

## The Apoptotic Effect of Extracellular Zinc Sequestration on HT29/219 and SW742 Cell Lines

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Received 25 April 2004; revised 26 October 2004; accepted 2 January 2005

### ABSTRACT

**Zn (II) is an important regulator of caspase-3, as well as an antioxidant, microtubule stabilizer, growth cofactor, and anti-inflammatory agent. Over the past 30 years, many researchers have demonstrated the important role of Zn (II) in a variety of physiological processes, including growth and development, maintenance and priming of the immune system, and in tissue repair and regeneration. In this study, we present evidence that chelation of extracellular zinc by diethylenetriaminepentacetic acid (DTPA) in different concentrations causes cell death in carcinoma cell lines, HT29/219 and SW742. Hoechst 33258 staining revealed that cell death was mainly by apoptosis. Additionally, significant increases in the activity of caspase-3 and -9 were observed in both cell lines. Caspase-8 activation was negligible in both cell lines. The cytotoxicity/apoptotic effect of DTPA was inhibited significantly by Zn (II), Cu (II) and N-Acetyl-L-Cysteine (NAC) ( $P<0.05$ ). Therefore, DTPA, the membrane-impermeable metal ion chelator, induces apoptosis through the depletion of extracellular zinc ion. Iran. Biomed. J. 9 (4): 169-175, 2005**

**Keywords:** Extracellular zinc, Apoptosis, Diethylenetriaminepentacetic acid (DTPA), Caspase, Colon carcinoma cell line

### INTRODUCTION

**Z**inc, a member of the IIb group of metal, is critical for the functional and structural integrity of eukaryotic cells and tissues. It is required for events as diverse as gene expression, DNA synthesis, enzymatic catalysis, hormonal storage and release, tissue repair, neurotransmission, memory and the visual processes. Our current understanding of the cellular biology of Zn (II) commenced with the identification of stoichiometric quantities of Zn (II) in metalloenzymes four decades ago. Since then, Zn (II) has been shown to be a structural and/or functional component of more than 200 metalloenzymes as well as numerous Zn (II) finger transcription factors [1].

In addition to house-keeping metabolic roles of Zn (II), this metal also plays critical roles in many physiological processes [1]. Furthermore, Zn (II) has properties advantageous for a role in cytoprotection as it protects proteins and nucleic acids from oxidation and degradation, while stabilizing

the microtubular cytoskeleton and cell membranes. Zinc is able to bind to cysteine, histidine and glutamate in proteins causing protein folds associated with novel functions (as in Zn (II) fingers), and exists in only one oxidation state (II), therefore it can not undergo redox reactions commonly responsible for the generation of potentially damaging oxy-radicals. In fact, by binding to thiol groups in proteins, Zn (II) can reversibly protect them from oxidation [2]. The anti-oxidant property of Zn (II) could probably be important in the regulation of apoptosis. From this viewpoint, it is interesting that Zn (II) shares features in common with anti-apoptotic protein Bcl-2 that emerged later in evolution and has also anti-oxidant properties. Both Zn (II) deficient rodents and Bcl-2 knockout mice that are growth-retarded, have severe immunodeficiency associated with massive thymic atrophy and depletion of CD4<sup>+</sup> T cells and exhibit graying fur [1].

The diverse functions of Zn (II) arise from tightly-bound pools within proteins that constitute a largely

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fixed pool of cellular Zn (II) (associated with metalloenzymes and zinc finger proteins) as well as more dynamic, labile pools which are loosely associated with proteins [1, 2], lipids [3], and cytoskeleton [4] or sequestered in vesicles [5]. These labile pools, which are believed to be important in cytoprotection and the regulation of apoptosis [2], amongst other things, are readily influenced by Zn (II) deprivation or supplementation [4].

