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Original Article

Induction of cytotoxicity in human cells exposed to thallium(I) and thallium(III)

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ABSTRACT

Cytotoxicity induced by thallium(I) acetate, thallium(I) sulfate, and thallium(III) chloride was studied using human peripheral lymphocytes and four different *in vitro* assays. In all cases, the cells were treated with 0.5, 1, 5, 10, 50, and 100 µg/mL of the three thallium compounds and were evaluated 2, 4, 8, and 24 h later. The three thallium compounds reduced cell viability and increased cell death *via* apoptosis and necrosis in a concentration- and time-dependent manner. Methyl tetrazolium reduction assay showed the highest sensitivity for recording thallium-induced cytotoxicity. When cells in the G_0 phase were treated with thallium compounds, the mitotic index decreased in a concentration-dependent manner. These results suggest that the cell membrane is the target of thallium. Both oxidation states induced cell death at the lowest concentrations tested (0.5 and 1 µg/mL) and with short-term exposure (4 and 8 h), which decreased the cellular proliferative capacity. These results indicate that mitochondria are the most sensitive to the effects of thallium.

KEYWORDS: viability, apoptosis, necrosis, proliferation, cytotoxicity, thallium.

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INTRODUCTION

Recent reviews of thallium (Tl) toxicity highlight the need to obtain relevant information about its two main oxidation states (I and III) using different methodologies, including *in vitro* assays aimed at determining the cytotoxic potential [1-3]. Tl is a metal that has acquired special attention because of its applications in different fields, its industrial uses, its environmental occurrence, and its high toxicity at very low concentrations [4, 5]. TI, which is a cumulative poison that is completely absorbed following ingestion, inhalation, or cutaneous contact, is distributed throughout the body by systemic circulation subsequent to its binding to transferrin in the blood cells. Its major route of elimination is through urine and feces, and in cases of poisoning it has also been detected in hair, and nails, which are considered good biomarkers of exposure; in this regard, recent reports concerning accidental and homicidal poisoning with Tl salt solutions have increased [1, 6, 7]. Smoking was recently confirmed as a Tl source, and TI levels in smokers are significantly higher than those in nonsmokers [8]. Tl compounds to which humans are exposed include acetates, chlorides, sulfates, and oxides [1]. Tl is used in homeopathic medicine to treat peripheral neuropathy, muscular incoordination, and skin diseases [9, 10]. Nevertheless, its traditional homeopathic usage has not been reviewed.

Several mechanisms have been proposed to explain the toxicity of Tl, including inhibition of

Na^+/K^+ ATPase pumps, depolarization of the mitochondrial membrane, interference with the antioxidant defense system of the cell, interaction of Tl cations with amino-sulfhydryl groups, and disruption of calcium homeostasis [1, 2]. Recently, three of its compounds, Tl(I) acetate, Tl(I) sulfate, and Tl(III) chloride, have been considered as cytotoxic and genotoxic compounds as they alter viability and proliferation and induce DNA and chromosomal damage in human cells following *in vitro* exposure [11, 12]. However, there is still much to explore regarding the mode of action of Tl, and combinations of several methods open the possibility to collect more information about the toxic potential of these compounds. Examples of these methods include a fluorochrome-mediated assay using dual 5,6-carboxyfluorescein diacetate/ ethidium bromide (CFDA/EB) or acridine orange/ ethidium bromide (AO/EB) staining, a methyl tetrazolium (MTT) colorimetric assay, and measurement of the mitotic index (MI) in cell cultures, which are the most commonly employed techniques for evaluating cell viability, cell death, and alteration in cell proliferation following exposure to xenobiotics. Thus, the aim of this study was to evaluate the cytotoxicity induced by Tl(I) and Tl(III) compounds using human peripheral lymphocytes and applying different *in vitro* assays to measure viability, cell death, and proliferation.

MATERIALS AND METHODS

All materials were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

Cell collection and treatments

Heparinized (heparin; Inhepar PiSA®, Mexico, Mexico) blood samples were obtained by venopuncture from two healthy nonsmoking males without histories of recent exposure to toxic chemicals or radiation. The lymphocytes were separated using a gradient method with Histopaque®-1077 as described by the manufacturer. Next, 2×10^6 cells were placed into an Eppendorf tube containing 1 mL of RPMI-1640 culture medium (Gibco, Grand Island, NY, USA) and Tl compound. Thallium(I) acetate ($TlCH_3COO$; CAS No. 563-68-8; purity 98%), Tl(I) sulfate (Tl_2SO_4; CAS No. 7446-18-6; purity 99.99%), and Tl(III) chloride ($TlCl_3 \cdot 4\ H_2O$; CAS No. 13453-33-3; purity 98%) were each dissolved separately in distilled water, then sterile-filtered through a 0.22-mm filter (Whatman, Maidstone, UK) and added to the culture medium at concentrations of 0.5, 1, 5, 10, 50, and 100 µg/mL (which corresponds to 1.9, 3.8, 19.0, 38.0, 189.8, and 379.6 µmol/L for $TlCH_3COO$; 1.8, 3.7, 18.5, 37.0, 184.9, and 369.8 µmol/L for Tl_2SO_4; and 1.3, 2.6, 13.1, 26.1, 130.6, and 261.2 µmol/L for $TlCl_3$).

