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RESEARCH ARTICLE

Inorganic tin compounds do not induce micronuclei in human lymphocytes in the absence of metabolic activation

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Abstract

The genotoxic evaluation (*in vitro* analysis) of a series of eight inorganic tin(II) and tin(IV) compounds [tin(II) acetate, tin(II) chloride, tin(II) ethylhexanoate, tin(II) oxalate, tin(II) oxide, tin(IV) acetate, tin(IV) chloride and tin(IV) oxide], for the detection of micronuclei in human blood lymphocytes, was performed in the absence of metabolic activation by the cytokinesis-block micronucleus assay. Human lymphocytes were treated for over one cell cycle (31 hours), with concentrations ranging from 1 to 75 μM (1, 5, 10, 20, 50 and 75 μM), of tin(II) and tin(IV) salts dissolved in dimethyl sulfoxide. The above-listed concentrations cover the values that have been detected in humans with no occupational exposure to tin compounds. The experimental results show the absence of genotoxicity for all inorganic compounds tested in the specific concentrations and experimental conditions. Cytotoxic effects of tin(II) and tin(IV) compounds were evaluated by the determination of cytokinesis block proliferation index and cytotoxicity percentage. Our observations on the cytotoxicity pattern of the tested tin(II) and tin(IV) compounds indicate that they are cytotoxic in several tested concentrations to human lymphocytes treated *in vitro*. The observed differences in cytotoxicity of each tested compound might reflect differences in their chemical structure.

Keywords

Cytotoxicity, genotoxicity, human blood lymphocytes, micronucleus assay, tin(II) and tin(IV) compounds

History

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Introduction

Tin is a gray-white metal existing in two well-defined chemical oxidation states, namely, divalent [tin(II) or stannous tin] and tetravalent tin [tin(IV) or stannic tin] (Blunden & Wallace, 2003). Undoubtedly, tin(II) chloride is one of the most important examples of compounds in the positive (II) oxidation state and has been mainly applied as a starting material for the preparation of inorganic coordination compounds. It is also known as a reducing agent in organic synthetic chemistry (Earnshaw & Greenwood, 1998). On the other hand, the more stable tin(IV) compounds have been extensively studied over the last few years because of the numerous industrial applications as wood preservatives and agrochemical fungicides, mainly organotin(IV) compounds, biocides and environmental disinfectants. Particularly, tri-*n*-butyltin(IV) compounds have been effectively used in antifouling paints for ships (Hoch, 2001).

The antibacterial and -cancer applications of numerous tin(IV) complexes have been described in detail (Basu Baul, 2008; Gielen et al., 2005; Hadjikakou & Hadjiliadis, 2009; Pizarro et al., 2010). It has been suggested that organotin(IV) compounds interact through the phosphate group of DNA sugar backbones (Tabassum & Pettinari, 2006), whereas their antitumor effect is exhibited through binding to thiol groups of proteins (Penninks & Seinen, 1984). An action mode through a gene-mediated pathway in cancer cells has been proposed (Chasapis et al., 2004; Gielen, 2003). Currently, organotin(IV) compounds are very important in cancer chemotherapy because of their apoptotic-inducing character (Cima & Ballarin, 1999; Pellerito et al., 2005), constituting a potential nonplatinum active class of metallopharmaceuticals (Tabassum & Pettinari, 2006). Despite the numerous compounds tested, the mechanism of antiproliferative activity is not well established (Hadjikakou & Hadjiliadis, 2009).

As a result of its wide use in industry, agricultural and other biocide activities, tin, in both inorganic and organic forms, is widely dispersed throughout the ecosphere, where it can accumulate in the food chain (Hodge et al., 1979) through the tin geocycle (Craig, 1988; Ridley et al., 1977; Wood, 1974).

Subsequent to an intensive literature search, we have realized that thus far there are only a few reports regarding the possible genotoxicity of tin compounds in human peripheral blood in *in vivo* and *in vitro* conditions (Ganguly, 1993; Ganguly et al., 1992; Hu et al., 1987; Talukder et al., 1989). The above-mentioned studies report increased chromosome aberrations (CAs), sister chromatid exchanges (SCEs) and

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micronuclei (MN) frequencies in tin miners and patients with lung cancer who had worked in Chinese tin mines (Hu et al., 1987). The Ganguly group report increased CAs and SCEs in healthy donors' lymphocyte cultures, ranging from 20 to 40 years of age, who have been exposed to stannic chloride (Ganguly et al., 1992; Talukder et al., 1989). Parallel studies from the same group reported that the percentages of CA and MN were higher in cultures from donors over 50 (Ganguly, 1993; Talukder et al., 1989).