There are several reports indicating that apoptosis can result directly from a decline in intracellular Zn (II) induced by culture of cells in either Zn (II)-free medium [6-8] or in the presence of membrane-permeant Zn (II) chelators such as N, N, N', N'-terakis-(2-pyridylmethyl) ethylenediamine (TPEN) [9, 10]. However, only a few investigations have investigated the role of extracellular zinc chelation in apoptosis [9]. This study was performed to evaluate the effect of extracellular Zn (II) chelation on the human colon cancer cell lines (HT29/219 and SW742).

## MATERIALS AND METHODS

Chemicals, culture media and related compounds were purchased from Sigma Co. (USA). Cell culture plastic ware was obtained from Nunc Co. (Denmark), caspase-3 and -8 colorimetric assay kits (Cat. No. 101K4019 and Cat. No. 80K4104, respectively) were from Sigma (Germany). Caspase-9 colorimetric assay kit (Cat. No. BF10100) was obtained from Sigma (USA).

**Cell culture.** HT29/219 (NCBI C154) and SW742 (NCBI C146) colon carcinoma and adenocarcinoma cells (obtained from National Cell Bank of Iran, NCBI) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. They were incubated at 37°C in a humidified CO<sub>2</sub>-incubator with 5% CO<sub>2</sub> and 95% air.

**Cytotoxicity assay.** To evaluate the cytotoxic effect of diethylenetriaminepentacetic acid (DTPA) on these cell lines, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), colorimetric assay was applied [9]. Briefly, asyn-chronously growing cells ( $1.5 \times 10^4$  cells/ml) were transferred into 96-well culture plates containing 200 µl of medium and incubated for 24 h. Various concentrations of DTPA (0-100 µM) were added and incubated for different time intervals (0-72 h as indicated) followed by MTT

assay. The percent of cell viability was calculated as the: (mean OD of treated cells/mean OD of control cells)  $\times$  100.

**Analysis of nuclear morphology.** Cells were plated in 8-well chamber slides and allowed to adhere. DTPA-treated cells were fixed with methanol-acetic acid 3:1 (v/v) for 10 min and then staining was carried out with Hoechst 33258 (200 µg/ml). Slides were washed with PBS (pH 7.4) and examined by an epifluorescence microscope (Micros, Austria). Apoptotic cells were defined on the basis of nuclear morphology changes such as chromatin condensation and fragmentation.

**Caspase-3, -8 and -9 activation assays.** Caspase-3 (using DEVD-pNA as substrate), caspase-8 (using Ac-IETD-pNA as substrate) and caspase-9 (using LEHD-pNA as substrate) colorimetric assay kits were used to investigate the activation of these caspases in the treated HT29/219 and SW742 cells. Briefly, to estimate caspase-3 and -8 activities, cells were lysed by incubation with cell lysis buffer on ice for 15 min and then centrifuged at 20,000  $\times$ g for 10 min at 4°C. For caspase-9 activation assay, cells were lysed by incubation with cell lysis buffer on ice for 10 min and then centrifuged at 10,000 g for 1 min at 4°C. Enzymatic reactions were carried out in a 96-well flat-bottom microplate. To each reaction samples, 5, 25 and 50 µl of cell lysate (100-200 µg total protein) were added for caspase-3, -8 and -9 respectively. Additional controls, one free from cell lysate and the other lacking substrate as well as caspase-3 and -8 positive controls were included. Protein content was estimated by Bradford method [11]. The activities were expressed as nmole/min/mg protein.

**Effect of various divalent metal ions on DTPA cytotoxicity activity.** To evaluate the effect of different divalent metal ions (calcium, magnesium, copper, zinc), the cell lines were treated with DTPA (100 µM) for 48 h in the presence of increasing concentrations of metal ions as indicated.

**Effect of N-Acetyl-L-Cysteine (NAC) on DTPA cytotoxicity effect.** To study the involvement of reactive oxygen species (ROS) in the induction of apoptosis, the cell lines were pre-treated with increasing concentrations of NAC for 24 h. The cell lines were then treated with DTPA (100 µM) for 48 h.