All experiments were performed in triplicate (except for the MI assay). The cells were incubated at 37 °C, and evaluated 0, 2, 4, 8, and 24 h after adding the metallic compound. The 0-h evaluation was performed within 0-15 minutes after adding the chemical compound. At all times, an untreated control was available. Cadmium(II) chloride (228 µg/mL of $CdCl_2 \cdot 2.5\ H_2O$; CAS No. 7790-78-5; purity 79.5-81%) was used as a positive control [13].

CFDA/EB assay

As described by Takasugi (1971) [14], the cells were collected by centrifugation, and 20 µL cells were resuspended in 20 µL of a solution containing 0.125 µg/µL CFDA and 0.025 µg/µL EB. After 5 min, the cells were washed three times with RPMI-1640 medium (1 mL at 37 °C), and the cell viability was analyzed using a fluorescence microscope (Nikon Optiphot-2, Nikon, Japan) with florescence equipment (510-560 nm; G2A filter) at a magnification of 40×. For each treatment, 600 random cells (100 cells from each triplicate experiment, with the samples of two donors) were evaluated, distinguishing viable cells (green fluorescence in the cytoplasm) from nonviable cells (compromised cells with green fluorescence in the cytoplasm and red fluorescence in the nucleus, and dead cells with red fluorescence in the nucleus).

AO/EB assay

The quantification of cell viability and apoptotic cells was performed *via* AO/BE uptake [15]. Following treatment, the cells were collected by centrifugation, and 20 µL of cells were resuspended in 1 µL of an AO/EB dye mix (100 µg/mL of AO and 100 µg/mL of EB) prepared in phosphate-buffered saline (PBS). The cells were plated on a

clean slide and analyzed immediately using a fluorescence microscope (G2A filter) at a magnification of 40×. For each treatment, 600 random cells (100 cells from each triplicate experiment with the samples of two donors) were evaluated, distinguishing viable cells from apoptotic and necrotic cells. Using the data obtained, we estimated the cell death index as follows: cell death index = (cells with apoptotic nuclei and bright green fluorescence + cells with apoptotic nuclei and bright orange fluorescence + necrotic cells that have a uniform orange color) / total number of cells scored) × 100. The morphological features of apoptotic cells such as cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation can most easily be identified using microscopy. Apoptotic cells differ substantially from necrotic cells in morphology. Cells undergoing the stages of necrosis burst and lyse their contents, resulting in "cell ghosts".

MTT assay

The MTT assay is based on the protocol described by Mosmann (1983) [16] with some modifications. Briefly, after treatment, the cells were incubated at 37 °C for 4 h with 0.25 mg/mL (10 µL of 5 mg/mL) of MTT dissolved in PBS. Washing with saline buffer medium (1 mL) was followed by the addition of DMSO (0.1 mL) and gentle shaking for 15 min so that complete dissolution of the formazan product was achieved. The cell samples were centrifuged, and the resulting solutions were transferred to 96-well plates, and the optical density was measured at 550 nm with a reference wavelength of 630 nm using a microplate spectrophotometer system (Epoch BioTek, USA). We performed 6 experiments for each treatment (the experiments were performed in triplicate with samples from two donors). The mitochondrial activities were calculated using the obtained data and are presented as percentages of the control values using the following formula: MTT reduction = (optical density of the treated group / optical density of the control group) × 100.

MI assay

After 24 h of treatment with 1, 10, or 100 µg/mL of Tl compounds, the culture medium was removed, and cell viability was evaluated using the trypan blue exclusion method. A total of 2×10^6 viable cells were placed in a 15-mL tube followed by culture in 5 mL of fresh Tl-free PB-MAX karyotyping medium (Gibco, Grand Island, NY, USA) at 37 °C for 72 h. In all cases, 0.02 µg/mL of colcemid (Gibco® KaryoMAX®; ThermoFisher, Waltham, MA USA) was added to each culture 2 h before harvest. All cultures were set up in duplicate. The cultures were harvested and fixed as described previously [11]. Slides were made using the flame-drying technique and later stained with Giemsa solution (Gibco® KaryoMAX®; ThermoFisher). All slides were coded for analysis, and 16,000 cells were counted to estimate the MI for treatment (4,000 for cultures with samples from two donors).

Statistical analysis

The results are shown as the means ± standard deviations. Significant differences in the CFDA/EB, AO/EB, and MTT assays were determined using the nonparametric x^2-test, while data from the MI assay were analyzed using the *z*-test. The probability values of $p < 0.05$ were considered significant, and $p < 0.01$ was considered highly significant.