The present work evaluated the possible genotoxic effects of a series of eight tin(II) and tin(IV) inorganic compounds, namely, tin(II) chloride, tin(II) acetate, tin(II) ethylhexanoate, tin(II) oxalate, tin(II) oxide, tin(IV) acetate, tin(IV) chloride and tin(IV) oxide, by the cytokinesis-block micronucleus (CBMN) assay, which can detect the induction of genetic damage in cultured cells of human blood lymphocytes (HBLs). This damage may be expressed as elevated MN frequencies in cultured lymphocytes. MN may originate from acentric chromosome fragments or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The simplicity, rapidity and sensitivity of the CBMN assay make it a valuable tool for genotoxicity screening. The assay detects the potential clastogenic and aneugenic activity of chemicals in cells that have undergone cell division after exposure to the test chemical (Kirsch-Volders et al., 2011; OECD, 2010). The use of cytochalasin B (Cyt-B), an inhibitor of actin polymerization, which prevents cytokinesis while permitting nuclear division (Fenech & Morley, 1985; MacLean-Fletcher & Pollard, 1980), leads to formation of binucleated (BN) cells, which are scored for the presence of MN (Fenech, 1993, 1997; Kirsch-Volders et al., 2003).

In the present study, we aimed to comparatively investigate the cytotoxic and genotoxic effects of the above-listed eight inorganic tin compounds using the CBMN assay in human lymphocyte cultures. The previously mentioned tin compounds are used as precursors for the experimental production of new organo-tin compounds with possible agrochemical and pharmaceutical applications.

Methods

Chemicals

Tin(II) acetate (CAS no.: 638-39-1), tin(II) chloride (CAS no.: 7772-99-8), tin(II) ethylhexanoate (CAS no.: 301-10-0; purity, ≥95%), tin(II) oxalate (CAS no.: 814-94-8; purity, 98%), tin(II) oxide (CAS no.: 21651-19-4; purity, >99%), tin(IV) acetate (CAS no.: 2800-96-6), tin(IV) chloride (CAS no.: 7646-78-8; purity, >99.9%) and tin(IV) oxide (CAS no.: 18282-10-5; purity, >99.9%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All chemicals were of the highest purity grade commercially available.

The aim of our study was to examine the genotoxic effect of the specific tin compounds in concentrations that correspond to the actual detectable values that have been detected in concentrations in humans (see below). These particular tin compounds were used as precursors for the manufacture of new complex organometallic compounds, with possible applications on the agrochemical and pharmaceutical industry.

Average tin intake (using data from seven countries) ranges from <1 up to 15 mg/day per person (WHO, 2005). Based on the above-mentioned data and taking into consideration (1) that blood represents approximately 7% of body mass or approximately 4.4 L in a 64-kg person (Cameron et al., 1999), (2) the mean weight of our donors (72 kg) and (3) the molecular weight of tin (118.71), the simulated tin concentration in the blood of our donors ranged approximately from 1.7 to 25.3 μM. Further, it has been mentioned that individuals with no occupational exposure history to tin compounds have been found with tin concentrations in their blood (detection limit: 2 μg/L) ranging from 2 to 9 μg/L (Hamilton et al., 1973; Kazantzis, 1994). The above-mentioned value corresponds to 0.075 μM. Therefore, the selection of our concentrations (1–75 μM) suffices for the aforementioned detectable tin concentrations in humans and our highest examined concentration is 1000 times higher, compared to the maximum value determined by Hamilton et al. (1973) and Kazantzis (1994).

CBMN assay in human lymphocytes *in vitro*

The CBMN assay was performed according to the standard procedure, with minor modifications (Kirsch-Volders et al., 2003; OECD, 2010).

Blood samples were obtained and kept under sterile conditions in heparinized tubes from two nonsmokers, healthy individuals (18 and 25 years old) not undergoing any drug treatment, who did not have any viral infection or X-ray exposure for at least 1 year.

Whole blood (0.5 mL) was added to 6.5 mL of Ham's F-10 medium (GIBCO, Grand Island, NY), 1.5 mL of fetal bovine serum (GIBCO) and 0.3 mL of phytohemagglutinin (GIBCO) to stimulate cell division.

