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ZINC DIFFERENTIALLY MODULATES DNA DAMAGE INDUCED BY ANTHRACYCLINES IN NORMAL AND CANCER CELLS

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Zinc is one of the most essential trace elements in human organism. Low blood level of zinc is often noted in acute lymphocytic leukemia (ALL). Treatment with zinc adjuvant is hypothesized to accelerate recovery from ALL, and in conjunction with chemotherapy, cure ALL. *Aim:* We determined the effect of zinc on DNA damage induced by doxorubicin and idarubicin, two anthracyclines used in ALL treatment. *Methods:* The experiment was performed on acute lymphoblastic leukemia cell line (CCRF-CEM) and lymphocytes from peripheral blood of healthy, adult subjects. To evaluate the level of DNA damage the comet assay in the alkaline version was used. *Results:* We observed a significant difference in DNA damage level between normal and cancer cells in the presence of zinc. Cancer cells exhibited a significant increase of DNA damage in the presence of zinc, while in lymphocytes no such effect was observed. *Conclusion:* Our results suggest that zinc may protect normal cells against DNA-damaging action of anthracyclins and increase this action in cancer cells.
***Key Words*: zinc, doxorubicin, idarubicin, DNA damage, acute lymphocytic leukemia.**

Zinc belongs to essential microelements in human organism. It plays a significant role in many physiological processes including neuronal function, wound healing, immunity or growth and development. At the cellular level zinc plays an important role in such fundamental processes as cell proliferation, DNA metabolism and repair, oxidative defense and cellular signaling pathways. It is a component of thousand of protein domains, including hundreds of zinc-containing enzymes and also many transcription factors [1, 2]. Zinc within the cell is under precise regulation and majority of it is bound to a number of proteins. The excess of free (labile) intracellular zinc may disturb essential molecular events, including protein folding [3, 4]. On the other hand, zinc deficiency may disturb cell oxidation status, deregulate mitochondrial functions, interfere with DNA repair and contribute to cancer induction and development [5, 6]. Zinc may also play an important role in healthy aging [7]. A decrease in zinc level in lymphocytes of elderly was observed to be with an increased level of oxidized proteins and a lower expression of anti-oxidative enzymes [8]. Restoration of zinc level reversed these unfavorable correlations.

The disturbance in zinc metabolism has been recognized in the pathogenesis of leukemias since 1949. Eby [9] described a 3-year-old girl, which obtained zinc at a rate of 3.18 mg/kg body weight/day, from the start of chemotherapy through 3 years of maintenance therapy. That treatment resulted in bone marrow remission from more than 95% to zero blast cell count in both hips within the first 14 days of treatment, which never relapsed. In addition to the reduction of blast cells, red blood cell production and other hemopoietic functions returned to normal at a clinically remarkable rate. There were no side effects of zinc or chemotherapy at any time, and zinc was hypothesized to have improved the patient's overall ability to withstand toxic effects of chemotherapy. Identical results occurred in 13 other children with ALL whose parents chose therapy with zinc adjuvant. This report identifies zinc treatment as being vital to rapid and permanent recovery from ALL [9]. Now no modern chemotherapy, with the exception of the Polish trial [10], includes zinc adjuvant even though zinc serum levels are usually low in leukemic children [9].

Doxorubicin and idarubicin are anthracyclines used as chemotherapeutic drugs in the effective treatment of a broad spectrum of leukemias and solid tumors [11, 12]. However, both doxorubicin and idarubicin have serious side effects, as brain damage, growth and development delay or cardiomyopathy [13]. Cardiotoxicity of anthracyclines is caused by a generation of ROS (reactive oxygen species), possibly with participation of iron ions or long-lived secondary alcohol metabolites [14, 15]. Free radicals and iron can damage cell membranes increasing its permeability and interact with macromolecules inside the cell. Cardiac cells are especially susceptible to free radical damage because of their high oxidative metabolism and relatively poor antioxidant defense. Moreover, anthracyclines have a very high affinity for cardiolipin, a phospholipid in the inner mitochondrial membrane resulting in anthracyclines accumulating inside cardiac cells [16]. Because of the side effects of anthracycline chemotherapy, many compounds are tested as potentially protective agents such as amifostine, dexrazoxane, selenium, vitamins E, A and C and memantine [16–18]. Doxorubicin and idarubicin exert their cytostatic effect by direct and indirect interaction with DNA [17, 19–21]. In the present work we determined the modulation of the DNA-damaging effect of these drugs by zinc in normal lymphocytes and CCRF-CEM cells.