RESULTS

CFDA/EB analysis

CFDA/EB analysis of cells exposed to different concentrations of Tl compounds is presented in Figure 1. Evaluations were performed 0, 2, 4, 8, and 24 h after treatment, and each experimental group was compared with its control and 0 h treatment samples. In general, Tl(I) acetate, Tl(I) sulfate, and Tl(III) chloride reduced viability in a similar manner, showing concentration- and time-dependent effects (Figure 1A, 1B, and 1C, respectively). No significant differences were found in the living cells of the control group at different assessment times or in comparison to the control with the 0 h sample of each concentration of the Tl compounds.

The three Tl compounds reduced viability from the 4th hour of exposure at concentrations of 50 and 100 µg/mL ($p < 0.05$), an effect that is observed at 24 h exposure for all concentrations applied ($p < 0.01$ compared with the control;

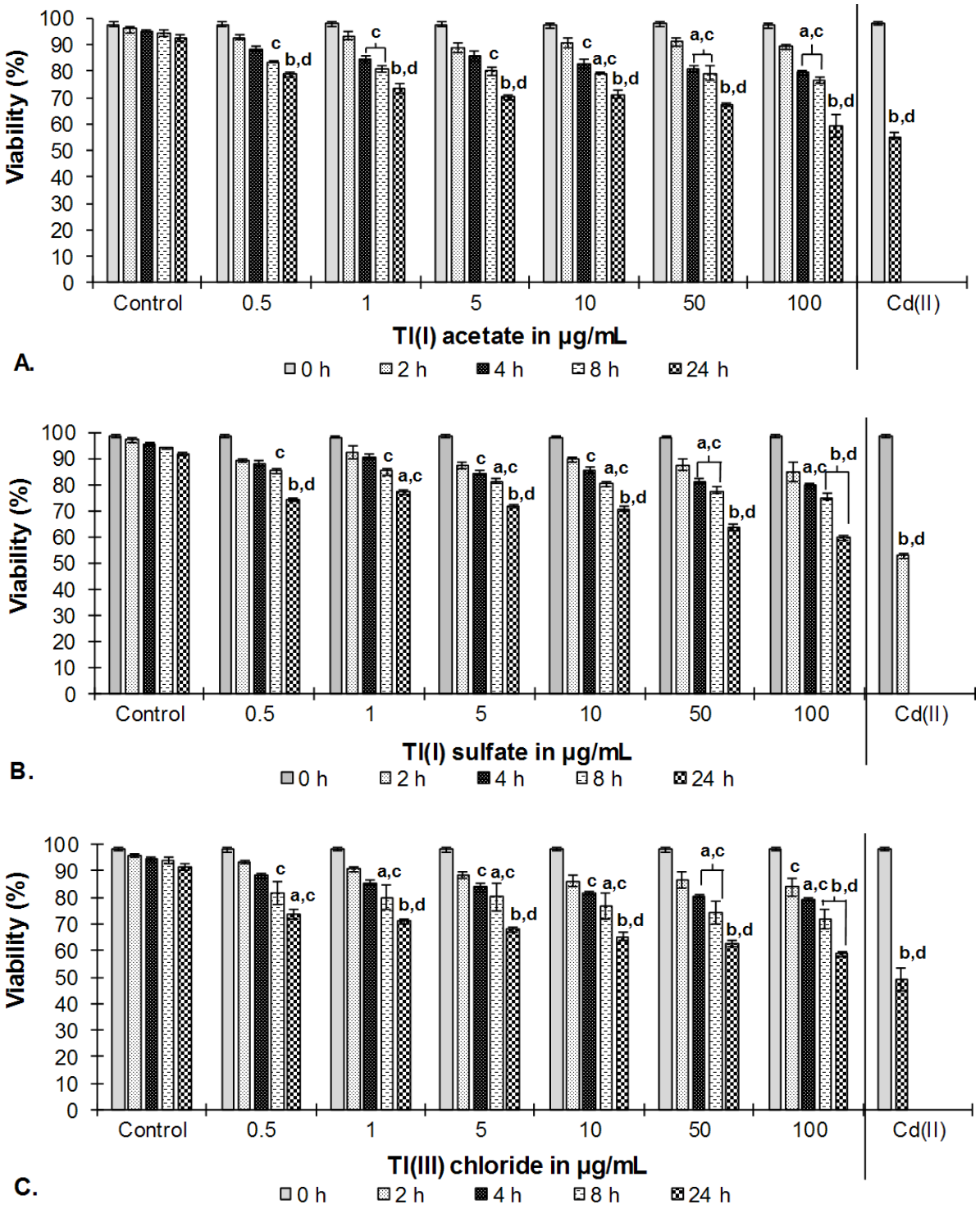

Figure 1. Viability of human lymphocytes treated with 0.5, 1, 5, 10, 50, and 100 µg/mL of Tl compounds using the CFDA/EB assay. Evaluations were performed immediately after treatment (0 h) and 2, 4, 8, and 24 h later. (A) Tl(I) acetate, (B) Tl(I) sulfate, and (C) Tl(III) chloride. In all cases, an untreated control was available, and Cd(II) chloride (228 µg/mL) was used as a positive control. The experiments were performed in triplicate using samples from two donors, and the data were expressed as the means ± standard deviation (n = 6). Statistical differences are shown as $^{a}p < 0.05$, $^{b}p < 0.01$ compared with the control, and $^{c}p < 0.05$, $^{d}p < 0.01$ compared with the 0-h exposure.