The appropriate chemical solutions were added to final concentrations of 1, 5, 10, 20, 50 and 75 μM. All the tested chemicals were dissolved in dimethyl sulfoxide (DMSO). DMSO concentrations in cultures did not exceed 0.75%. The appropriate solution volumes were added 41 hours after culture initiation. For each tested chemical, two identical sets of two independent experiments were conducted for all aforementioned concentrations as well as for positive and negative controls. The reported results represent the pooled data from the 2 donors' replicated cultures (Kirsch-Volders et al., 2003). Mitomycin C (Sigma-Aldrich Chemie GmbH) at final concentration of 1.5 μM served as positive control (Stivaktakis et al., 2010).

Subsequently, 6 μg/mL of Cyt-B (Sigma-Aldrich Chemie GmbH) was added to the culture medium 44 hours postculture initiation and 3 hours after the addition of the appropriate chemical solutions. This concentration of Cyt-B was selected so as to obtain a higher percentage of BN cells and a lower baseline MN frequency (Surrallés et al., 1992).

Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 72 hours. Seventy-two hours postculture initiation, cells were harvested and collected by centrifugation. A mild hypotonic treatment with a 3:1 (v/v) solution of Ham's medium and Milli-q H_2O, for 3 minutes at room temperature, was followed by at least three times for 10-minute fixation with a freshly prepared 5:1 (v/v) solution

of methanol/acetic acid. Cells were finally stained with 7% Giemsa (Papapaulou et al., 2001; Vlastos et al., 2010; Vlastos & Stephanou, 1998).

In total, 4000 BN cells with preserved cytoplasm were scored per experimental points. Standard criteria were used for scoring MN (Fenech, 1997; Fenech et al., 2003). To determine possible cytotoxic effects, the cytokinesis block proliferation index (CBPI) was calculated by counting at least 2000 cells for each experimental point (500 cells per culture of each donor). CBPI is given by the following equation: $CBPI = M_1 + 2M_2 + 3(M_3 + M_4)/N$, where M_1, M_2, M_3 and M_4 correspond to the numbers of cells with one, two, three and four nuclei and N is the total number of cells (Surrallés et al., 1995). The percentage of cells that are affected by toxicity (% cytotoxicity) is determined as follows: % cytotoxicity = 100–100 [$CBPI_T$–1/$CBPI_C$–1] (OECD, 2010).

Statistical analysis

All results are expressed as the mean frequency ± standard error. The statistical analysis of the MN data was conducted using the *G* test for independence on 2 × 2 tables. The chi-square (χ^2) test was used for the analysis of CBPI among each treatment. Statistical decisions were based on a significance level of 0.05. The statistical software used for data analysis was the Statistical Package for Social Sciences for Windows (version 17.0; SPSS, Inc., Chicago, IL).

Results

CBMN assay

Eight inorganic tin compounds were applied to *in vitro* analysis of their possible genotoxic effects by the CBMN assay in HBLs.

The results of MN analysis (mean values for MN induction) obtained from human peripheral blood lymphocyte (PBL) cultures treated with different concentrations (1, 5, 10, 20, 50 and 75 μM) with the tin(II) and tin(IV) compounds are shown in Figures 1–8. No statistically significant differences of MN frequencies were observed in all examined compounds at all tested concentrations, compared to controls. However, data of certain concentrations, although not indicating statistically significant differences, show an increase in MN frequency [i.e. tin(II) oxide at 50 and 75 μM, tin(IV) chloride above the concentration of 20 μM and tin(II) chloride at 50 μM].

The reported negative and positive (72.5 ± 29%) control frequencies of MN in our experiments (Figures 1–8) are in accord with published values in the used cytogenetic end point (Clare et al., 2006).

Cytotoxic index evaluation

Cytotoxic effect was evaluated by the CBPI index. The results of this index analysis are shown in Figures 1–8.

