



***In vitro* ZnCl₂ cytotoxicity and genotoxicity in human leukocytes: Zero-order kinetic cellular zinc influx**

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ABSTRACT. Zinc (Zn) is an essential trace element for cellular viability, but concentrations above physiologic level may lead to cellular damage. The purpose of the present study was to evaluate the *in vitro* ZnCl₂ genotoxicity and cytotoxicity in human leukocyte cells. This was assessed in an unprecedented way that correlated the level of intracellular Zn after cell exposition with the cellular damage. The exposure to increased Zn concentrations (2.5–20 µg mL⁻¹), showed significantly reduced cellular leukocyte viability. However, significant DNA damages were observed only when the Zn exposure concentrations were from 10–20 µg mL⁻¹. The Zn intracellular levels found in leukocytes was from 72.25–268.9 pg cell⁻¹, starting to induce cytotoxicity and genotoxicity at concentrations of 95.68 and 126.2 pg cell⁻¹, respectively. The relationship between the exposure concentration and intracellular levels of Zn suggests that the influx of Zn, in the form of ZnCl₂, occurs in human leukocytes under zero-order kinetics.

Keywords: cell toxicity, zinc, human leukocyte, cellular uptake.

Citotoxicidade e genotoxicidade do ZnCl₂ em leucócitos humanos *in vitro*: influxo celular de zinco com cinética de ordem zero

RESUMO. O Zinco (Zn) é um elemento traço essencial para a viabilidade celular, mas em concentrações acima dos níveis fisiológicos pode conduzir a danos celulares. A proposta do presente estudo foi avaliar a citotoxicidade e genotoxicidade do ZnCl₂ em leucócitos humanos *in vitro*. De maneira sem precedentes, foi acessado o nível de Zn intracelular após exposição e relacionado com o nível de dano celular. A exposição a crescentes concentrações de Zn (2,5–20 µg mL⁻¹), mostraram significante redução da viabilidade celular dos leucócitos. Entretanto, danos significativos ao DNA foram encontrados somente a partir das concentrações de exposição ao Zn de 10–20 µg mL⁻¹. Os níveis intracelulares de Zn encontrados nos leucócitos foram de 72,25–268,9 pg célula⁻¹, começando a induzir citotoxicidade e genotoxicidade nas concentrações de 95,68 and 126,2 pg célula⁻¹, respectivamente. A relação entre a concentração de exposição e os níveis intracelulares de Zn sugerem que o influxo de Zn, sob a forma de ZnCl₂, ocorre em cinética de ordem zero em leucócitos humanos.

Palavras-chave: citotoxicidade celular, zinco, leucócitos humanos, captação celular.

Introduction

Zn is an essential trace element for all human cells that is involved in the development, proliferation and differentiation of cellular growth modulation (CARDOSO et al., 2012). In addition, Zn regulates the activity of some enzymes (YANG et al., 2013), such as superoxide dismutase (KAMER et al., 2012) and catalase (YORULMAZ et al., 2013).

In humans there is, on average, a total of 2 to 3 g of Zn, which is found in majority in muscle and bone tissues (PLUM et al., 2010). In relation to the

central nervous system, Zn is distributed unevenly, with higher concentrations on the blood brain barrier, hippocampus, hypothalamus, cerebral cortex and olfactory bulb, where it has a known role in cognition and memory processes (PIECHAL et al., 2012). At the cellular level, Zn is well distributed, being found in the nucleus, cytosol and associated with membranes. Two groups of proteins that regulate the Zn influx and efflux from the cytosol mediate cellular homeostasis. The zinc importer group (Zip) contains 14 proteins and the zinc transporter group (ZnT) is composed of 10 proteins (PLUM et al., 2010).

Although Zn is an essential micronutrient for body physiology, it is well known that Zn can also induce cellular damage (YORULMAZ et al., 2013). Different cell types have been exposed to Zn concentrations from 25 to 300 μM , which showed great variability in cytotoxicity and genotoxicity levels (SLIWINSKI et al., 2009; PLUM et al., 2010). Given these unpredictable cause-effect-concentrations observed in Zn exposures, there is need for studies to clarify the minimal Zn concentrations capable of inducing damage to different cell types, in order to corroborate with the elucidation of these events.