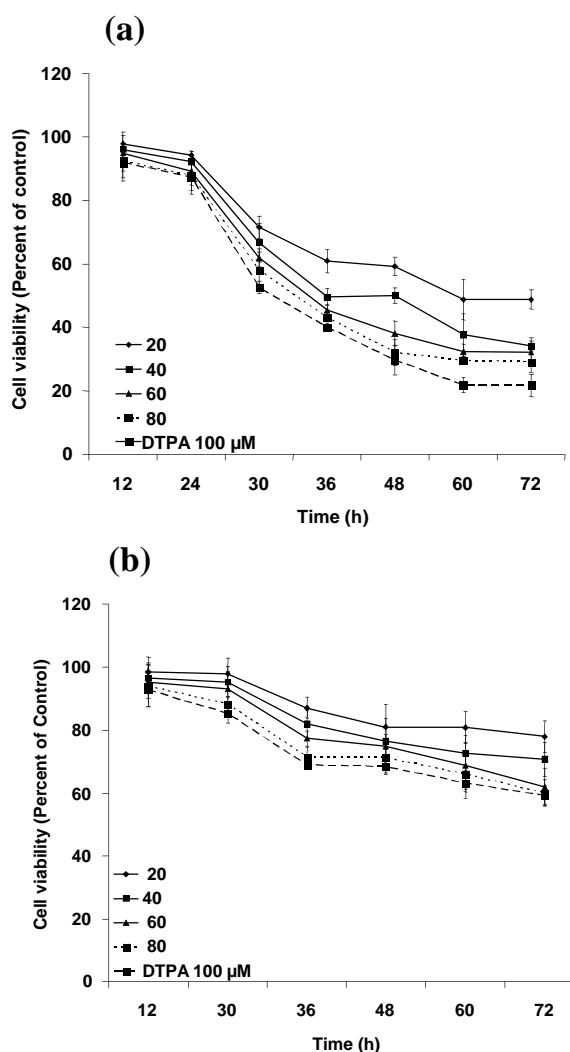
**Quantification of intracellular labile zn (II) by zinquin fluorescence:** To study the effect of decreasing the labile intracellular concentration of zinc in the induction of apoptosis by DTPA, zinquin was used to estimate the intracellular concentration of labile zinc ions [12, 13]. Briefly, after being treated for 24 hours by DTPA, cells in suspension were incubated with zinquin (25  $\mu$ M) for 30 min ( $10^6$  cells for each test). Zinquin was diluted freshly in PBS (pH 7.4) and added immediately to the cells. There was no serum in the medium (PBS containing 1 mg/ml ovalbumin). After 30 min at room temperature, the samples were transferred into fluorometry grade cuvettes (600  $\mu$ l). A Shimadzu RF 5000 spectrofluorometry (Shimadzu, Japan) was used in read mode (excitation was 365 nm and emission 490 nm and slit widths 10 nm). A set of control tubes contained a membrane-permeable Zn chelator TPEN (25  $\mu$ M) to give non-specific fluorescence. Control tube fluorescence activity then were subtracted from total fluorescence of each measurement to gain the specific fluorescence of each tube.

**Statistical analysis.** The results were expressed as the mean  $\pm$  SD and statistical differences were evaluated by one way ANOVA.  $P < 0.05$  was considered significant.

## RESULTS

**Cytotoxicity assay.** To determine cytotoxicity of DTPA, viability test was applied using MTT assay. As it is shown in Figure 1a, treatment of HT29/219 cells with DTPA resulted in a significant cell death at concentrations higher than 40  $\mu$ M in 12 h. With the increase in incubation time (from 24 to 72 h), the effective dose response decreased to 20  $\mu$ M ( $P < 0.05$ ) (Fig. 1a). In the treated SW742 cell line, however, DTPA induced a significant cell death ( $P < 0.05$ ) at concentration higher than 60  $\mu$ M in 30 h. At 20  $\mu$ M, however, this compound caused a significant cell death ( $P < 0.05$ ) when cells were treated for a longer period of time (from 36 to 72 h) (Fig. 1b).