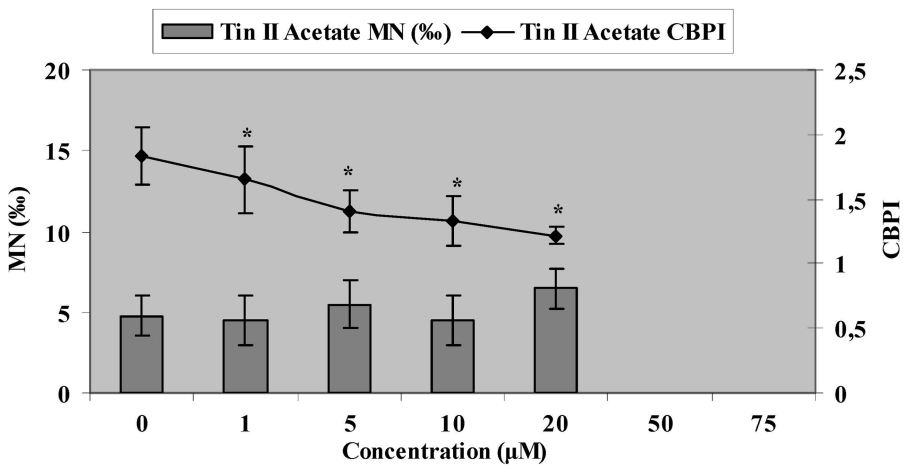

Figure 1. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin II acetate. Binucleated cells (4000) scored per experimental point; $^*p < 0.001$ [*G* test for MN; χ^2 for CBPI].

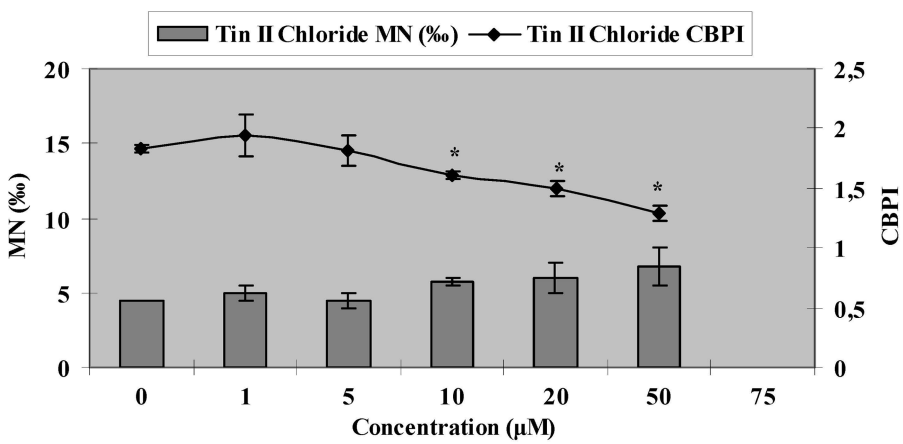

Figure 2. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin II chloride. Binucleated cells (4000) scored per experimental point; $^*p < 0.001$ [*G* test for MN; χ^2 for CBPI].

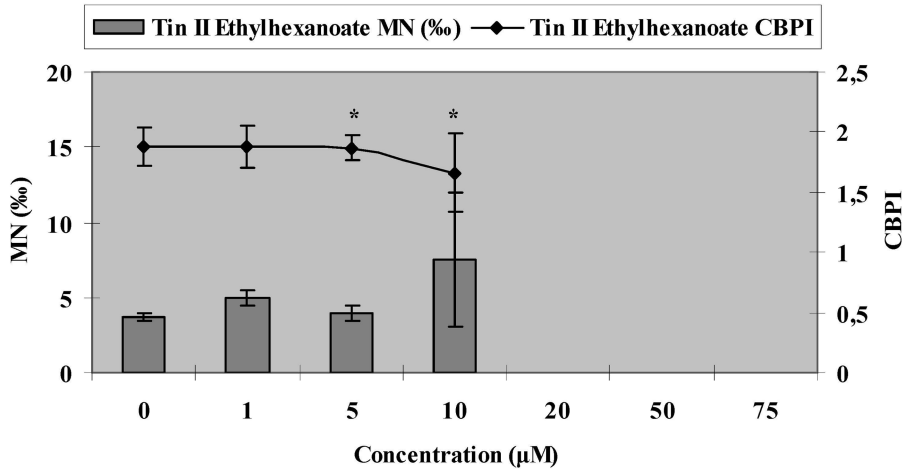

Figure 3. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin II ethylhexanoate. Binucleated cells (4000) scored per experimental point; *$p < 0.001$ [G test for MN; χ^2 for CBPI].