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Abbreviations used: ALL – acute lymphocytic leukemia; CCRF-CEM – acute lymphocytic leukemia cell line; DAPI – 4',6-diamino-2-phenylindole; FSB – fetal bovine serum; NMP – normal melting point agarose; LMP – low melting point agarose; PBS – phosphate-buffered saline; ROS – reactive oxygen species; S.E.M. – the average error of the mean.

MATERIALS AND METHODS

Chemicals. RPMI 1640 medium, streptomycin and penicillin, normal melting point (NMP) and low melting point (LMP) agarose, phosphate-buffered saline (PBS), DAPI (4',6-diamino-2-phenylindole), hydrogen peroxide (H_2O_2) and histopaque 1077 were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Fetal bovine serum (FSB) and L-glutamine was obtained from GIBCO. Doxorubicin, idarubicin and zinc salts — $ZnCl_2$ and $ZnSO_4$ were from Sigma-Aldrich Co (St. Louis, MO, USA). All chemicals were of the highest commercial grade available.

Cell culture. The human ALL cell line (CCRF-CEM) was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in the presence of a 5% CO_2 atmosphere at 37ºC.

Isolation of lymphocytes. Lymphocytes were isolated from peripheral blood of male healthy donors at the age between 20 and 35 by centrifugation in a density gradient of histopaque 1077 (15 min, 280 × g, 4°C). Each experiment was performed on blood obtained from an individual donor. The pellet containing lymphocytes was washed twice in PBS and resuspended in RPMI 1640 medium to give about 1×10^5 cells per ml and further processed. The study was approved by Ethic Committee of Medical University of Lodz.

Cell viability. The viability of the cells, lymphocytes and CCRF-CEM cells, was determined by trypan blue exclusion assay. The cells were incubated for 1 h at 37°C with doxorubicin at concentration from the range 0.25–1 μM and with idarubicin at concentration from the range 0.01–1 μM, washed and suspended in RPMI 1640 medium. An equal volume of 0.4% trypan blue reagent was added to the cell suspension and the percentage of viable cells was evaluated under a field microscope. Assays were performed in triplicate with lymphocytes from three donors and in triplicate with CCRF-CEM cells.

Cell treatment. Doxorubicin and idarubicin were diluted in PBS and added to the cell suspension to give final concentration in the range 0.01–1 and 0.25–1 μM for idarubicin and doxorubicin, respectively. Zinc salts were diluted in PBS and added to the sell suspension to give a final concentration 10 μM. Each experiment contained three modes of exposure to anthracyclines: cells with anthracycline (doxorubicin or idarubicin) only, and anthracycline with zinc salt — $ZnSO_4$ and $ZnCl_2$, separately. Cells were incubated for 1 h at 37ºC. Each experiment included a positive control, which was hydrogen peroxide at 10 μM for 10 min on ice.

Comet assay. The comet assay in the alkaline version was performed according to the procedure of Singh et al. [22], with modification [23]. A freshly prepared suspension of the cells at the density $1–2 \times 10^6$ cells/ml in 0.75% LMP agarose dissolved in PBS was spread on microscope slides precoated previously with 0.5% NMP agarose. The cells were lysed for 1 h in 4°C in a solution of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10. After lysis, the slides were placed in the comet assay electrophoresis system. DNA was allowed to unwind for 20 min in the unwinding solution consisted of 300 mM NaOH and 1 mM EDTA, pH > 13. Electrophoresis was conducted in the electrophoresis buffer containing 30 mM NaOH and 1 mM EDTA, pH > 13, at the temperature not exceeding 12°C for 20 min under 0.75 V/cm and 28 mA. The slides were washed in the water, drained and stained with 2 μg/ml DAPI and covered with cover slips. All steps mentioned were conducted under dimmed light.

Slides were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) equipped with COHU 4910 digital video camera (Cohu, San Diego, CA, USA) supplied with a UV1 filter block, and connected to a personal computer equipped with an image analysis system Lucia — Comet v.4.51 (Laboratory Imaging, Praha, Czech Republic). Fifty randomly selected images from each experiment were measured. The DNA in comet tail was taken as an index of DNA damage. This parameter is positively correlated with the level of DNA breakage and/or alkali labile sites and negatively correlated with the level of DNA cross-links [24]. Each experiment was performed at least 3 times. In the case of lymphocytes, each experiment was performed at least 3 times with the blood from at least 3 different donors.

