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# Effect of Zn treatment on wild type and MT-null cell lines in relation to apoptotic and/or necrotic processes and on MT isoform gene expression

Alessandro Santon [1], Alessia Formigari [1], Vincenzo Albergoni, Paola Irato *

*Department of Biology, University of Padova, via U. Bassi, 58/B, 35131 Padova, Italy*

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**Abstract**

It has been shown in various systems that zinc is able to antagonize the catalytic properties of the redox-active transition metals iron and copper, although the process is still unclear. Probably, the protective effect of Zn against oxidative stress is mainly due to the induction of a scavenger metal binding protein such as metallothionein (MT), rather than a direct action. To support this hypothesis, in this study, the effects of Zn, Cu, Fe, Zn+Cu and Zn+Fe treatments were investigated in a fibroblast cell line corresponding to an SV40-transformed MT-1/-2 mutant (MT−/−), and in wild type (MT+/+), by valuing metal concentrations and apoptotic and/or necrotic processes. We also investigated the synthesis of MT and the levels of both MT-1 and MT-2 mRNAs. In MT+/+ cells, co-treatment with Zn+Fe caused a decrease in Fe content compared to treatment with Fe alone. After Zn and Zn+Cu exposure the expression of MT-1 and MT-2 isoforms increased with a concomitant increase in MT synthesis. Annexin V-FITC and propidium iodide staining revealed necrotic or apoptotic cells in terminal stages, especially after Fe treatments. Immunofluorescent staining with an anti-ssDNA Mab and annexin detected a lower signal in co-treated cells compared to the single treatments in both cell lines. The intensity and quantity of fluorescence resulting from anti-ssDNA and Annexin V staining of MT null cells was higher compared to wild type cells. These results suggest that Zn alone does not completely exert an anti-oxidant effect against Cu and Fe toxicity, but that induction of MT is necessary.


*Keywords:* Metallothionein; Apoptosis; Heavy metals; Mouse embryo; Fibroblast

## 1. Introduction

The presence of copper (Cu), zinc (Zn) and iron (Fe) is essential for different physiological functions, even though excess Cu and Fe increases free radical levels thus enhancing the biological damage free radical mediated [1,2]. Zn can protect cells against oxidative damage by binding to vicinal sulphydryl groups, therefore, preventing intramolecular disulfide formation. It may also protect cells by inhibiting the production of reactive oxygen species (ROS) such as hydroxyl ($^{\bullet}OH$) or superoxide ($O_2^{\bullet -}$) by transition metals [3]. This occurs by competition between Zn and other transition metals such as Fe and Cu for binding sites [4]. Some studies have reported on the ability of Zn to interact with Cu and Fe, decreasing the metal content in cells and tissues and retarding oxidative process [5].

Furthermore, it has been suggested that free metal ions such as Cu and Fe, which are involved in the production of free radicals, play an important role in the regulation and induction of apoptosis and necrosis.

It is well known that apoptosis can be regarded as a response to cellular damage by free radicals and is distinct from necrosis both in terms of morphology and in its mechanism of induction. During apoptosis, the loss of phospholipid asymmetry occurs, which results in exposure of phophatidylserine (PS) residues at the outer plasma membrane and may serve as a trigger for phagocytosis. The exposure of PS occurs early during apoptosis and precedes the loss of plasma membrane integrity [6]. Apoptosis may also be detected by the exposure of PS by allowing FICT labeled Annexin V to bind to PS, which is exposed at the outer membrane leaflet.

* Corresponding author. Tel.: +39 049 8276315; fax: +39 049 8276300.
*E-mail address:* paola.irato@unipd.it (P. Irato).
*URL:* http://dept.bio.unipd.it/ (P. Irato).
[1] A. Santon and A. Formigari contributed equally to this work.

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Biochemical and cytochemical studies have demonstrated that DNA of apoptotic chromatin has increased sensitivity to thermal denaturation. Thus, by using a gentle DNA denaturing agent such as formamide and heating to 75 °C, DNA denaturation can be induced only in condensed apoptotic chromatin [7]. In fact, formamide-induced DNA denaturation and staining with an anti-ssDNA Mab can detect condensed apoptotic chromatin and is useful for identification of apoptotic cells. The formamide-Mab procedure specifically stains apoptotic cells and clearly distinguishes apoptosis from necrosis.

The influence of Zn on apoptosis is a well-known phenomenon. In both, in vitro and in vivo models, Zn supplementation prevents apoptosis induced by a variety of agents [8]. One anti-apoptotic mechanism of Zn is its capacity to minimize oxidative damage to cellular organelles, thereby suppressing major signalling pathways leading to caspase activation and apoptosis [9]. Moreover, Zn was first described as an inhibitor of calcium magnesium-dependent endonuclease DNA fragmentation [10]. On the contrary, other transitional metals like Fe or Cu can cause oxidative DNA and protein damage by radicals generated in the Fenton reaction. Furthermore, Zn is a strong inducer of metallothioneins (MTs) that are able to bind Cu and other metals. MTs are small proteins (6000–7000 daltons) that are rich in cysteine residues and lack both aromatic amino acids and histidine [11]. Due to their multifunctional roles, MTs have been proposed as a chelator of heavy metals and excessive essential metals, and to act as scavengers for reactive oxygen species (ROS) [12]. Their synthesis is induced by heavy metals and several other factors. Therefore, induction of MTs, which bind several heavy metals with high affinity, is a common protective mechanism [13]. This induction depends on the metal-regulatory transcription factor (MTF-1), but the mechanism by which transcription of *mt* genes is activated has not been fully understood.