Figure 1). However, when comparing the data with the 0-h exposure samples, we found statistically significant differences in the samples exposed for 4 h to 1 µg/mL of Tl(I) acetate (Figure 1A), in the samples exposed for 4 h to 5 µg/mL of Tl(I) sulfate (Figure 1B) and in the samples exposed for 2 h to 100 µg/mL of Tl(III) chloride (Figure 1C), an effect that was maintained at 24 h exposure for all concentrations.

The CFDA/EB value of the positive control showed that Cd(II) chloride at a concentration of 228 µg/mL reduced viable cells 24 h after treatment, with cell viability ranging from 55-49%. A time-dependent response was observed for all time points (0, 2, 4, 8, and 24 h) for Cd(II) (data not shown), indicating that the lymphocytes were maintained under appropriate conditions. The same experimental conditions were maintained for the AO/BE and MTT assays.

AO/EB analysis

Morphological changes in the cell structure were observed by AO/EB double staining after exposure to Tl compounds. Using apoptosis (early and late) and necrosis data, the cell death index was calculated as a parameter of cytotoxicity. Cells treated with the highest concentrations of Tl(I) and Tl(III) showed some significant differences from the 2nd hour of exposure ($p < 0.05$), and the differences increased with concentration and exposure time ($p < 0.01$ compared with the control group; Figure 2). Generally, the three Tl compounds tested induced apoptosis, and the number of necrotic cells, early apoptotic cells and late apoptotic cells increased, where the increase followed the same trend (data not shown). Cells treated with Tl(I) acetate (Figure 2A) showed a significant increase in the cell death index at all concentrations tested following 4 h exposure and following 8 h or 24 h exposure for Tl(I) sulfate (Figure 2B) or Tl(III) chloride (Figure 2C) compared with the control. However, when the data was compared with the 0-h exposure samples, significant differences were found at all concentrations following 2-h exposure to Tl(I) sulfate and 4-h exposure to the other two Tl compounds.

Regarding the AO/EB assay, in the cell cultures treated with Cd(II) chloride at a concentration of 228 µg/mL, necrotic, and apoptotic damage increased, where the increase followed the same trend.

MTT analysis

The MTT assay was used to measure cellular metabolic activity. The results demonstrated a time-dependent effect of Tl compounds on human lymphocytes treated *in vitro* (Figure 3). MTT reduction was observed from the 2nd hour of exposure to Tl(I) acetate and Tl(III) chloride, while Tl(I) sulfate showed significant differences from the 4 h exposure compared with the respective control and 0 h groups ($p < 0.01$) (Figure 3A, 3B, and 3C, respectively). Treatments with the three Tl compounds at each tested concentration showed time-dependent effects. However, these results showed no concentration-dependent effects.

When human lymphocytes were exposed to Cd(II) chloride at a concentration of 228 µg/mL, a clear cytotoxic effect was observed.

MI analysis

For MI evaluations, we selected three concentrations based on the data collected in the CFDA/EB, AO/EB, and MTT assays. The concentrations used were 1, 10, and 100 µg/mL of each Tl compound. The MI percentage decreased following all Tl treatments in a concentration-dependent manner (Figure 4): Tl(I) acetate reduced the MI to 20, 37, and 55%, Tl(I) sulfate reduced the MI to 18, 34, and 58%, and Tl(III) chloride reduced the MI to 17, 28, and 48% compared with the respective control groups (Figure 4A, 4B, and 4C).

The results obtained for the MI value of the positive control showed that Cd(II) chloride at a concentration of 228 µg/mL inhibited cell division; the MI decreased approximately 59-65%. These results confirmed the potential cytotoxicity of metals and, similar to other assays, indicate that the human cell cultures and treatments were handled properly.