Data analysis. Values were expressed as mean ± S.E.M. from at least 3 separate experiments, i.e. data from 3 experiments were pooled and the statistical parameters were calculated. The data were analyzed using Statistica package (StatSoft, Tulsa, OK). If no significant differences between variations were found, as assessed by Snedecor-Fisher test, the differences between means were evaluated by applying the Student t test. Otherwise, the Cochran-Cox test was used.

RESULTS

We showed that the incubation of normal lymphocytes and leukemic cells with doxorubicin caused a significant ($p < 0.001$) induction of DNA damage (Fig. 1). After incubation with doxorubicin we observed comets with shorter tails and with smaller amount of tail DNA with increasing concentration of the drug. We suggest that this effect may be due to the induction of both DNA cross-links and its fragmentation by the drug. The level of DNA damage induced by doxorubicin was higher in lymphocytes than in cancer cells. However, in the cancer cells we observed a higher difference in the level of DNA damage between the cells incubated with the lowest and the highest concentration of doxorubicin. Therefore, the processes leading to DNA damage — DNA fragmentation and DNA cross-links are more effective in cancer cells and for this reason we suggest that they are more sensitive to doxorubicin than normal lymphocytes. Idarubicin also induced DNA damage

in both types of cells, but it was more pronounced in cancer cells ($p < 0.001$) (Fig. 2).

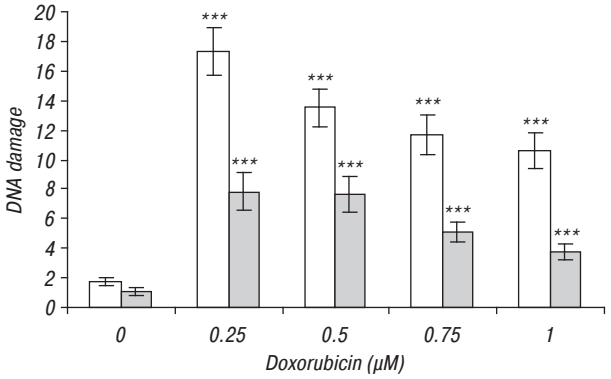

Fig. 1. DNA damage, measured as the comet tail DNA in the comet assay, in lymphocytes (white) and CCRF-CEM cells (grey) incubated for 1 h at 37°C with doxorubicin. The result of positive control (10 μM H_2O_2 at 4°C for 10 min) was as follows: for lymphocytes 15.03 ± 1.00 ($p < 0.001$) and for CCRF-CEM cells 10.55 ± 1.00 ($p < 0.001$)

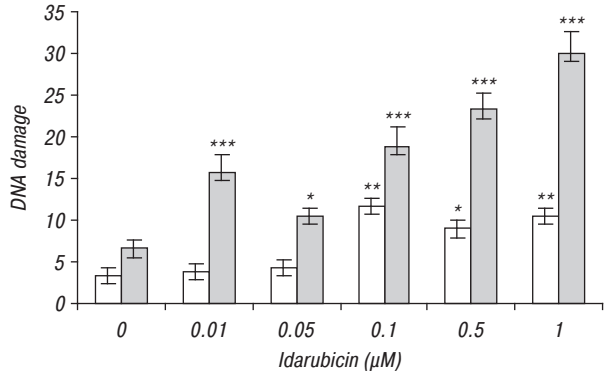

Fig. 2. DNA damage, measured as the comet tail DNA in the comet assay, of lymphocytes (white) and CCRF-CEM cells (grey) incubated for 1 h at 37°C with idarubicin. The result of positive control (10 μM H_2O_2 at 4°C for 10 min) was as follows: for lymphocytes 15.03 ± 1.00 ($p < 0.001$) and for CCRF-CEM cells 10.55 ± 1.00 ($p < 0.001$)

We showed a different effect of zinc on the genotoxicity of doxorubicin and idarubicin in normal and cancer cells. The incubation of lymphocytes with zinc and doxorubicin at 0.75 μM and 1 μM caused a significant decrease ($p < 0.05$) of DNA damage induced by the anthracycline (Fig. 3*a*). In the case of CCRF-CEM cells we observed a significant increase ($p < 0.001$) of DNA damage induced by doxorubicin at the same concentrations in the presence of zinc (Fig. 3*b*). The addition of zinc to lymphocytes during incubation with idarubicin caused a decrease of DNA damage ($p < 0.05$) induced by this antibiotic (Fig. 4*a*). On the contrary, the addition of zinc to CCRF-CEM cells evoked the significant increase of DNA damage ($p < 0.001$) (Fig. 4*b*).