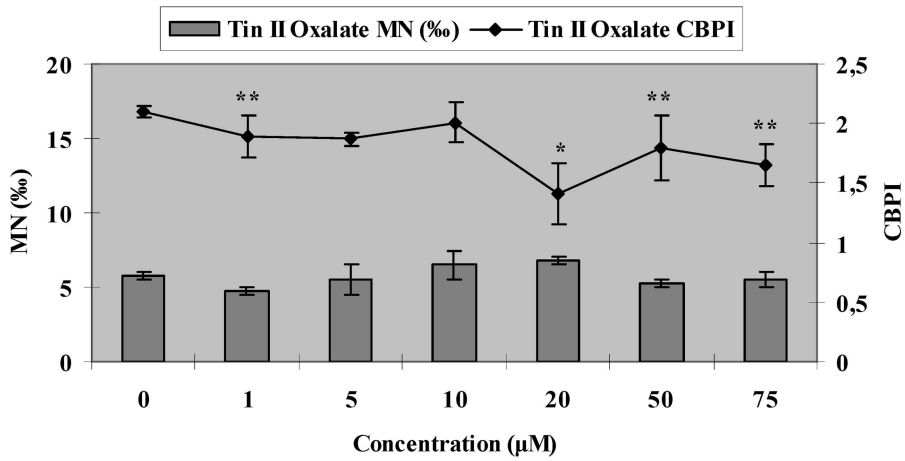

Figure 4. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin II oxalate. Binucleated cells (4000) scored per experimental point; *$p < 0.01$; **$p < 0.001$ [G test for MN; χ^2 for CBPI].

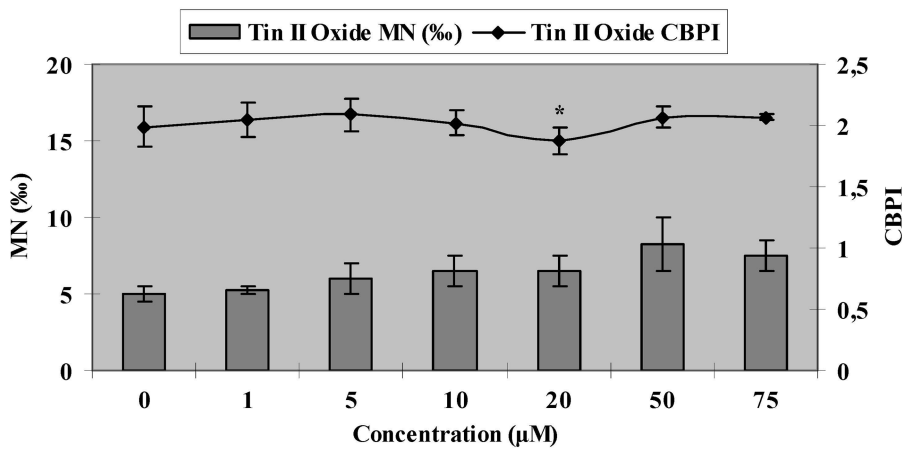

Figure 5. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin II oxide. Binucleated cells (4000) scored per experimental point; *$p < 0.001$ [G test for MN; χ^2 for CBPI].

Regarding this index, tin(II) and tin(IV) acetate induced a statistically significant decrease ($p < 0.001$) on CBPI values at concentrations of 1, 5, 10 and 20 µM, compared to controls. In the case of treatments by tin(II) and tin(IV) acetate at the highest concentrations of 50 and 75 µM, the data could not be measured as a result of cytotoxicity (Figures 1 and 6).

Tin(II) chloride induced a statistically significant decrease ($p < 0.001$) on CBPI value at concentrations of 10, 20 and 50 µM and the tin(IV) chloride at all tested concentrations, except the highest of 75 µM. In the latter case (75 µM), the data could not be measured as a result of cytotoxicity (Figures 2 and 7).

In the case of tin(II) oxide, statistically significant differences ($p < 0.001$), in comparison to control, on CBPI value were noted only at 20 µM, whereas in the case of tin(IV) oxide, similar differences were observed at 5 and 75 µM (Figures 5 and 8).

For the tin(II) ethylhexanoate compound, an intense decrease at the concentrations of 5 and 10 µM was observed,

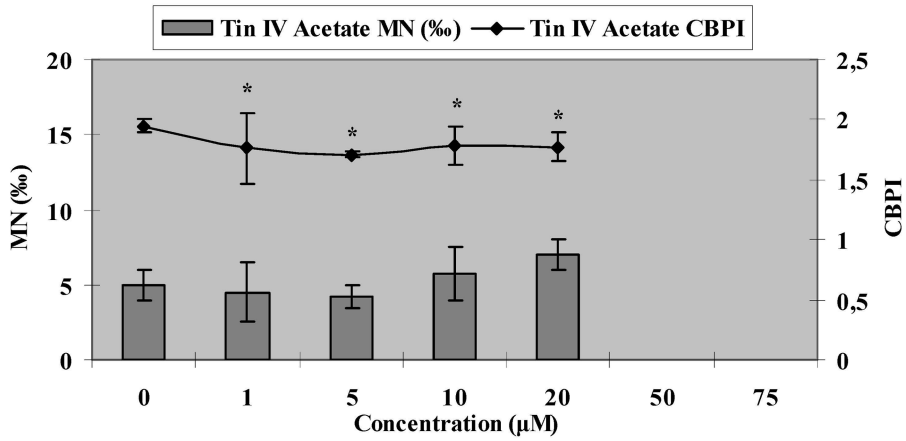

Figure 6. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin IV acetate. Binucleated cells (4000) scored per experimental point; $*p < 0.001$ [G test for MN; χ^2 for CBPI].