DISCUSSION

The CFDA/EB assay is one of most commonly used tools for rapid evaluation under a microscope. The results of the present study revealed that

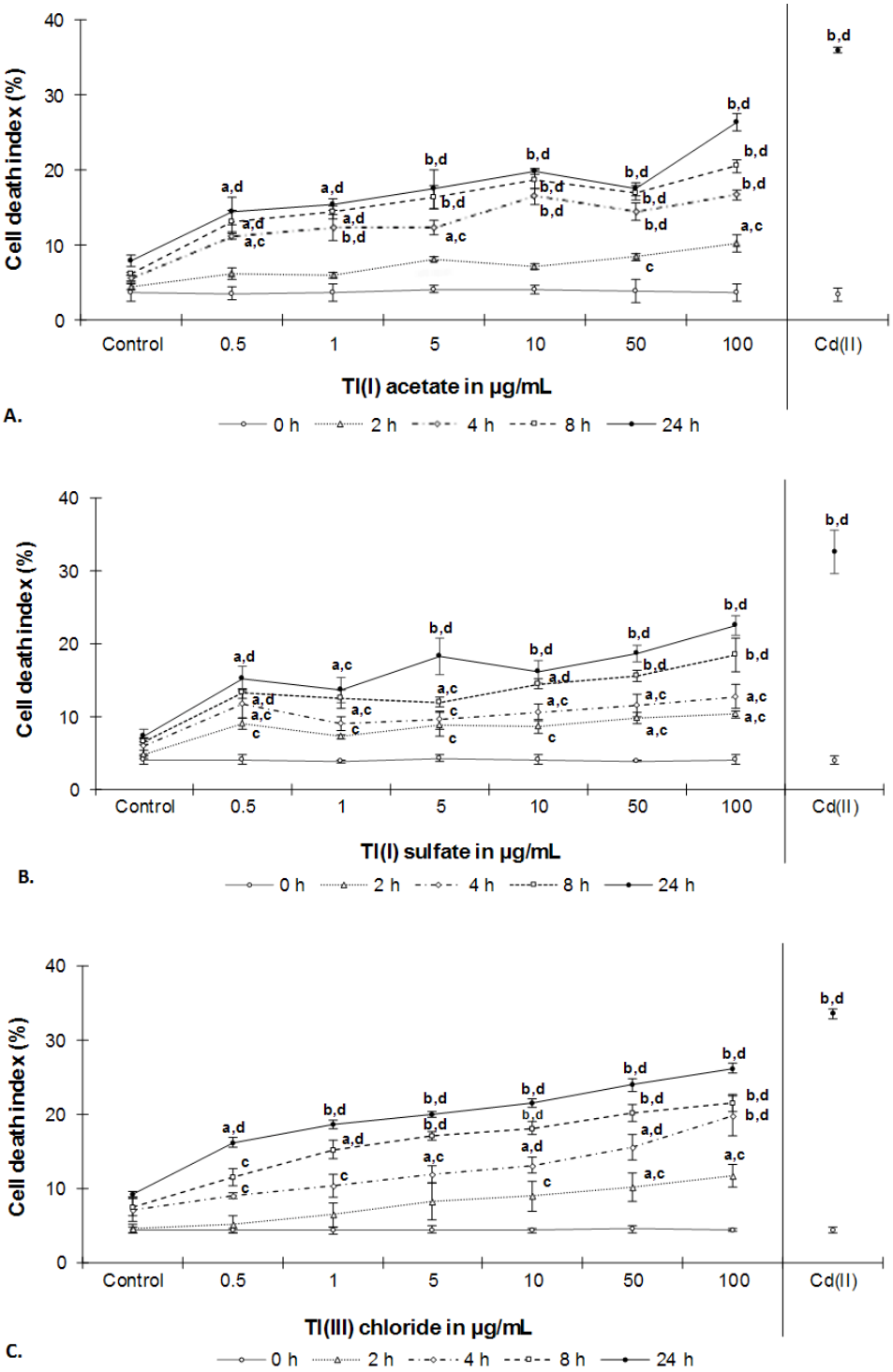

Figure 2. Death index of human lymphocytes treated with 0.5, 1, 5, 10, 50, and 100 µg/mL of Tl compounds using the AO/EB assay. Evaluations were performed immediately after treatment (0 h) and 2, 4, 8, and 24 h later. (A) Tl(I) acetate, (B) Tl(I) sulfate, and (C) Tl(III) chloride. In all cases, an untreated control was available, and Cd(II) chloride (228 µg/mL) was used as a positive control. The experiments were performed in triplicate using samples from two donors, and the data were expressed as the means ± standard deviation (n = 6). Statistical differences are shown as $^{a}p < 0.05$, $^{b}p < 0.01$ compared with the control, and $^{c}p < 0.05$, $^{d}p < 0.01$ compared with the 0-h exposure.

