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EVALUATION OF CYTOTOXIC EFFECT OF ZINC ON MOLT-4 CELL-LINE BY MITOCHONDORIAL THIAZOL TETRAZOLIUM ASSAY

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Abstract

Zinc has important effects on structural and functional activities of many proteins and enzymes involved in biological activities especially regulation of immune- system. Deficiencies of this element may result in many diseases and immunological disorders. Symptoms of zinc toxicity include nausea/vomiting, fever, cough, diarrhea, fatigue, neuropathy and dehydration. Further signs include growth retardation, altered iron function, anemia, copper deficiency, decreased immune function, decreased HDL (high density lipoprotein), increased LDL (low density lipoprotein), and increased HgbA1C. This study was carried out to examine the in vitro effects of different concentration of zinc on viability and death of T-Lymphoid (Molt-4) cell line. In this study, the cell line was exposed to different concentrations of zinc (10nM to 500µM) followed by incubation (37°C, 5% CO_2) at various time points (12 to 72 h). The cells were then evaluated with trypan blue exclusion dye and Mitochondorial Thiazol Tetrazolium Assay (MTT) and light microscopy. The results of this study showed almost different responses to different amounts of zinc by The T cell line (Molt-4). Zinc concentrations below 100µM at different incubation time points had little or no effect on cell line compared to the controls. Higher concentrations of zinc (>100µM) viability diminished to 70% at 12 h and less than 50% at 24 to 72 h of incubation times. We conclude that Zinc has dose-dependent cytotoxicity on Molt-4 cells.

Keywords:

Zinc, Molt-4 Cells, Viability, Cell death, Zinc toxicity.

Introduction

Clinical and experimental observations have highlighted the importance of zinc (Zn) in maintaining immunological integrity (1,2). Zn is a cofactor in more than 300 enzymes involved in various immune- functions (3,4). Human growth and development is strictly dependent on Zn (5,6). The total body content of this trace element is 2-4g and plasma concentration is only 12-16 µM/L. Zn deficiency will lead to poor health and impaired immune response, excessive intake can also be harmful to health (7,8).

Symptoms of zinc toxicity include nausea/vomiting, fever, cough, diarrhea, fatigue, neuropathy and dehydration.

Further signs include growth retardation, altered iron function, anemia, copper deficiency, decreased immune function, decreased HDL (high density lipoprotein), increased LDL (low density lipoprotein), and increased HgbA1C (5). Martin et al. [4] maintained the human cell lines of lymphoid (Molt-3 and Raji) and myeloid (HL-60) origin in vitro under Zn – sufficient (to 50µM) or Zn–defficient conditions. Under these coditions, cell proliferation, viability and mode of cell

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death were assessed. All cell types showed decreased proliferative capacity and viability with Zn deficiency. But when Zn was increased (to 50µM) no effect was observed on cell proliferation and viability compared to the controls (9). The results of Michiko et al. (10) with use of both PI and FITC-Labeled showed that when Molt-4 cells were exposed to various concentrations of Zn for 48h or 300µM their viability decreased . With exposure to removed 100µM and 200µM Zn for 48h the viability was 75% and 10%, respectively. While it was reduced to 80% and 20% after exposure to 300µM for 10 and 24 h, respectively. For the first time in Iran we showed the effects of different Zn concentrations on viability, cell proliferation and morphology of Molt-4 cell in vitro. We compared our results with the work of other researchers to find if Zn can be used to modulate immune-functions.

Materials and mthods

The human T cell acute lymphoblastic leukemic cell line, Molt-4 cells (purchased from Institute Pasteur of Iran) was cultured in RPMI-1640 medium contaning 10% calf serum.The cells were maintained at 37° C in a 5% CO_2 incubator and with passage every day. Cell culture was done under sterile conditions and below laminar-hood. With removal of Molt-4 cells from flask stock, by use of Trypan Blue 0.4% in a suspension of Molt-4 cells, viability was more than 97%, (Viability (%)= live cells / (live cells + dead cells) × 100). The other steps were removal of 75µL (15000 cell) from this suspension, then transfered to 96-well plates. We added 10µL of different Zn concentrations (10 nM -500 µM) to all wells except controls. Plates were shaked and mixed under the laminar hood and sterile conditions. Then cells were maintained at 37°C in a 5% CO_2 incubator. At the end of incubation times (12-72 h) viability and cell proliferation were determined using both the trypan blue exclusion dye and MTT assay[1]. Also cell morphology was evaluated by Wright-Giemsa staining.