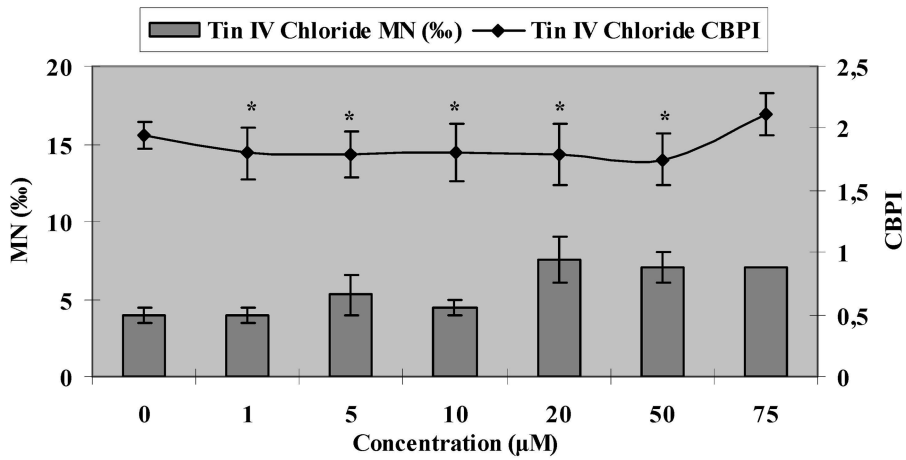

Figure 7. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin IV chloride. Binucleated cells (4000) scored per experimental point; $*p < 0.001$ [G test for MN; χ^2 for CBPI].

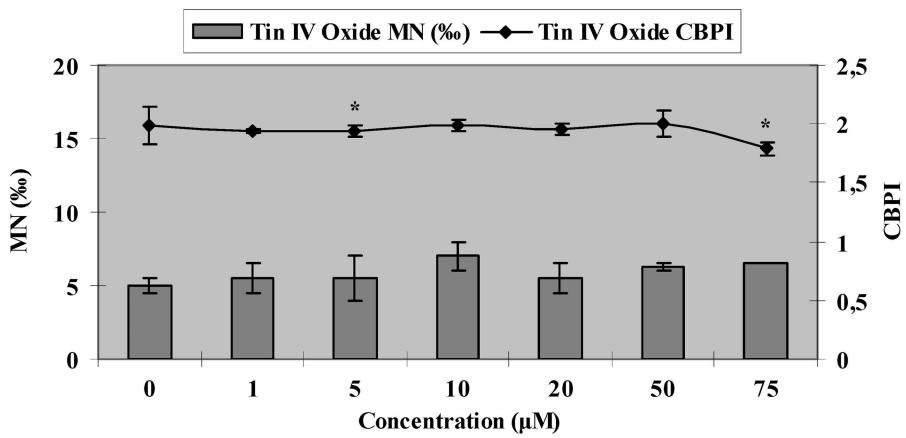

Figure 8. Induction of MN and CBPI values in human lymphocytes treated for 31 hours with tin IV oxide. Binucleated cells (4000) scored per experimental point; $*p < 0.001$ [G test for MN; χ^2 for CBPI].

whereas at the other concentrations (i.e. 20, 50 and 75 μM), the data could not be measured as a result of cytotoxicity (Figure 3).

Finally, in the case of tin(II) oxalate, a significant decrease ($p < 0.001$), at the concentrations of 1, 50 and 75 μM as well as at the concentration of 20 μM ($p < 0.01$), was observed (Figure 4).

Evaluation of the percentage of cytotoxicity for the tin(II) acetate produced a dose-dependent increase, ranging from 33.05% at the concentration of 1 μM to 65.05% at the concentration of 20 μM. The concentrations of 50 and 75 μM were not scorable as a result of extremely low numbers of BN cells.