In mammalian cells, two major forms are expressed, MT-1 and MT-2, and most studies have reported that cells lacking MT-1 or MT-2 are more sensitive to a wide range of stressors, such as oxidative stress and excess of heavy metals. Colangelo et al. [13], in their experiments on MT+/+ cells, have shown that MT biosynthesis is readily induced by Cd treatment, with a concomitant decrease in sensitivity to injury by ROS. Indeed, in an MT−/− cell line, pre-treatment with Cd did not reduce but actually enhanced oxidative stress.

Mahboobi et al. [14] correlated the presence (MT+/+) or the absence of MT (MT−/−) in cells pre-treated with different concentrations of $ZnCl_2$, $CuSO_4$, and $CdCl_2$ for different time intervals to the response to a number of different anti-tumor drugs with different mechanisms of action. However, the antioxidant effect of Zn supplementation in relation to apoptotic and/or necrotic processes and MT isoform gene expression has not yet been clarified.

Herein we investigated the effects of Cu and Fe on cytotoxicity in a fibroblast cell line corresponding to an SV40-transformed MT-1/-2 mutant (MT−/−) and in wild type (MT+/+) as well as the role of Zn in cellular protection. Co-treatment in knock-out lines has the aim of clarifying the mechanism of action of Zn and verify if it directly reduces the apoptotic damage caused by Cu and Fe, or indirectly, by induction of MT-1 and MT-2 isoforms.

## 2. Materials and methods

### 2.1. Cell culture

The simian virus 40 (SV40) transformed mouse embryo fibroblast for MT-1 and MT-2 double nullizygous (knock-out) mice and normal mice of the same strain were provided by Dr. John Lazo, University of Pittsburgh were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) at 37 °C in an atmosphere of 5% $CO_2$.

### 2.2. Treatment with heavy metals

For heavy metal treatment, cells were exposed to complete medium containing: 50 μM $ZnSO_4$, 75 μM $CuSO_4$, 200 μM $FeSO_4$, 50 μM $ZnSO_4$ plus 75 μM $CuSO_4$ and 50 μM $ZnSO_4$ plus 200μM $FeSO_4$ for 24 h. These concentrations were estimated with the MTT cell proliferation assay, which is based on the cellular conversion of a tetrazolium salt into a formazan product [15]. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of living cells present (data not shown). Higher concentrations of these metals caused evident cytotoxic effects, whereas lower amounts were not able to induce MT. Control cells were allowed to grow in complete medium under the same experimental conditions.

### 2.3. Determination of metals and MT contents

Cells were cultured as described above (2.1 and 2.2). Cells at 75% confluency were washed twice with phosphate buffered saline (PBS) and harvested using 0.25% trypsin in 1 mM EDTA. Cells were sedimented by centrifugation at 180×*g* for 5 min, washed twice with PBS and re-suspended in 20 mM Tris–HCl buffer (pH 7.5) supplemented with 0.006 mM leupeptin, 0.5 mM phenylmethylsulphonylfluoride (PMSF) and 0.01% β-mercaptoethanol. Then, cells were sonicated twice on ice for 30 s in a Labsonic U instrument. Homogenates were centrifuged at 48,000×*g* for 50 min at 4 °C, and the resulting supernatants were used for metal and MT quantification. Concentrations of Cu, Zn and Fe were determined by atomic absorption spectrophotometry with an air-acetylene flame (Perkin-Elmer mod. 4000). MT concentrations were determined by the silver saturation method [16]. The sample values were normalized to the total protein content, assayed by the Folin phenol reagent method [17] using bovine serum albumin as the standard.

### 2.4. RNA purification

Total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Total RNA was determined by UV absorbance at 260 nm and its purity was estimated by the absorbance ratio $A_{260}/A_{280}$ nm. In addition, RNA integrity was confirmed by ethidium bromide staining of ribosomal RNA following gel electrophoresis.