Cytotoxic assay by mtt reduction

This was carried out using the MTT assay described by Mosmann et al. (11).

The assay principle is based on the cleavage of the tetrazolium salt (MTT), in the presence of an electron coupling reagent, by active mitochondria.The produced water-insoluble formazan salt has to be solubilized in an additional step. Cells grown in a 96-well plate, are incubated with the MTT solution for approximately 4 h. After this incubation period, a water-insoluble formazan dye is quantitated using a spectrophotometer (ELISA reader).The revealed absorbance directly correlates to the cell number.

After end-points of incubation time (12-72h) at 37°C and 5% CO_2, the Molt-4 cells were loaded with 10 freshly prepared and Millipore filtered MTT(5mg/ml PBS) and incubated for 4 h at 25°C. After 4 h incubation 100 µL of isopropanol was added to each well, and the O.D. (optimal density) of product was evaluated in an ELISA reader at 540 nm wevelength after 15 min [12,13] (Viability (%) = O.D. of test / O.D. of control x 100).

Results

Effects of Zinc on cell growth, viability and morphology of Molt-4 cell line.

With different incubation times (12-72h) of Molt-4 cells suspension with medium (RPMI-1640 and 10% FCS) in presence of different Zn concentrations (10 nM -500 µM) at 5% CO_2 and 37°C, viability and cell proliferation assessed (by TB and MTT Assay) and data were analysed by Dunnet test. The results are shown in Table1.

[1] MTT assay=3-[4,5-dimethylthiazol-2yl]-2,5diphenile tetrazolium bromide)

Table 1: Effects of different Zn Concentrations on viability Molt-4 cell line by MMT assay after (a) 12 h, (b) 24 h, (c) 36 h, (d) 48 h, (e) 60 h and (f) 72 h

(a) 12 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	97	96	95	95	94	94	93	80	76	72	72	98
P- value	0.999	0.990	0.891	0.883	0.820	0.820	0.815	<0.05	<0.05	<0.05	<0.05	--

(b) 24 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability(%)	96	96	95	95	95	93	93	78	52	22	21	97
P- value	0.987	0.997	0.895	0.850	0.841	0.753	0.742	<0.05	<0.05	<0.05	<0.05	--

(c) 36 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	96	94.5	94	93.2	93	92.6	92.5	79	26	21	20	96
P- value	1.000	0.993	0.980	0.851	0.821	0.810	0.798	<0.05	<0.05	<0.05	<0.05	–

(d) 48 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	96	95	94.2	94.1	94	93.7	93.5	27	20	16	14	96
P- value	0.994	0.991	0.913	0.900	0.885	0.850	0.810	<0.001	<0.001	<0.001	<0.001	–

(e) 60 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	94	93.5	93	93	92.9	92.6	92	18	15	13	11	95
P- value	0.990	0.950	0.920	0.914	0.903	0.815	0.814	<0.001	<0.001	<0.001	<0.001	–

(f) 72 h

Zn(μM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	94	93	93	92.	92	92.	92.	15	13	12	4	95
P- value	0..991	0.945	0.940	0.917	0.907	0.886	0.870	<0.001	<0.001	<0.001	<0.001	

When Molt-4 cells were exposed to high concentrations of Zn(>100μM), their viability diminished to 70-80% after 12 h incubation and 5-15% after 72h incubation , respectively.

Zn concentration below 100μM of Zn at different incubation time periods had no effect on cell line when compared with the controls. To confirm that excessive Zn induces death cell line were stained with both trypan blue and Wright -Giemsa. Culturing of Molt-4 cells in the presence of lower concentration (<100 μM) and measurment of viability and total cells in end-points showed no effect of Zn on proliferation capacity.

At high concentrations of Zn (>100 μM) proliferative rate in the test groups was lower than the control groups ($p<0.05$).