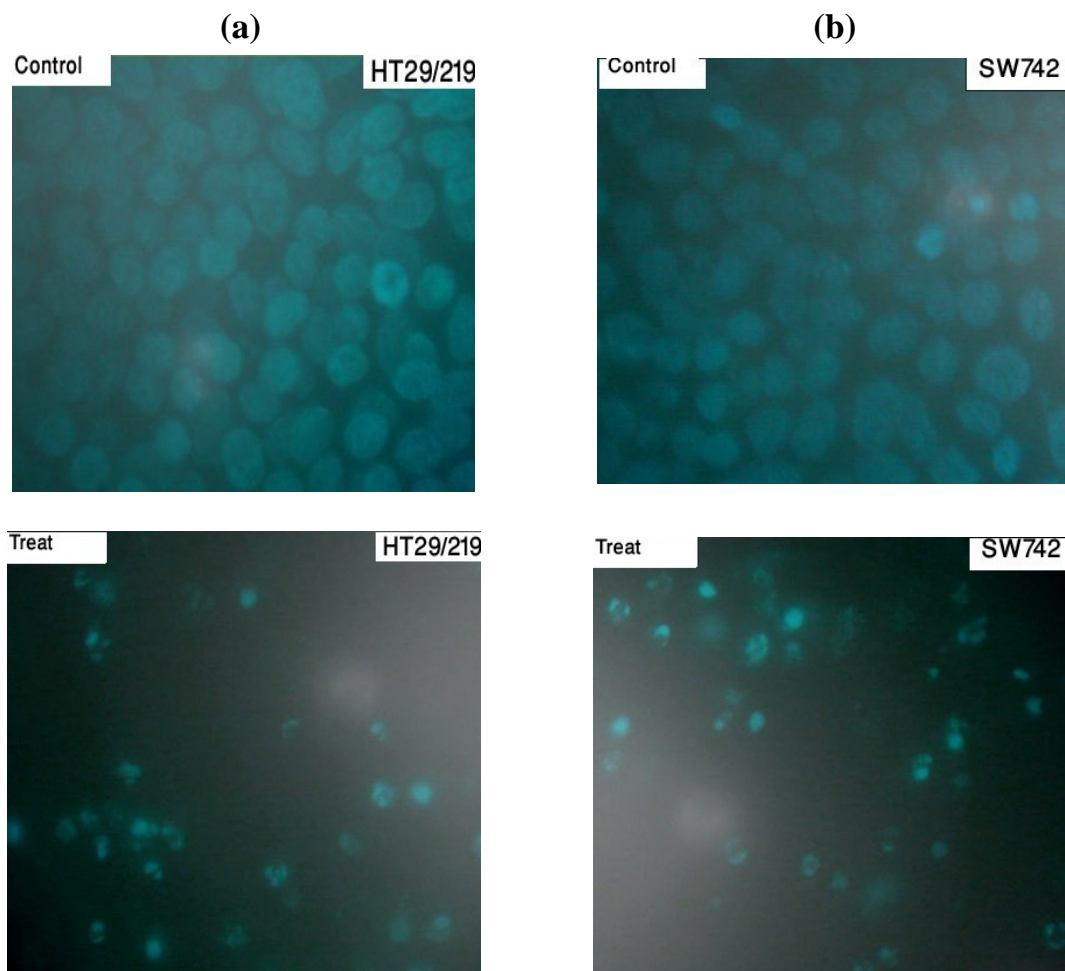
**Detection of nuclear morphology by Hoechst 33258.** The cell morphology was first examined by phase contract microscopy. In order to confirm the apoptotic cell death, control and treated cells were stained with Hoechst 33258. The DTPA complex caused typical apoptotic changes in the nuclear