### 2.5. Reverse transcription-PCR (RT-PCR) and product analysis

First-strand cDNA was synthesized with 1 μg total RNA, 50 pmol oligo (dT)18, 10 U Improm II Reverse transcriptase and 20 U RNase inhibitor in a final 20μl reaction mixture containing 5× reverse transcriptase buffer, 25 mM $MgCl_2$ and 10 mM dNTP. The reaction mixture was incubated at 25 °C for 5 min, at 42 °C for 1 h and at 70 °C for 15 min. PCR was carried out in a 50-μl reaction mixture containing 10 mM dNTP, 10 μM of each sense and antisense primer and 2.5 U of Taq DNA polymerase, in addition to 1 μl of cDNA. The sequence of sense and antisense primers for mouse MT-1 were 5′-tctcggaatggaccccaactg-3′ and 5′-tttacacgtggtggcagcgc-3, respectively, for mouse MT-2 were 5′-cgatctctcgtcgatcttca-3′ and 5′-ggagaacgagtcagggttgt-3′, respectively and for β-actin were 5′-ccagggtgtgatggtgggaatg-3′ and 5′-cgcacgatttccctctcagctg-3′, respectively. After an initial denaturation at 94 °C

for 2 min, amplification was carried out for 25 cycles of 30 s at 94 °C for denaturation, 30 s at 54 °C for annealing, and 30 s at 72 °C for extension with a final extension step at 72 °C for 5 min. The PCR products were analyzed by electrophoresis using a 2.0% (w/v) agarose gel, which was stained with ethidium bromide and visualized under UV light. The MT-1 and MT-2 mRNA levels were determined by RT-PCR analysis followed by densitometry scanning. All MT-1 and MT-2 RT-PCR products were normalized to the corresponding β-actin RT-PCR.