The same pattern was observed for the tin(II) chloride at the concentrations of 10, 20 and 50 μM (26.28, 40.68 and 64.75%, respectively). At the concentrations of 1 and 5 μM, cytotoxicity was not calculated because the CBPI was equal to or higher than control value. The highest concentration (75 μM) was not scorable as a result of extremely low number of BN cells.

For the tin(II) ethylhexanoate, the cytoxicity (%) was calculated only at the concentration of 10 μM (45.20%),

whereas for the highest concentrations (20, 50 and 75 μM) it was not scorable and for the lowest concentrations (1 and 5 μM) was not calculated because the CBPI was equal to or higher than the control value.

In the case of tin(II) oxalate, cytotoxicity ranged from 18.03% (1 μM) to 45.78% (75 μM).

Cytotoxicity of tin(II) and tin(IV) oxide was calculated only at the concentrations of 20 (12.03%) and 75 μM (21%), respectively. At the other concentrations, cytotoxicity was not calculated because the CBPI was equal to or higher than the control value.

Cytotoxicity levels for tin(IV) acetate ranged from 14.90% (1 μM) to 22.58% (20 μM), whereas the highest concentrations, of 50 and 75 μM were not scorable as a result of extremely low numbers of BN cells.

Finally, the cytotoxicity of tin(IV) chloride, in all concentrations, ranged around the level of 20%, except the lowest concentration (1 μM), where it was calculated at 29.90%, whereas the higher concentration (75 μM) was not evaluated because the CBPI was equal to or higher than the control value.

The OECD guideline (2010) recommends that the highest concentration to be examined should induce a cytotoxicity of $55 \pm 5\%$. This recommendation is approached in the first four examined tin compounds. Based on what we have mentioned above, concerning the selection of the examined concentrations, in combination that all compounds are tested up to the same maximum concentration, the difficulty in dissolving the compounds, tin(II) oxide and tin(IV) oxide (Westrum & Thomassen, 2002) and the fact that the solvent limit in the culture should not exceed 1% (Kirsch-Volders et al., 2003; Titenko-Holland et al., 1997), we decided to use these particular concentrations in all the remaining four tin compounds, which do not reach the upper toxicity limit. Therefore, the $55 \pm 5\%$ cytotoxicity may not always be achieved.

Discussion

Inorganic tin may undergo oxidation reduction, ligand exchange and precipitation reactions in the environment. The biomethylation of inorganic tin has been demonstrated in pure bacterial cultures, sediments and decaying plant material. Inorganic tin compounds may be bioconcentrated by organisms, but data are limited (WHO, 2005).

For the general population, the diet is the main source of exposure to inorganic tin. The Joint FAO/WHO Expert Committee on Food Additives recently concluded that mean tin intakes in seven countries ranged from <1 up to 15 mg/day per person, but maximum daily intakes could reach 50–60 mg for certain individuals who routinely consume canned fruits, vegetables and juices from unlacquered cans. Drinking water is not a significant source of inorganic tin and might contribute approximately 0.012–0.02 mg/day. In humans and laboratory mammals, absorption of inorganic tin from the gastrointestinal tract is low (generally less than 5%), but is influenced by dose, anion (compound solubility) and the presence of other substances. Unabsorbed ingested tin is mostly (95–99%) excreted in the feces within 48 hours. Absorbed tin is mainly distributed to the bone, but also to the lungs, liver and kidneys. Limited evidence suggests that inorganic tin does not readily cross the blood–brain barrier. Absorbed tin is mainly excreted in the urine, with some additional biliary excretion occurring. In mice, the biological half-life of absorbed inorganic tin was approximately 30 days (WHO, 2005).

In general, metal genotoxicity is caused by three predominant mechanisms: (1) interference with cellular redox regulation and induction of oxidative stress; (2) inhibition of major DNA repair systems resulting in genomic instability and accumulation of critical mutations and (3) deregulation of cell proliferation (Beyersmann & Hartwig, 2008).

In our study, the *in vitro* analysis of the above-mentioned tin(II) and tin(IV) compounds, usually used for the synthesis of organotin agropharmaceuticals, for their possible genotoxic effects by the CBMN assay in HBLs, indicated that there were no statistically significant differences. However, data for tin(II) oxide at 50 and 75 μM, tin(II) chloride at 50 μM and tin(IV) chloride above the concentration of 20 μM showed an increase in MN frequency that was not statistically significant.

Shelby et al. (1993) reported that tin(II) chloride gave no evidence of genotoxic potential in a well-conducted *in vivo* study involving its administration by injection to mice. In the meantime, significant elevations of CAs, SCEs and MN counts in PBLs of tin miners and in patients with lung cancer who had worked in the Yunnan tin mines (China) showed the possible carcinogenic effect of tin (Hu et al., 1987).