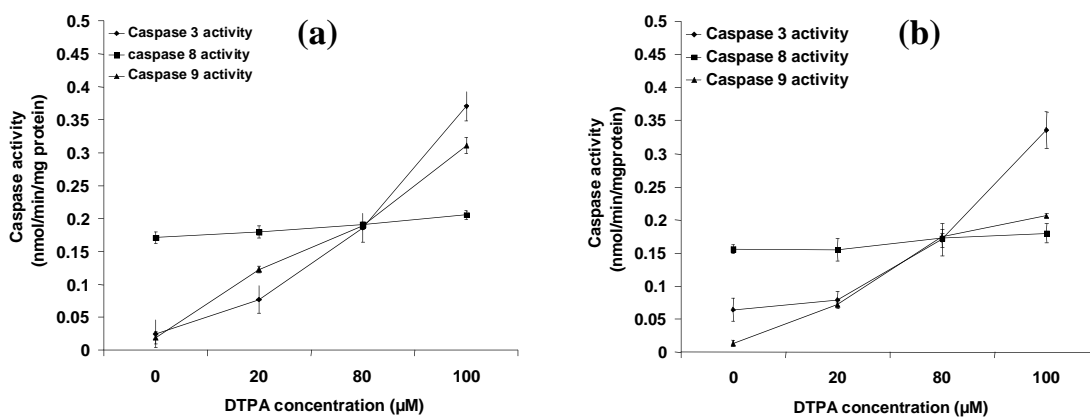
**Fig. 1.** Effect of DTPA on the growth of (a) HT29/219 and (b) SW742 cell lines. The cells were treated with different concentrations of S100A8/A9 and DTPA for 12 to 72 h and the viability was assessed by MTT assay. Results are expressed as percentage of corresponding control and represent the mean  $\pm$  SD of 4 repeats.

morphology, with pronounced condensation of chromatin and DNA fragmentation (Fig. 2a, HT29/219 cell line and Fig. 2b, SW742 cell line).

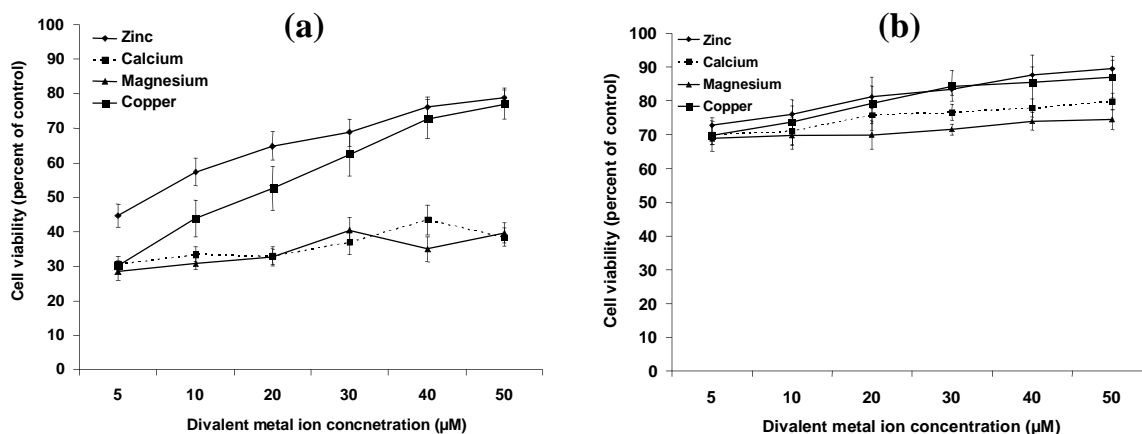
**Caspase-3, -8 and -9 activation.** To explore the possible biochemical mechanisms underlying DTPA-induced apoptosis, the activation of caspase-3, -8 and -9 were assayed. The results demonstrated that the activity of caspase-3 and -9 were significantly ( $P < 0.05$ ) increased in both cell lines upon treatment with DTPA. However no changes in caspase-8 activity were observed (Fig. 3).