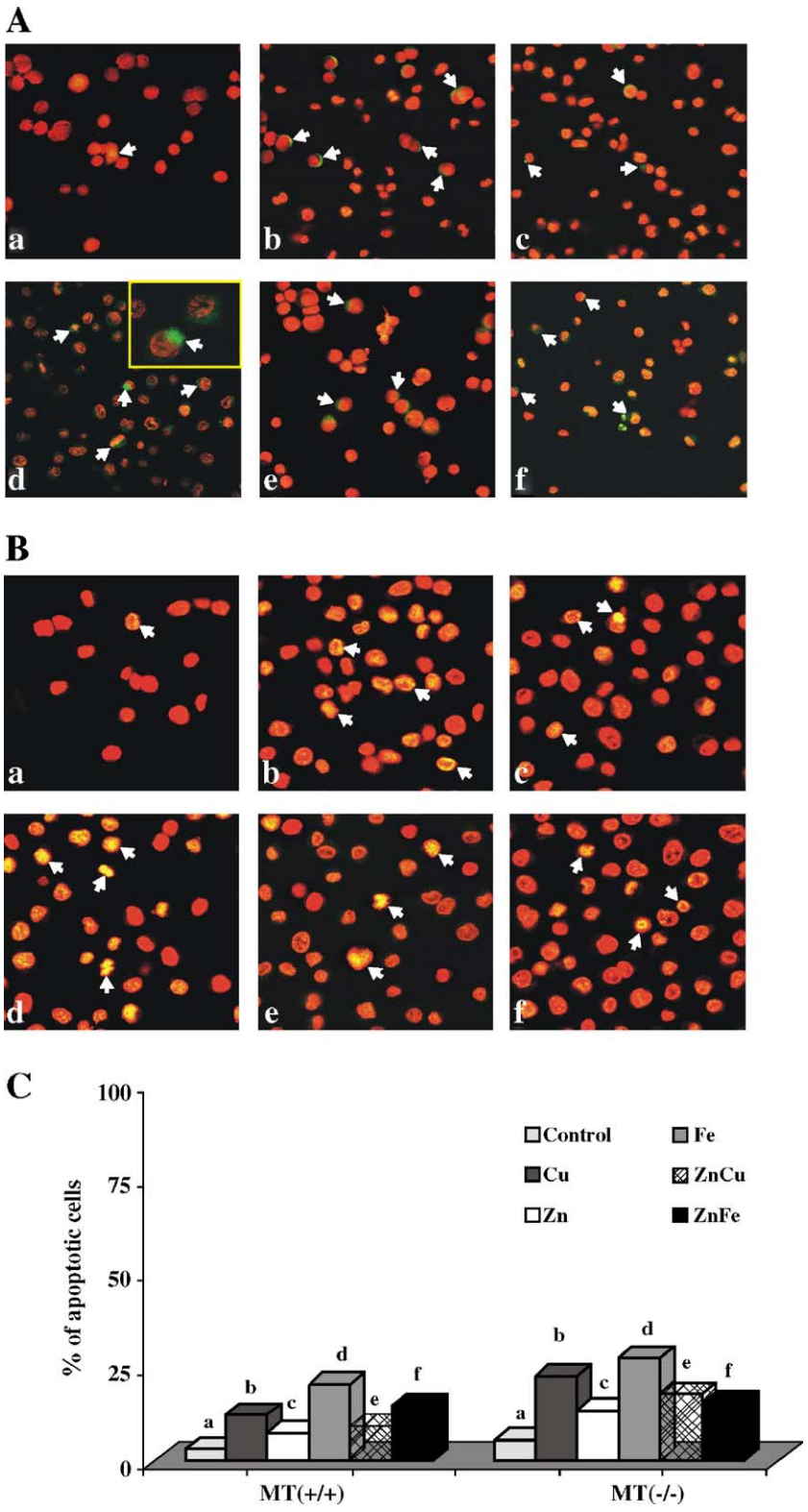


Fig. 1. Localization of apoptotic cells (arrows) using Mabs against ssDNA and counterstained with propidium iodide in wild type (MT+/+) (A) and in MT-null (MT−/−) cells (B) cell lines untreated (a) and treated with Cu (b), Zn (c) Fe (d), Zn+Cu (e) and Zn+Fe (f) for 24 h. ×60 objective (a–f). (C) Percentage of apoptotic cells. The different letters correspond to statistically significant differences for $P<0.05$.

### 2.6. DNA denaturation and staining with the Mab against ssDNA

Cells were fixed in methanol-PBS (6:1) for 24 h at −20 °C. Cells fixed were re-suspended in 250 μl formamide and heated in a water bath at 75 °C for 10 min. Cells were placed on ice for 10 min. Subsequently, cells were washed with 2 ml of 1% FBS in PBS, re-suspended in 100 μl Mab F7-26 (dilution 1:10 in PBS containing 5% FBS) and incubated at room temperature for 15 min. Cells were then rinsed with PBS and stained with fluorescein-conjugated anti-mouse IgM (dilution 1:50 in PBS containing 1% FBS) at room temperature for 15 min. Cells were then rinsed with PBS and re-suspended in 0.5 ml of PBS containing 1 μg/ml propidium iodide. The cells were prepared as a wet-mount on glass slides. The slides were observed under a Radiance 2000 confocal fluorescence microscope. The total cells and fluorescent cells were counted for each field examined (at least 10 fields per plate). The percentage of Mab-labeled cells was calculated as the number of FITC-stained cells divided by the total number of cells counterstained with propidium iodide. Data from at least three independent experiments were obtained and standard errors of measurement were calculated.

### 2.7. Annexin-V assay

Cells were washed with PBS and incubated with annexin-V FICT for 25 min at room temperature. Cells were then washed with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and fixed with 4% paraformaldehyde for 5 min at 4 °C. Then, cells were counterstained with propidium iodide (1μg/ml) for 5 min at room temperature and were observed under a Radiance 2000 confocal fluorescence microscope.

### 2.8. Statistical analysis

The experiments were set up in triplicate and the analyses of each sample were repeated at least twice. Results are reported as the mean±S.D. Statistical analysis was performed with the Primer statistical program. Statistical differences were calculated with one-way analysis of variance (ANOVA) with a Student–Newman–Keuls follow-up test. Significant difference was considered when $P<0.05$.

## 3. Results and discussion

In this study, we analyzed the effects of treatment with Zn, Cu, Fe, Zn+Cu and Zn+Fe by valuing MT and metal content (Zn, Cu and Fe), levels of both MT-1 and MT-2 mRNAs, and localization both of apoptotic and necrotic cells