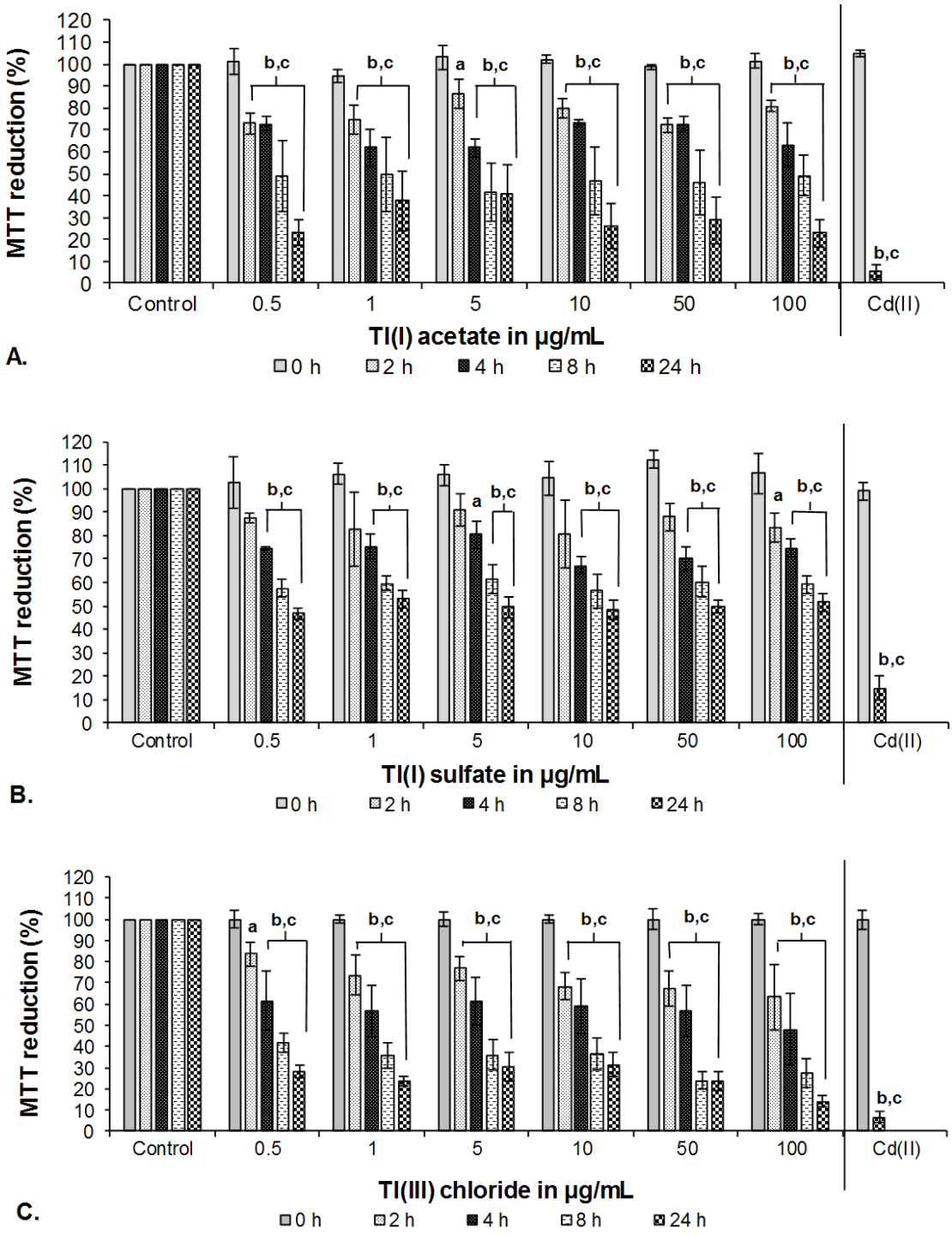

Figure 3. Mitochondrial activity assessed by MTT reduction of human lymphocytes treated with 0.5, 1, 5, 10, 50, and 100 µg/mL of Tl compounds using the colorimetric MTT (tetrazolium) assay. Evaluations were performed immediately after treatment (0 h) and 2, 4, 8 and 24 h later. (A) Tl(I) acetate, (B) Tl(I) sulfate, and (C) Tl(III) chloride. In all cases, an untreated control was available, and Cd(II) chloride (228 µg/mL) was used as a positive control. The experiments were performed in triplicate using samples from two donors, and the data were expressed as the means ± standard deviation (n = 6). Statistical differences are shown as $^{a}p < 0.05$, $^{b}p < 0.01$ compared with the control, and $^{c}p < 0.01$ compared with the 0-h exposure.