**Fig. 2.** Detection of typical for apoptosis nuclear condensation and fragmentation upon stimulation of a) HT29/219 and b) SW742 cells with DTPA by Hoechst 33258 staining.



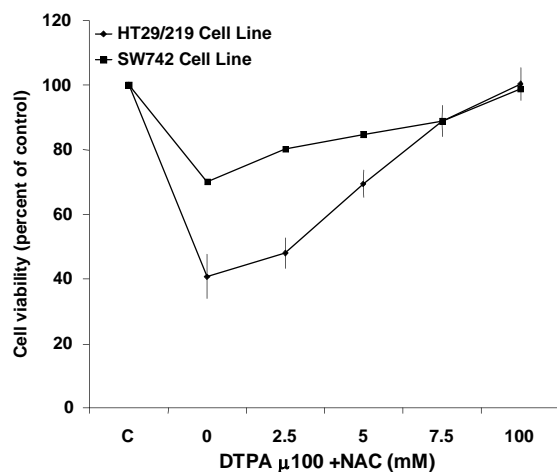
**Fig. 3.** Enzymatic measurement of activity of caspase family of proteases. Activity of caspase-3 (DEVDase activity), caspase-8 (IETDase activity) and caspase-9 (LEHDase activity) in (a) HT29/219 and (b) SW742 cells following treatment with human DTPA for 36 h were quantified by an enzymatic assay (see method section for details). Results are expressed as activity of the enzyme and represent the mean  $\pm$  SD of 4 repeats.



**Fig. 4.** The effect of divalent metal ions on the cytotoxicity of DTPA in HT29/219 (a) and SW742 (b) colon cell lines. Cells were treated with DTPA (100  $\mu$ M) for 48 h, the cell death was detected by MTT-assay. Results represent the mean  $\pm$  SD of 4 repeats.

**The effect of divalent metal ions, Ca (II), Mg (II), Zn (II), and Cu (II) on the DTPA-induced cytotoxicity.** To identify the influence of divalent metal ions on the cytotoxicity effect of DTPA, HT29/219 and SW742 cell lines were treated with DTPA (100  $\mu$ M) in the presence and absence of different concentrations of Ca (II), Mg (II), Zn (II), and Cu (II). A significant increase in cell viability was observed when treated cells were co-incubated with Zn (II) or Cu (II) (Fig. 4 a and b).

**The effect of NAC on the DTPA-induced cytotoxicity.** As shown in Figure 5, pretreatment with NAC prevents the cell cytotoxicity in both cell lines treated by DTPA ( $p < 0.05$ ).

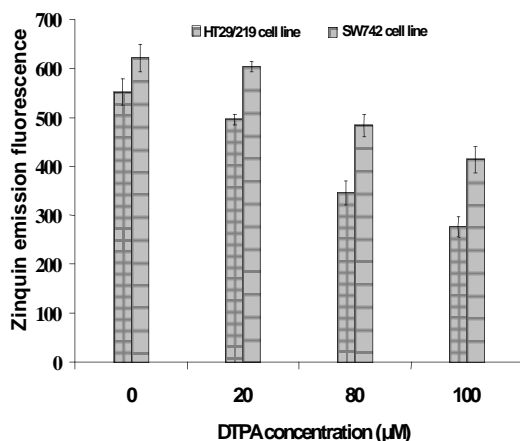


**Fig. 5.** The effect of N-Acetyl-L-cysteine on the cytotoxic effect of DTPA in HT29/219 and SW742 colon cell lines. The cells were treated with the indicated stimuli for 48 h, the cell death was detected by MTT-assay. Results represent the mean  $\pm$  SD of 4 repeats.