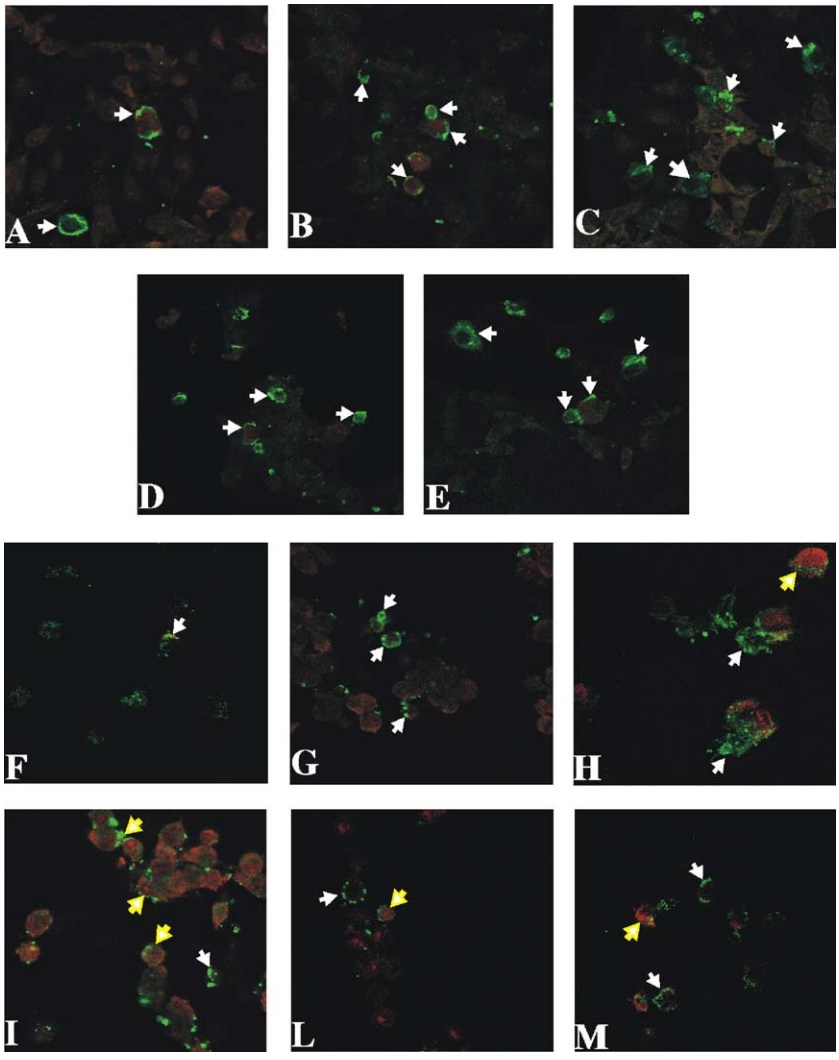


Fig. 2. Localization of apoptotic (white arrows) and necrotic (yellow arrows) cells by annexin-V FICT and counterstained with propidium iodide in the wild type (MT+/+) cell line treated with Zn (A), Cu (B), Fe (C), Zn+Cu (D) and Zn+Fe (E) and MT-null (MT−/−) cell lines untreated (F) and treated with Cu (G), Zn (H) Fe (I), Zn+Cu (L) and Zn+Fe (M) for 24 h. ×60 objective (A–M).

using the high affinity binding of Annexin V to PS, and determining the apoptotic index by Mab against ssDNA in MT−/− and MT+/+. A major objective was to clarify the antioxidant role of Zn in the fibroblast cell line corresponding to an SV40-transformed MT-1/-2 mutant that does not express mt-1/-2 genes. It has been reported that Zn exerts its effect in an indirect manner by stabilizing the structure of the cell membrane and by maintaining high MT tissue concentrations for extended periods of time [3,5]. The purpose of our study was that to verify if Zn can exert its protective action even in absence of MT expression. It is known that Zn has a high capacity to induce the MT. It has been shown that both MT-1 and MT-2 isoforms are also essential for a normal wound repair in the CNS, to reduce oxidative stress and neuronal apoptosis and their deficiency impairs neuronal survival. In particular, Penkowa and Hidalgo [18] examined the expression and roles of neuroprotective MT-1 and MT-2 in the rat CNS, in experimental autoimmune encephalomyelitis (EAE), administered Zn-MT-2 intraperitoneally during autoimmune disease. They demonstrated that only Zn-MT-2 and not Zn content decreased oxidative stress. Probably, Zn used concentration (0.35 μg/10 g body wt) was too low to induce MT synthesis [19]. Moreover, an extracellular action of MT is also proposed in the recovery of the CNS from injury [20]. On the other hand, the ability of Zn in the stabilization of sulfhydryls or reduction in the formation of $^{\bullet}OH$ from $H_2O_2$ and superoxide through the antagonism of redox-active transition metals such as Fe and Cu has been shown in various systems [21,22]. Holme et al. [23] report that $ZnCl_2$ blocked the degradation of pro-caspase and the induction of apoptosis, on contrary Lin et al. [24] suggest that in vivo Zn-induced oxidative stress may result in apoptosis.

### *3.1. Effect of co-treatment of Zn+Cu and Cu alone on wild type and MT-null cell lines*

Our recent findings showed that both Cu accumulation and Zn supplementation increases the tissue content of MT, whereas Zn treatment decreases MTox (oxidized-MT) in LEC rat liver. In this context, the large amount of MT, induced by Zn and Cu, is probably the manifestation of a defense mechanism against Cu accumulation, while the low levels of MTox can be considered as an index of low oxidative stress resulting in reduced cell death [25].

Apoptotic cells were localized using Mab anti single-strand DNA (ssDNA) and the combination of FITC-conjugated annexin V, which preferentially binds the newly exposed PS on apoptotic cells, and dual staining with propidium iodide that permits the differentiation of apoptotic from necrotic cells by confocal microscopy analysis. The exposure of PS has been demonstrated to occur during the effector phase, probably downstream of changes in the mitochondria, as Bcl-2 inhibited the release of mitochondrial proteins and PS exposure [26].

In fact our results indicate that in MT+/+ cells co-treatment with Zn–Cu reduced both apoptotic and necrotic cells of 24% compared to treatment with Cu alone as shown in Figs. 1 and 2. However, we also observed that in the MT−/− cells there was a decrease of 22% in the cell death index after Zn exposure. In the MT+/+ cells, the apoptotic index reduction in the co-treated cells is probably in relation to the increased synthesis of MT induced by Zn (Table 1 and Fig. 3) and its role antioxidant and scavenger versus ROS. On the other hand, in the MT−/− cells, the apoptotic index reduction can be in relation only with the Zn treatment. The Zn potential regulatory roles earlier in the apoptotic pathway have been show in some cell types. In human premonocytic cells induced to undergo apoptosis by hydrogen peroxide, 1 mM Zn supplementation increased the ratio of Bcl-2 to Bax, a proapoptotic protein, resulting in the inhibition of active caspase-3 and a reduction of apoptotic cells [27]. Aiuchi et al. [28] reported that Zn, at concentrations above 100 μM, inhibited the processing of caspase-3 in HL-60 cells that had been treated with geranylgeraniol. However, Perry et al. [29] demonstrated a dose-dependent inhibition of caspase-3 cleavage of PARP from 30 to 100 μM zinc.