The viability of the cells was checked concurrently in all experiments at tested concentrations of anthracyclines and zinc salts (data not shown). In the concentrations range of anthracyclines used in all experiments the viability of both types of cells was greater than 80%. We observed the greatest decrease in the cell viability in the case of CCRF-CEM cells incubated with idarubicin and zinc salts. The viability of CCRF-CEM cells was 60.0 ± 4.0% ($p < 0.001$) in cell samples incubated with idarubicin and $ZnCl_2$.

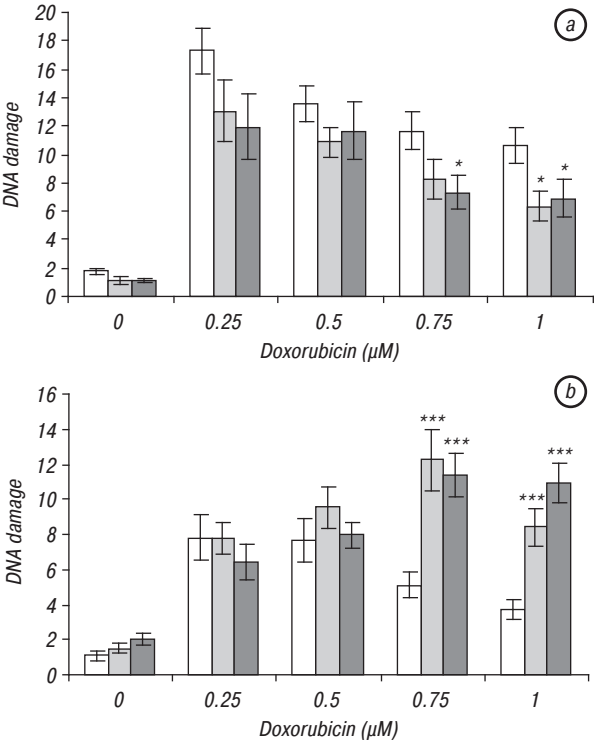

Fig. 3. DNA damage, measured as the comet tail DNA in the comet assay, of lymphocytes (*a*) and CCRF-CEM cells (*b*) incubated for 1 h at 37 °C with doxorubicin in the presence of $ZnCl_2$ (light grey) and $ZnSO_4$ (dark grey). The result of positive control (10 μM H_2O_2 at 4 °C for 10 min) was as follows: for lymphocytes 15.68 ± 1.00 ($p < 0.001$) and for CCRF-CEM 9.25 ± 1.00 ($p < 0.001$)