Preliminary results of Talukder et al. (1989) indicated that stannic chloride, at concentrations of 10 and 20 μg/mL, statistically increased the frequency of CA, MN and SCEs after 48 and 70 hours of exposure. In pooled data from both sexes, they observed significantly increased MN and CA frequencies in donors more than 50 years of age, as compared to younger donors. In the meantime, they did not clarify their smoking habit, whereas they stated significantly increased SCEs frequencies in smokers. Further, incubation of human lymphocytes from 11 male donors with tin(IV) chloride at 2 or 4 μg/mL for 70 hours resulted in 2- to 3-fold increases in incidences of CAs and SCEs (Ganguly et al., 1992). Treated human lymphocytes, from 52 donors of both sexes, with 4 μg/mL for 48 hours revealed significant elevations of CA and MN formations. A linear relation between induction of CA and MN frequencies and the age of the studied subjects was reported (Ganguly, 1993). The previously reported MN experiments, conducted 20 years earlier, used a protocol with logical modifications in relation to the recently approved OECD (2010) guideline. Under this consideration, the observed differences in our study could possibly be explained as being a result of a modified treatment schedule, exposure period and donors' selection. Ganguly's group studies included a 48- and 70-hour exposure period, where tin(IV) salt was added at the initiation of the cultures, preceding cell activation by the mitogen. Meanwhile, our data are concentrated at an exposure period of 31 hours and the chemicals were added after the activation of lymphocytes by the mitogen.

Another study, which investigated the effect of divalent tin on DNA, reported that tin(II) chloride induces the denaturation of double-stranded DNA, leading to alterations of all

four nucleotide triphosphates (de Mattos et al., 2005). Stannous-ion-induced DNA damage was suggested to occur by formation of reactive oxygen species (ROS) (Dantas et al., 1996; McLean et al., 1983), as found for other metal ions that contribute to genetic instability by inducing different types of oxidative DNA damage (Beyersmann & Hartwig, 2008).

Viau et al. investigated the genotoxic and comutagenic effect of stannous chloride in V79 cells. Inhibitory effects of tin(II) on the repair of methyl methane sulfonate-induced DNA damage suggest that this metal can also interfere in DNA repair systems and thus may contribute to increased mutations by shifting the balance from error-free to -prone repair processes (Viau et al., 2009).

Overall, some evidence suggests that the DNA damage induced by inorganic tin compounds may be a secondary effect associated with ROS. The mechanism resulting in chromosome damage has not been determined, although it is known that certain inorganic salts can give positive results in such assays as the result of changes in the ionic strength or pH of the test medium (WHO, 2005).

The toxicology pattern of both inorganic tin and organotins (compounds possessing at least one Sn-C bond) has been previously discussed by many researchers, as stated by Blunden & Wallace (2003). Pellerito et al. reported that organotin(IV) compounds exert their toxic effects involving processes such as apoptosis and necrosis. The precise balance of these actions and their outcomes may differ radically from one cell type to another and among different organisms (Pellerito et al., 2006).

Cytotoxic effects of tin(II) and tin(IV) compounds were evaluated by the determination of CBPI. Our observations on the cytotoxicity pattern of the tested tin(II) and tin(IV) compounds indicate that they are cytotoxic in several tested concentrations to human lymphocytes treated *in vitro*. The observed differences in cytotoxicity of each tested compound might reflect differences in their chemical structure.

Our observations on the cytotoxicity of tin(IV) chloride come into agreement with the previously reported positive *in vitro* cytotoxicity results of tin(IV) chloride in human lymphocyte cultures, where mitotic index and cell-cycle kinetics (replicative index) were depressed (Ganguly, 1993; Ganguly et al., 1992; Talukder et al., 1989). In addition, the limited observed cytotoxicity of tin oxide compounds in our results comes into agreement with their action in the environment, which exert low toxicity in organisms largely as the result of their low solubility, poor absorption, low accumulation in tissues and rapid excretion (WHO, 2005).

Conclusions

In the present study, the tested tin(II) and tin(IV) salts, under the protocol used, did not induce MN formation in human PBLs without metabolic activation. On the contrary, our results revealed increased cytotoxicity in several concentrations for all of the tested tin compounds.

Declaration of interest

The authors report no conflicst of interest. The authors alone are responsible for the content and writing of this paper.

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