On this basis, we analyzed the metal contents in both MT+/+ and MT−/− cells. As shown in Table 1, Cu concentrations were significantly higher in both MT+/+ and MT−/− cells treated with Zn+Cu compared to cells treated with Cu alone (2.0 and 1.3 fold, respectively). In the MT+/+, the increase in Cu content could be attributable to the formation of a Cu–MT complex. It is well-known that Zn is a more potent inducer of MT than Cu; however, MT has a much higher affinity for Cu than Zn, resulting in Cu sequestration into MT. Moreover, the MT content in Zn+Cu treated cells was significantly higher (1.25 fold) than in Cu treated cells. The MT concentrations in

Table 1
Cu, Zn, Fe, and MT levels in wild type (MT+/+) and MT-null (MT−/−) cell lines untreated (control) and treated with Cu, Zn, Fe, Zn+Cu and Zn+Fe for 24 h

| Treatment | MT+/+ | | | |
|---|---|---|---|---|
| | Cu | Zn | Fe | MT |
| Control | 0.01±0.00*[a] | 0.26±0.02*[a] | 0.06±0.00*[a] | 6.10±0.32* |
| Cu 75 μM | 0.05±0.00○[a] | 0.20±0.01○[a] | 0.07±0.00○[a] | 6.20±0.70* |
| Zn 50 μM | 0.03±0.00‡[a] | 0.57±0.01†[a] | 0.05±0.01*[a] | 9.25±1.27○ |
| Fe 200 μM | 0.01±0.00*[a] | 0.28±0.01*[a] | 0.47±0.03●[a] | 6.12±0.09* |
| Cu 75 μM+Zn 50 μM | 0.10±0.00●[a] | 0.86±0.03‡[a] | 0.03±0.00‡[a] | 7.79±0.46† |
| Zn 50 μM+Fe 200 μM | 0.03±0.00‡[a] | 0.60±0.00●[a] | 0.33±0.02¤[a] | 7.34±0.08† |
| Treatment | MT−/− | | | |
| | Cu | Zn | Fe | MT |
| Control | 0.01±0.00*[a] | 0.27±0.01*[a] | 0.11±0.01*[b] | – |
| Cu 75 μM | 0.10±0.01†[b] | 0.28±0.01*[b] | 0.02±0.01‡[b] | – |
| Zn 50 μM | 0.01±0.00*[a] | 0.67±0.02●[b] | 0.03±0.01‡[b] | – |
| Fe 200 μM | 0.01±0.00*[a] | 0.30±0.01*[b] | 0.52±0.07●[b] | – |
| Cu 75 μM+Zn 50 μM | 0.13±0.01‡[b] | 0.32±0.00†[b] | 0.05±0.00‡[b] | – |
| Zn 50 μM+Fe 200 μM | 0.01±0.00*[a] | 0.50±0.01‡[b] | 0.71±0.01¤[b] | – |

Values are indicated as the mean±S.D. and expressed as μg/mg total proteins. The different symbols correspond to statistically significant differences among the different treatment groups, whereas the various letters correspond to statistically significant differences between the two cell lines for $P<0.05$.

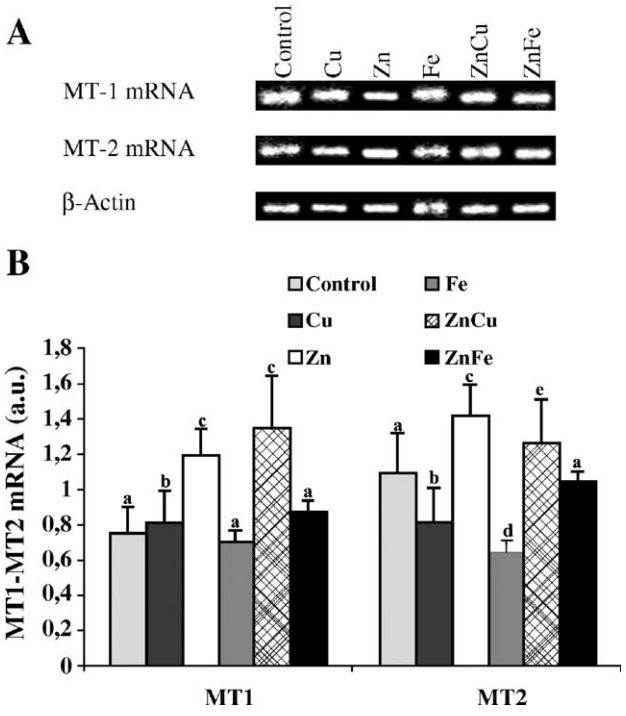


Fig. 3. Expression of metallothionein (MT) isoforms (arbitrary units) in a wild type (MT+/+) cell line after treatment with Cu, Zn, Fe, Zn+Cu and Zn+Fe for 24 h. (A) PCR products in agarose gel electrophoresis. (B) Levels of MT-1 and MT-2 mRNAs under various treatment conditions. The results are expressed as the mean±S.D. The different letters correspond to statistically significant differences for $P<0.05$.