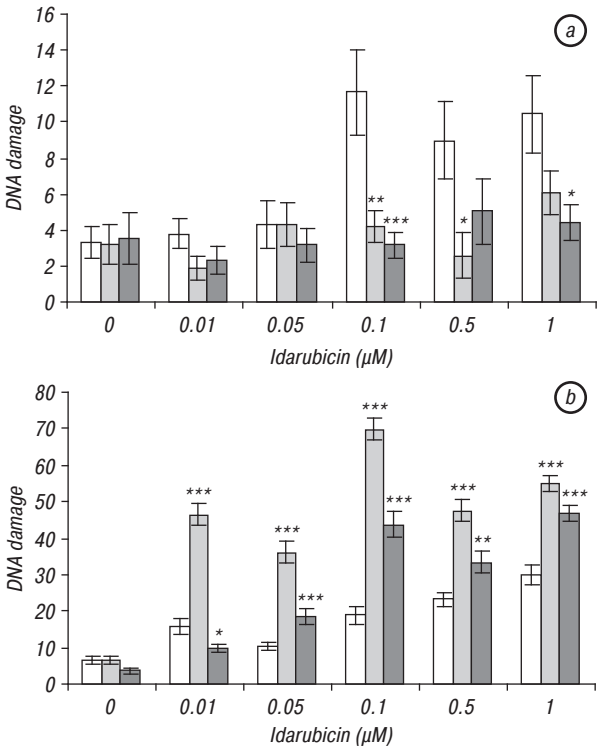

Fig. 4. DNA damage, measured as the comet tail DNA in the comet assay, of lymphocytes (*a*) and CCRF-CEM cells (*b*) incubated for 1 h at 37 °C with idarubicin in the presence of $ZnCl_2$ (light grey) and $ZnSO_4$ (dark grey). The result of positive control (10 μM H_2O_2 at 4 °C for 10 min) was as follows: for lymphocytes 14.88 ± 1.00 ($p < 0.001$) and for CCRF-CEM cells 12.16 ± 1.00 ($p < 0.001$)

DISCUSSION

Anthracyclines, including doxorubicin and idarubicin are drugs applied in haematological malignancies. Both antibiotics display antitumor properties through a number of molecular pathways. Both act as topoisomerase II poisons and possess mutagenic and recombinagenic properties [25]. Topoisomerase II generates transient double strand breaks in DNA. Anthracyclines intercalate into DNA and perturb the re-ligation step of topoisomerase II resulting in the formation of the ternary drug-DNA-topoisomerase II cleavable complex. This reaction results to a fragmentation of DNA [26]. Anthracyclines can also generate adducts with DNA. It was shown that doxorubicin, activated by the endogenous formaldehyde to an active Schiff base, was able to form monoadducts within the 5`-GCN-3` sequences [21]. Doxorubicin can also induce cross-links in DNA [19] and inhibit the DNA methyltransferase DNMT1 [27]. Moreover, anthracyclines can generate free radicals through activation by a number of cellular oxydoreductases. Generation of free radicals is considered as one of the major mechanisms of anthracyclines cyto- and genotoxicity [28]. Genotoxic action of anticancer drugs in normal tissues may lead to the induction of secondary malignancies; therefore, the problem of the identification of mechanism(s) of such action is justified.

Treatment with zinc adjuvant is hypothesized to accelerate recovery from ALL, and in conjunction with chemotherapy, might cure ALL [9]. Not any side effect of zinc or chemotherapy assisted by zinc was reported and the element is considered as an agent to improve the patient's overall ability to withstand toxic effects of chemotherapy [9]. In the present work, we determined the effect of zinc on genotoxicity of doxorubicin and idarubicin in normal lymphocytes and CCRF-CEM cells. To study DNA damage the alkaline comet assay was used. It is a sensitive and relatively inexpensive and noninvasive technique used for the detection of DNA damage and repair. DNA damage detected by the alkaline comet assay includes single-strand breaks (SSBs), alkali labile sites (e.g., apurinic/apyrimidinic (AP) sites), double-strand breaks (DSBs) and modified bases as well as DNA-DNA and DNA-protein cross-links. One of the major advantages of this assay is the capacity to analyze DNA damage and repair in individual cells. Furthermore, small numbers of cells are required for this assay.

Previously, we showed that zinc did not induce DNA damage in normal cells, but did so in cancer cells of human myelogenous leukemia (K562 cell line) [29]. We observed a key difference between the action of zinc in normal and cancer cells in the presence of H_2O_2, since the element exerted a protective effect against cyto- and genotoxic action of H_2O_2 in the former, whereas it increased such action in the latter. Moreover, we observed that zinc inhibited the repair of DNA damage induced by H_2O_2 in cancer cells [29].

Our results suggest a significant difference between normal and cancer cells in DNA damage extent induced by anthracyclines, doxorubicin and idarubicin in the presence of zinc. Cancer cells exhibited a significant increase of DNA damage in the presence of zinc (Fig. 3*b,* 4*b*), while no such effect was observed in lymphocytes (Fig. 3*a,* 4*a*). Zinc can be active as a modulator of cellular oxidative defense [30] and the addition of zinc may decrease prooxidative action of anthracyclines. It was shown that some cancer cell lines were sensitive to external zinc and the addition of zinc could disturb a proliferation, trigger senescence or induce apoptosis in these cells [31]. Our results suggest that zinc may protect normal cells against the DNA-damaging action of anthracyclins and increase this action in leukemic cells. Therefore, zinc can be useful in ALL chemotherapy as a modulator of drug genotoxicity.

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