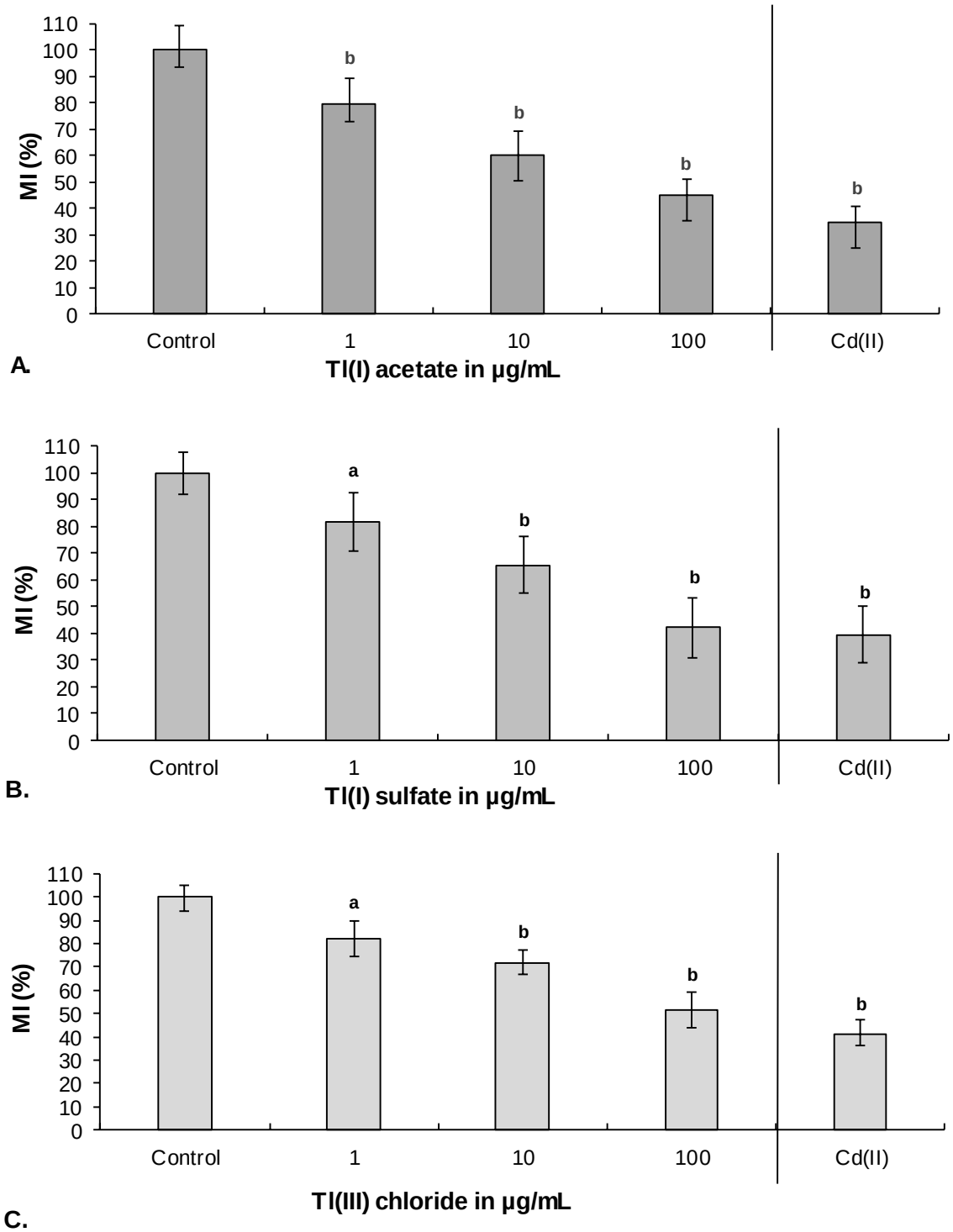

Figure 4. Decrease in the mitotic index (MI) in human lymphocytes treated with 1, 10 and 100 µg/mL of Tl compounds using a proliferation assay. The cells were treated for 24 h and were incubated for 72 h in Tl-free medium. (A) Tl(I) acetate, (B) Tl(I) sulfate, and (C) Tl(III) chloride. In all cases, an untreated control was available, and Cd(II) chloride (228 µg/mL) was used as a positive control. Each bar shows the mean and standard deviation of two independent experiments with 1 replicate (n = 4). Statistical differences are shown as $^{a}p < 0.05$, $^{b}p < 0.01$ compared with the control.

treatment with Tl(I) acetate, Tl(I) sulfate, or Tl(III) chloride reduced cell viability in a concentration- and time-dependent manner. Viability was significantly compromised at 4 h at a concentration of 5 µg/mL, and at 8 and 24 h with all tested concentrations; however, a 50% cytotoxic effect was not reached in this assay. These data, together with the observations of other authors, show that the cell membrane is an important target of Tl-induced toxicity.

Tl(I) and Tl(III) destabilize membrane fluidity, induce reactive oxygen species, cause glutathione and adenosine triphosphate depletion, and increase lipid peroxidation *in vitro* at 5-250 µmol/L Tl metallic ions [17-20]. Possible mechanisms of the reduced viability of human lymphocytes exposed to Tl compounds are oxidative stress, lipid packing and organization of the bilayer, and the lack of mitochondrial adenosine triphosphate, which results in intracellular acidosis and osmotic injury and leads to plasma membrane lysis, as shown in murine cells [18, 19].