the MT+/+ cells treated with Zn and Zn+Cu were 1.5 and 1.3 fold, respectively, higher than in control cells, whereas no differences were observed between Cu treated cells compared to control cell cultures (Table 1). In accordance with a large amount of MT synthesis, Fig. 3 shows that both MT-1 and MT-2 mRNAs were highly induced following Zn and Zn+Cu treatments (1.6 and 1.8 fold, respectively, for MT-1, and 1.3 and 1.2 fold for MT-2 compared to control cells), which agrees with the observed selectivity of zinc-induced MTF-1 binding to an MRE sequence [30]. In particular, treatment of mammalian cells with Zn in vivo promotes rapid nuclear translocation of MTF-1 [31] and causes a dramatic increase in MTF-1 DNA binding activity measured in vitro concomitant with the occupancy of MREs in the MT-1 promoter in vivo and the activation of MT-1 gene expression [32]. However, multiple binding factors may further modify these metal-regulated processes [33]. The increased expression of MT-1 and MT-2 mRNAs in MT+/+ cells was associated with increased protection against Cu toxicity as well as the harmful effects of free radicals generated by the Fenton reaction.

Jiang et al. [34] demonstrated that Zn-induced MT was able to bind intracellular Cu, quench the redox cycling activity of Cu, inhibit Cu-dependent oxidative stress in membrane phospholipids, and prevent Cu dependent apoptosis in HL-60 cells. However, it is important to point out that the Cu-bound MT is redox inactive in the absence of oxidative stress [35].

On the other hand, in MT−/− cells, the Cu increase in the co-treated group is probably related to the increase in GSH content and SOD activity (data not shown). In fact, Cu is present in the catalytic site of enzyme. Moreover, the presence of free metal with redox properties, such as Cu, may support the formation of free radicals and, as a consequence, the induction of SOD. We also observed that the level of Cu in MT−/− cells after both Cu and Zn+Cu treatment was significantly higher than in MT+/+ cells (Table 1). On this basis, we postulated that Cu-MT is more rapidly degraded by lysosomes than other biomolecules involved in Cu accumulation. The sequestration of excess Cu binding MT by lysosomes [36] may be of general importance for the homeostasis of Cu in cells. In MT−/− cells, we observed a reduction in Zn content in Zn+Cu treated cells (about 50%) compared to cells treated with Zn alone. This reduction may be related to competition between metals at uptake. Membrane transporters in the mechanism of metal homeostasis are also gaining attention and one explanation is that the presence of large amounts of Zn and Cu and in the absence of MT such as in MT−/− cells, the induction and synthesis of ZnT increases. On the other hand, in wild type cells we observed an increase in the Zn content in co-treated cells compared to cells treated with zinc alone. Furthermore, we also observed that in MT+/+ cells treated with Zn+Cu, the Zn content was significantly higher with respect to that in MT−/− cells (2.7 fold). We hypothesize that in null type cells the transcription factor, MTF-1, which responds to changes in intracellular Zn, not mediating MT gene induction, is involved to a greater extent in the ZnT-1 gene transcription facilitating Zn efflux from the cytoplasm [37]. This hypothesis must be supported by experiments on Zn transporters and on the interactions between Cu and Zn at these specific binding sites. Genes encoding mammalian zinc transporters have been cloned and the corresponding proteins have also been studied [38]. For example, ZnT-2 promoters have multiple metal response elements, as does the MT promoter, and this may support the competition between Cu and Zn [39]. However, it is also possible that, due to the accumulation of Cu, changes in subcellular distribution of metals occurred, since we evaluated the metal content only in the supernatant fraction and not in the total homogenate.

Given the present data, we favor the hypothesis that the protective effect of Zn against oxidative stress is mainly due to the induction of MT, rather than a direct action, even if the role of Zn alone in cellular protection can not be minimized.