Discussion

Zinc at high concentrations has been shown to inhibit characteristic events in the late stages of apoptosis such as DNA fragmentation or induction of hypodiploid cells (15,16), while relatively low concentrations of the metal (80-200 μM) induced apoptosis in mouse thymocytes (17,18). We have reported that relatively high doses of zinc (200-500 μM) induce necrosis in human prostate carcinoma cells (19). Zinc at 100-300 μM induced necrosis and apoptosis, the cell death being independent of caspase activation. Furthermore, the induction of apoptosis was not inhibited by caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO. The concentration of zinc used here was

only about 10-fold higher than one that found in serum or tissue (20).

Furthermore, the metal binds to serum proteins in the medium, and the concentrations required for cortical neuronal death could be reduced using a medium lacking serum (21). Perry et al. showed that 100 µM zinc caused the complete inhibition of etoposide-induced Poly(ADP-ribose) polymerase proteolysis, an apoptotic event in Molt-4 cells (14). Michiko et al. did not observe any zinc inhibition of cell growth at concentrations lower than 100 µM(10). These conflicting observations could be due to the fact that zinc-induced cell death mainly comprises necrosis, with some apoptosis occurring independent of caspase activation.

Apoptosis is characterized by morphological and physiological changes such as cell shrinkage, abnormal chromosome condensation, apoptotic body formation, and DNA fragmentation (22). However, all cell strains did not exhibit the same series of events. For example, low-molecular weight fragmented DNA corresponding to nucleosomal ladders was not detected in topoisomerase II inhibitor-induced apoptotic Molt-4 cells (23). In the present study, the release from cytochrome c, and induction of annexin-positive, 7A6 antibody-reactive cells and abnormal chromosome condensation were observed, while activation of caspase-3 and caspase-8 were not detected and induction of hypodiploid cells was low. There are at least two pathways for the activation of caspase-3. Upon anti-Fas treatment, autoproteolytic activation of caspase-8 occurs, which in turn activates other caspases such as caspase-3 and caspase-6 (24). Michiko et al. did not detect any zinc-induced increase in caspase-8 activity (10). Another mechanism might involve the release of cytochrome c from the mitochondria, an event which induces apoptosis by activating caspase-9 and caspase-3 (25). Since we detected zinc-induced release of cytochrome c, the two processes are not essentially linked. Although caspase activation and abnormal chromosome condensation are characteristic features of apoptosis, both could be induced through separate pathways (26).

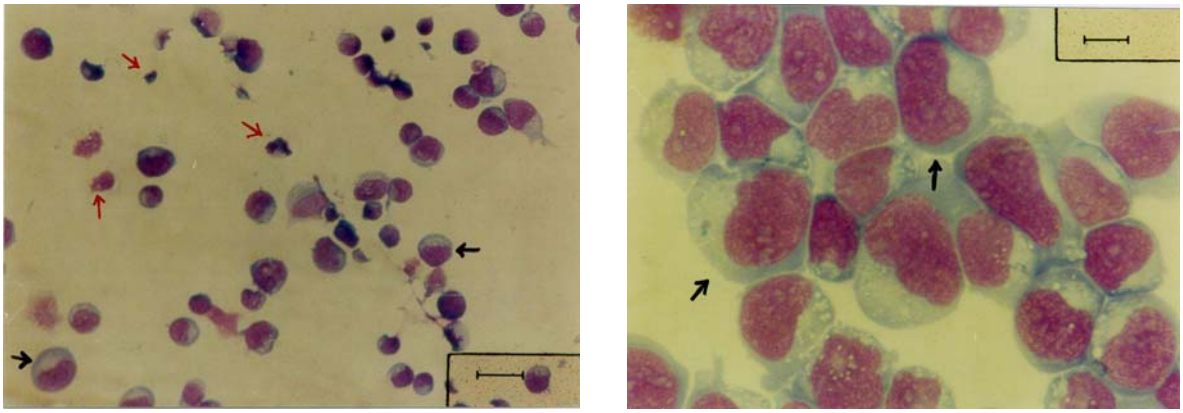

Fig.1: Molt-4 Cells (x100) (left), The effects of Zn on morphology of Molt-4 Cells, 200µM/12 h Wright-Giemsa staining) (x40) (right).