Herein, we report the *in vitro* minimal Zn genotoxicity and cytotoxicity concentrations in human leukocytes. These screenings were performed at concentrations from 2.5-20 $\mu\text{g mL}^{-1}$, being significantly lower than the ones cited in the literature. Furthermore, the intracellular Zn levels were determined after cellular exposure in order to relate them to the observed cell damage and understand the kinetic entrance of Zn in human leukocytes under experimental controlled conditions.

Material and methods

Ethic aspects

The experimental protocols performed in this study, including the collection of venous blood samples, were submitted to and approved by the Universidade Federal de Santa Maria (Rio Grande do Sul State, Brazil) Research Ethics Committee under registration number 0089.0.243.000-07.

Material preparation

The material that was used for *in vitro* assays was new and disposable to avoid Zn contamination. Control samples of the reagents were tested to identify metal contamination. Before use, all glassware was immersed for 24 hours in 20% aqueous HNO_3 (v v^{-1}) and subsequently rinsed with deionized water.

Both the comet and cell viability assays were performed in triplicate, while six replicates were analyzed to quantify the intracellular Zn content. The following screening strategy was performed for all assays: (1) Negative control – adjusted leukocyte suspension (LS) (104 cell mm^{-3}) in pH 7.4 PBS buffer; (2) positive control – LS plus 4 mM H_2O_2 ; tests – composed of LS with either 18.5 μM ($2.5 \mu\text{g mL}^{-1}$) or 37.0 μM ($5.0 \mu\text{g mL}^{-1}$) or 73.0 μM ($10 \mu\text{g mL}^{-1}$) or 110 μM ($15 \mu\text{g mL}^{-1}$) or 147 μM ($20 \mu\text{g mL}^{-1}$) Zn (ZnCl_2 , analytical grade, Merck®, Darmstadt, Germany). This concentration range was

chosen based on specialized literature data that related the metal concentration with cellular or DNA damage (SLIWINSKI et al., 2009; GUAN et al., 2012; SHARIF et al., 2012). All sample groups were incubated for 1 hour at 25°C under slow stirring of which the direction was inverted each 10 min. Thereafter, the above-mentioned tests were performed, as described below, with the exception of the intracellular Zn quantification. In this case, the samples were additionally washed five times with PBL buffer pH 7.4 and centrifuged at 2,000 rpm for 10 min. at room temperature.

Intracellular Zn content: Inductively coupled plasma mass spectrometer (ICP-MS)

The intracellular Zn quantification in the leukocytes was performed using ICP-MS analysis. Special care related to the material and treatment of the samples (human leukocyte) was taken into account. The water used was previously distilled and deionized on an ion-exchange column and purified using a Milli-Q system (Millipore®, Billerica, USA) with resistivity of $18.2 \text{ M}\Omega \text{ cm}^{-1}$. The reagent used for the decomposition of LS (HNO_3 , 65%, 1.4 kg L^{-1} , Merck®, Darmstadt, Germany) was a redistilled analytical grade (model duoPUR distillation Subboiling Distillation System, Milestone®, Sorisole, Italy).

Argon (99.999% purity, White Martins, Brazil) was used for plasma generation. A hydrochloric acid solution was prepared by diluting the concentrated acid in water. The sodium tetrahydroborate (NaBH_4 , PA, minimum purity of 97%, Nuclear®, Brazil) solution was prepared by dissolving the solid reagent in a 0.1% (w v^{-1}) NaOH solution (minimum purity of 99%, Vetec®, Brazil). Zn was added from a stock solution of ZnCl_2 (1000 mg L^{-1}). All materials were decontaminated by immersion in an aqueous HNO_3 20% (v v^{-1}) solution for 24 hours and subsequently rinsed with distilled/deionized water.

The leukocyte decomposition was done by the wet method in an open system (block digester - Tecnal, model TE 040/25 for 40 quartz tubes, aluminum body and thermostat). The samples were weighed and transferred to glass tubes ($2.5 \times 40 \text{ cm}$), followed by addition of 4 mL of concentrated HNO_3 and 1 mL of HCl 1N. The temperature was elevated to 50°C for 20 min. and 90°C for 4 hours and 30 min. For the efficiency of the digestion procedure, glass funnels were placed on the upper end of the pipes, aiming to facilitate condensation and reflux of gases and vapors generated during digestion. Upon cooling to room temperature, the solutions were transferred to graduated polypropylene vials and the volume was completed

to 25 mL with ultra-purified water. The samples were adequately diluted in 5% HNO₃ (v v⁻¹) by the factor required to get within the concentration range of the calibration curve and analyzed by ICP-MS (PerkinElmer SCIEX, Model Elan DRC II, Thornhill, Canada) to determine the extracted Zn content. Certified reference material was used for the measurements, obtaining consistent concentrations (98-101%) with CRM.