Different methodologies exist to assess cell death by apoptosis or necrosis [21]. The advantage of AO/EB is that it differentiates viable from nonviable cells and cells that are entering apoptosis (early apoptotic) or that are already undergoing apoptosis (late apoptotic). One of the differentiating factors of this assay is that AO allows the visualization of cells without damage to the cell membranes that are undergoing death by apoptosis. This aspect is not considered in tests that measure damage to the plasma membrane, such as Trypan blue or CFDA/EB assays, which explains the cytotoxicity induction in lymphocytes exposed to Tl compounds at 2 h with concentrations of 50 or 100 µg/mL in the AO/EB assay. Cell death increased in concentration- and time-dependent manners with the three Tl compounds, and the degree of cytotoxicity showed the following tendency: Tl(I) acetate > Tl(I) sulfate > Tl(III) chloride. The induction of cytotoxicity occurred from the lowest concentrations tested (0.5 and 1 µg/mL) and following short-term exposure (4 and 8 h).

Our data are consistent with several authors who demonstrated the induction of apoptotic and necrotic cell death as a result of exposure to Tl compounds in different *in vitro* and *in vivo* assays [1]. Thallium initiates apoptosis by the release of Ca^{2+} from intracellular compartments, which opens the mitochondrial membrane transition pore to cause the release of cytochrome *c*, the inhibition of oxidative phosphorylation, and the activation of caspases [22-24]. Although Tl(I) primarily stimulates mitochondrial destabilization and triggers cell death *via* the classical pathway of apoptosis, Tl(III) also uses the extrinsic apoptosis pathway [25].

MTT reduction occurs principally in active mitochondria in viable cells, and the present assay was more sensitive in detecting the harmful effects of both oxidation states of Tl at the low concentrations tested (0.5 and 1 µg/mL) and following short-term exposure (2 or 4 h). In all cases, the differences were accentuated when the data were compared with 0-h exposure, which indicates the sensitivity of the MTT assay compared with that of the CFDA/EB or AO/EB assays. This response was likely due to cellular contact with Tl ions, which activates mechanisms of survival, such as detoxification and DNA repair, and results in the increased flow of energy and metabolism, as occurs with other metallic cations [26].

The three Tl compounds decreased metabolic activity in a time-dependent manner, and the induction of cell toxicity (Tl(I) acetate = Tl(III) chloride > Tl(I) sulfate) was dependent on the chemical structure, oxidation state, and Tl ion concentration. Some studies proposed that Tl ions were intracellularly distributed in the cytosol, nuclei, lysosomes and mitochondria [27] and affected different functions in mitochondria that lead to decreased reductant capacity [24, 28].

The cell has mechanisms governing the decisive division between proliferative and antiproliferative states, and 24-h exposure to Tl(I) or Tl(III) compounds in cell culture is sufficient to decrease MI in a concentration-dependent manner with a cytotoxicity tendency of Tl(I) > Tl(III). Previous reports demonstrated increased chromosomal damage and inhibited mitosis in cultured lymphocytes stimulated with phytohemagglutinin and treated with Tl compounds [11, 12]. In the current study, Tl, in its two main oxidative states, was equally cytotoxic for cells in G_0 and for proliferating cells, which indicates that Tl leads to a strong injury,

even after compound withdrawal. This cytotoxicity is reflected by decreased viability as well as the decreased proliferative capacity of cells that survive after exposure to the metallic compound. Notably, both oxidation states were potentially harmful, regardless of whether the oxidative-reduction process from Tl(I) to Tl(III) (or vice versa) occurred in the cells.

A wide range of concentrations was assayed to determine a detailed dose-response relationship and to include concentrations of Tl found in the blood or urine of poisoned people, which reaches concentrations up to 5 µmol/L or greater [3, 11, 29]. High concentrations must be tested because Tl accumulates in different tissues and organs, and cells in these sites are in contact with the Tl ions at concentrations higher than the concentrations detected in biological fluids. Overall, the results of the present study suggest what is happening in workers or people intoxicated with Tl. The degree of cell damage induced by Tl was comparable to that induced by other highly cytotoxic metals such as Cd.

CONCLUSIONS

Together, our data show that Tl(I) acetate, Tl(I) sulfate, and Tl(III) chloride induce cytotoxic effects in human lymphocytes, as measured by several assays. In general, the three Tl compounds reduced viability (CFDA/EB assay) and increased cell death *via* apoptosis and necrosis (AO/EB assay) with concentration- and time-dependent effects. On the other hand, the MTT assay using the three Tl compounds showed that at each tested concentration, a time-dependent response occurred with a higher sensitivity in recording Tl-induced cytotoxicity than the CFDA/EB or AO/EB assay. When cells in the G_0 phase were treated with Tl compounds, the MI decreased in a concentration-dependent manner. These results suggest that Tl(I) and Tl(III) compounds induce cell death and decrease the proliferative capacity of the cell. Additionally, the cell membrane is a target for Tl, and mitochondria are sensitive to the effects of this metal.

FUNDING

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CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest. The authors are responsible for the content and writing of the paper.

ABBREVIATIONS

CFDA	:	5,6-carboxyfluorescein diacetate
EB	:	ethidium bromide
AO	:	acridine orange
MTT	:	methyl tetrazolium
MI	:	mitotic index

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