingly, our results demonstrated that when damaged cells were allowed to completely repair zineb- and azzurro-induced DNA damage, the repaired cell population did not completely recover the morphological features of the control cells, i.e. increased nuclear diameter, even when no free DNA migrating fragments were observed. The same phenomenon has been observed by Fortini et al. [26] in mammalian cells after a complete repair of either X-ray and alkylation-induced DNA damage. Accordingly, these authors suggested that such morphological differences could be committed to differences in the packaging of DNA in those cells undergoing repair when compared to untreated cells [26].

To date, only 14 fungicides have been evaluated for their carcinogenicity to humans by the International Agency for Research in Cancer [1,27–29]. Among those analysed, zineb has been overall evaluated and ranked into category 3 (i.e. insufficient available data to evaluate carcinogenicity to humans) since the degree of evidence for its carcinogenicity properties have been determined as not adequate data and/or inadequate evidence in humans and other animal systems, respectively [30]. We know that either an in vitro pulse treatment of zineb as well as one of its currently formulation, azzurro, produce DNA lesions that can be repaired within a 12 h period suggesting that the risk of genetic damage is, then, reduced, at least for some mammalian cells in culture. On the other hand, a completely different behaviour of zineb-induced lesions is found after a continuous cellular exposure during longer time periods. The presence of chromosomal aberrations in lymphocytes from crop workers occupationally exposed to zineb found by Pilinskaya [14] and our previous studies demonstrating the ability of a continuous in vitro treatment in inducing chromosomal aberrations, sister chromatid exchanges and a delay in cell-cycle kinetics in human lymphocytes [15] could indicate the inability of this cell type to repair, at least not totally, the lesions during the G_0 period. Furthermore, they could also suggest the persistence of such lesions in the genetic material, potentially comprising DNA breakage at sites of oncogenes or tumour suppressor genes which may play a crucial role in the induction of malignancies.

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