**Detection of intracellular labile zinc by zinquin.** To survey the mechanisms underlying human DTPA-induced apoptosis, the intracellular concentration of zinc ion was detected using zinquin emission fluorescence assay. The results confirmed that the intracellular concentration of zinc ions was decreased in both cell lines that treated with DTPA (Fig. 6).

## DISCUSSION

Zn (II) is a part of the oxidant defense system that protects cells from radicals and other oxidant species that are produced as a consequence of normal cell metabolism. Consistent with an essential role of zinc in oxidative defense, zinc deficiency causes oxidative damage to lipids, proteins, and DNA [14-16]. *In vitro* studies have shown that depletion of intracellular Zn (II) by treatment of cells with TPEN resulted in apoptosis [17-21]. Our data show that the membrane-impermeable zinc chelator and DTPA, caused cytotoxicity in HT219/219 and SW742 cells, although HT29/219 cells were significantly more sensitive than SW742 cells ( $P < 0.05$ ). These differences might be due to intracellular labile Zn (II) in HT29/219 and SW742 cell lines (Fig. 6). The cytotoxicity was mainly found to be apoptotic type using Hoechst 33258 staining. It is noteworthy to mention that DTPA concentration in our studies was much higher than the previous study, which was performed on the EL-4 cell line [9]. This observation confirms that EL-4 cells are more sensitive than HT219/219 and SW742 toward extracellular zinc chelation.



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**Fig. 6.** The effect of DTPA on intracellular zinc concentration by zinquin in the HT29/219 and SW742 colon cell lines.

We suggest that by activation of caspase-9, the apoptotic route may be through mitochondrial pathway. The finding that caspase-8 activity was not increased clearly indicates that the caspase-8/death receptor pathway was not involved in the DTPA-induced apoptosis. Recently, it has been shown that pro-caspase-3 is stabilised in the presence of zinc ions, either directly through binding to Zn (II) [22, 23] or indirectly through the effect of Zn (II) on redox-controlled processes [24]. One possible target of Zn (II) is Mch-2a/caspase-6 which is known to cleave and activate the proenzyme form of caspase-3. This complex is highly sensitive to Zn (II) [25-27]. Therefore, according to zinquin quantification of intracellular labile Zn (II) (Fig. 6), extracellular chelation of zinc by DTPA decrease the intracellular labile of this ion in HT29/219 and SW742 cell lines, thus resulting in the activation of caspase-3. Caspase-3 seems to be rapidly and directly activated by zinc chelation without any upstream event such as receptor mediation or mitochondrial pathway, suggesting that zinc protect cells from apoptosis by maintaining caspase-3 inactive [28]. Slight decreases in intracellular zinc could act as a starter of apoptosis or at least as a triggering factor amplifying the effect of various apoptotic compounds.

Independently, intracellular Zn (II) depletion causes a significant cellular stress by itself, since these bivalent cations are critical for the function of several transcription factors and enzymes. Cellular stress is known to activate the mitochondrial apoptotic-dependent pathway [29, 30]. ROS is involved in the initiation of apoptotic signalling.

We showed that NAC prevents apoptosis induced by DTPA when cells were pre-treated with the antioxidant. Therefore, facilitation in pro-oxidant state could contribute to the molecular mechanism by which DTPA exerts its apoptotic effect. It is believed that Zn (II) could protect the cells against oxidative damage via its role in Cu/Zn (II) superoxide dismutase (Cu/Zn (II) SOD), an enzyme which removes the superoxide anion radical [31]. Zinc can induce the synthesis of metallothionein, a protein that can chelate redox-active metals and scavenge hydroxyl radicals via its cysteine residues [30]. Zinc may be able to also exert its effect by binding to its membrane sites that might otherwise bind other redox-active metals i.e., Cu (II) and Fe (II) [30]. These results suggest that extracellular zinc ion depletion-induced apoptosis depends on caspase-3 and -9 activation. In addition to extracellular zinc, depletion causes an increase of the intracellular ROS, which might be due to intracellular zinc concentration.

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