### *3.2. Effect of co-treatment of Zn+Fe and Fe alone on wild type and MT-null cell lines*

It is well known that Fe is potentially deleterious as it can induce the formation of ROS through reduction of $H_2O_2$ to $^{\bullet}OH$ via the Fe-catalyzed Haber–Weiss reaction [40,41]. We observed that in MT+/+ Fe-treated cell death decreased of 28% after Zn exposure. Sreedhar et al. [42] suggest that Zn reduces the Fe-induced hydroxyl radical production by an increase of GSH and MT levels and maintaining the sulphydryl group stability. It has been also demonstrated that MTs are able to protect the cells against apoptosis/necrosis induced by oxidative stress by iron binding [43]. Probably Zn inhibits Fe-induced hydroxyl radical generation through Fenton reaction by

inducing the levels of free radicals quenchers such as MT and by competitive inhibition of iron uptake. In fact as expected, and in agreement with the findings of many others MT+/+ cells exposed to Zn showed MT upregulation, as demonstrated by silver saturation assay. This method revealed that a significant amount of MT protein was present in MT+/+ cells treated with Zn+Fe (1.3 fold higher than in control cells) despite the fact that the low endogenous levels of MTs mRNA were found using RT-PCR analysis. It is likely that this is related to the different induction and degradation times of ZnFe-MT with respect to Zn-MT and ZnCu-MT. The possibility of multiple mechanisms on fibroblast ZnFe-MT accumulation should be considered in studies specifically examining this aspect. We also observed that MT-2 mRNA levels in Zn+Fe treated cells was significantly higher than MT-1mRNA (1.2 fold). This supports the hypothesis that a particular metal could enhance the expression of a specific isoform of MT. However, the contribution of other factors such as MREs (metal response elements) and/or transcription factors should be investigated in more detail to better clarify their role.

Furthermore, we found that Zn+Fe exposure caused Zn accumulation with concomitant decrease (about 40%) in Fe content. The reduction of Fe content in Fe+Zn cells compared to Fe cells could be attributed to competitive inhibition of iron uptake by the divalent metal transporter in the presence of zinc. Divalent metal transporter 1 (DMT1) is predominantly a Fe transporter. However, functionally, DMT1 can mediate import of many other divalent metal ions, including Zn [44]. However, in both cell death assays, we observed that Zn exposure in combination with Fe, compared Fe exposure alone, greatly reduced both apoptotic and necrotic index. The reduction of cell death in the presence of Zn could be due to reduction in the oxidative stress. In fact, the SOD/GPX ratio, index of oxidative stress, was significantly lower in the Fe+Zn treated cells than treated Fe cells (data non shown). These results suggest that the protection exerted by Zn during Fe-mediated cell death could be due to both increased levels of MT and reduction of Fe content. On the other hand, also in the mutant cells we found that cell death index was lower (about 44%) after treatment with Fe+Zn compared to treatment with Fe alone. These results indicate that other mechanisms could be involve in the cell protection against oxidative stress induced by iron. In the MT−/− cells, we did not observe competition between two metals at level of uptake; in fact Fe content was higher in the Fe+Zn treated cells than Fe treated cells. Furthermore, in this cell line the MTs are not produced. Probably the absence of MT could result in the redistribution of zinc from one target within a cell to another so that the amount of free Zn increase inside the cell.

The induction and inhibition of apoptosis involves signal transduction pathways, and the response is modified by the concentration of free intracellular divalent metal ions. Indeed, the trace metal zinc is an essential component of many transcription factors and signal transduction molecules that regulate aspects of cellular metabolism, and zinc levels influence the regulation of mitosis and apoptosis. In particular, zinc supplementation in thymocytes inhibits apoptosis induced by glucocorticoids and γ-irradiation, and in vivo zinc deficiency leads to marked thymic atrophy (acrodermatitis enteropathica) and immunodeficiency [9]. Pre-treatment with Zn may induce cytoprotective pathways that reduce susceptibility to apoptosis. Thus, it seems possible that sufficiently lowering zinc concentrations in the cytosol may directly release the inhibition of caspases by zinc [45].

We also observed that in mutant cell line Zn seems to be a more efficient inhibitor of Fe-induced cell death relative to Cu-induced cell loss. In fact, the cell death Fe- and Cu-induced was lower of 44% and 22%, respectively. This response could be correlated to Zn different content in the two groups. We found that the Zn content was about 1.5 fold higher in the cells treated with Zn+Fe compared cells treated Zn+Cu.

However, we observed that the apoptotic index in MT−/− cells was significantly increased compared to that observed in MT+/+ cells under all treatment conditions (Fig. 1). It is also interesting to point out that in MT+/+ cells co-treatment reduced both apoptotic and necrotic cells compared to treatment with Fe alone as shown in Figs. 1 and 2 (40% for Zn+Fe compared to Fe). Moreover, previous reports have indicated that Zn has a key role in the regulation of apoptosis in a number of cell types [46]. Indirect evidence for additional sites for Zn regulation include the dependence of zinc-finger transcription factors that control the expression of cellular genes involved in apoptosis, such as nuclear factor-κB [47]. In conclusion, Zn most likely has multiple regulatory roles in protecting cells from apoptosis. Further studies are necessary in order to elucidate the specific regulatory mechanisms underlying zinc protection against apoptosis in many cell types. On this basis we believe that Zn alone could be able to reduce in part oxidative damage, but that concomitant action of MT and Zn alone play key role in the protection against Fe toxicity as well as the harmful effects of free radicals generated by the Fenton reaction.

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