Annexin V, a protein with high affinity for phosphatidylserine, can bind to exposed phospholipids in apoptotic cells. Phosphatidylserine externalization is a feature of apoptosis induced by various drugs (27), and its recognition by macrophages promoted phagocytosis (28). Such externalization has been shown to be an early apoptotic event prevented by inhibitors of caspase or Bcl-2 (29). However, Michiko et al. found that zinc-induced annexin-positive cells appeared at a late stage rather than an early stage of apoptosis. Moreover, the induction was not prevented by caspase inhibitors, in contrast to the case of etoposide-induction (10). Like zinc-induced phosphatidylserine externalization, the externalization in anti-CD2 and staurosporine-treated cells was not inhibited by caspase-3 inhibitors. Thus a distinct mechanism of induction of annexin-positive cells is presumably involved, depending on cell death-induced agents. Recently, thymocytes undergoing necrosis were found to be associated with externalization of phosphatidylserine (30). The enzymes responsible for this process occurring at early and late stages of apoptosis have yet to be identified, although lipid scramblase (31) and aminophospholipid translocase (32) probably play roles in it. An unusual observation in this study was that many necrotic cells exhibited abnormal chromatin condensation, since necrosis is generally not associated with induction of condensation, except with glutamate-induced necrosis in mouse cortical neurons (33).

Michiko et al. obtained evidence that zinc causes mixed types of cell death, necrosis, and apoptosis, the latter occurring in annexin-positive and 7A6-reactive cells without the activation of caspase-3 and caspase-8, and the induction of hypodiploid cells (10). Zinc-induced phosphatidylserine externalization is independent of caspase activation, in contrast with reports of anti-cancer drugs or cytokine-induced externalization (34). The latter evidence suggests that phosphatidylserine externalization occurs in distinct pathways, caspase dependently and independently.

In this study, we have shown that cell death can be induced by Zn concentrations more than 100µM in Molt-4 cells. Then high concentrations of Zn have detrimental effects on cell viability. The results of Michiko et al. (by methods PI and FITC) showed that when Molt-4 cells were exposed to 100 and 300 µM of Zn viability was 75 and 10%, respectively. At exposure to 300µM Zn for 12 and 24 h, viability was reduced to 80 and 20%, respectively (10). In our study by MTT assay, after exposure to 100µM and 200µM for 48 h, the viability was93.5% and 52%,respectively.This contrast with findings of Michiko H.Therefore, methods PI &FITC are better than MTT assay. But MTT can provide reproducible and accurate measurements of viability and cell proliferation and the results compare favourably with the other tests: (a) it is safe, economical, simple, fast and sensitive enough to handle a large number of samples in a short period of time and it can be used for studying as high as 15000 cells. Zn at less than 100µM at all periods (12-72 h) had no effects on viability and proliferation of cells, when compared to the control groups. Data analysis with package SPSS and Dunnet test showed that there is no significant difference between viability and proliferation of Molt-4 cells in the presence of <100µM compared to the control groups. In the presence of Zn>100µM there was a significant difference. Perry et al. showed that 100µM induced cell death in Molt-4 cells (14). But we did not observe any Zn inhibition of cell proliferation and viability in 100µM and lower than 100µM. Michiko et al. showed that Zn with 100µM concentration had no in vitro effect on cell proliferation and cell

viability (10). These results indicate that zinc induces both necrosis and apoptosis, without caspase-3 activation (10).
Martin et al. showed that Zn up to 50μM at in vitro has no cytotoxic effects on Mol-3 (9) .Our results are in line with conclusions of Michiko and Martin. Data analysis showed significant difference between proliferation and viability (MTT) in test group and controls. At high Zn concentrations cell proliferatin and viability were reduced , when compared with the controls. Wright-Giemsa stain and MTT assay showed that Zn induces cell death above 100μM.Cell death is characterized by morphological changes, such as shrinkage, abnormal chromosomal condensation. These changes were not observed in the controls. We conclude that Zn has dose-dependent cytotoxicity. Low concentrations have no effect on Molt-4 cells, but high concentrations decrease viability and cell proliferation compared to the controls.

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