The intracellular Zn content was calculated, considering the number of 104 cell mm⁻³ from LS, taking into account the Zn concentration that the cells were exposed to and the value determined by ICP-MS analysis for the same, which was assumed as the content of Zn taken up by cells. The data found from replicates were taken into account to generate the data to their respective groups.

Cytotoxicity

The Zn cytotoxicity was evaluated by a cell viability test, using the Trypan Blue method to determine the loss of membrane integrity (BUROW et al., 1998). Initially, the cells were exposed to the previously established Zn concentration. After the incubation, 100 µL of LS were mixed for 8 min. with 100 µL of 0.4% Trypan Blue solution. The cell viability was determined microscopically (400×magnification) in a Neubauer Chamber and two categories of cells were scored: (1) living cells, which appeared uncolored or light blue; and (2) dead cells, with a blue color. For this assay, 300 cells were counted for each sample analyzed.

Genotoxicity

For the genotoxic evaluation, the comet alkaline assay (single cell gel electrophoresis assay) was performed according to the methodology described by Montagner et al. (2010) and in accordance with the comet assay guidelines (TICE et al., 2000). The microscope slide analysis was performed, counting 100 cells per slide and classifying them into damage levels from 0 (no damage) to 4 (maximum damage). The average obtained for each treatment allowed calculating the respective damage rate, considering the DNA damage scale from zero (100 cells x 0) to four (100 cells x 4) as proposed by Singh et al. (1988).

Statistical analysis

All data obtained from the tests carried out were treated by one-way analysis of variance (ANOVA), followed by Tukey's Multiple Comparison Test, when pertinent, accepting $p < 0.05$.

Results and discussion

Evaluation of intracellular Zn content in human leukocytes

One of the challenges in the Zn toxicological study is to know the Zn quantity capable of entering toward the cytoplasm from cell exposed to this metal. Furthermore, it is important to know if there is a constant cell entry percentage or if this entry is disproportional considering the different concentrations of exposure. Figure 1 shows an increasing intracellular concentration of Zn in human leukocytes exposed to ZnCl₂ at concentrations from 2.5 µg mL⁻¹ to 20 µg mL⁻¹, when compared to negative and positive controls ($p < 0.05$).

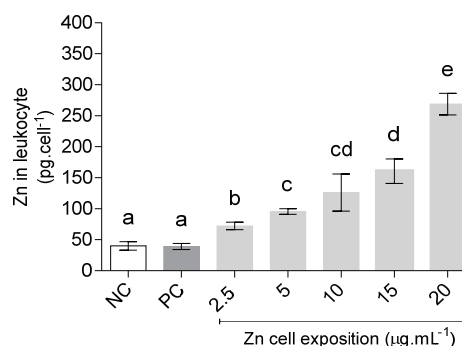


Figure 1. Intracellular content of Zn in human leukocytes cell exposed to different concentrations of the metal. Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and expressed average \pm SEM, with $p < 0.05$. a, b, c, d, e - the superscript letters indicate statistical difference between groups; NC = negative control, PC = positive control.

Figure 2 shows the percentage of Zn cell influx equivalent to a leukocyte cell related to the different ZnCl₂ concentrations of exposure, which allows evaluating the profile of Zn entry in leukocyte cell. As can be observed there is a decrease in the percentage of Zn cell input. This may be associated with the phenomenon of Zip protein saturation, the complex responsible for the cellular Zn influx (PLUM et al., 2010). Although there is decay in the entry percentage (Figure 2), the absolute increase of intracellular Zn concentration with increasing concentration of metal exposure (Figure 1) suggests that the influx kinetics is zero-order.

Cytotoxicity profile

The cytotoxicity and genotoxicity evaluation has been subject of researches among scientists in order to elucidate the Zn concentrations capable of inducing cellular damage in different tissues. Our results demonstrate that Zn exposure ranging from

5 to 20 $\mu\text{g mL}^{-1}$ significantly decreased leukocyte cell viability when compared to the negative control ($p < 0.001$). Moreover, in the case of the two higher concentrations, 15 and 20 $\mu\text{g mL}^{-1}$ ($p < 0.001$), the cytotoxicity was higher than of the positive control (Figure 3).

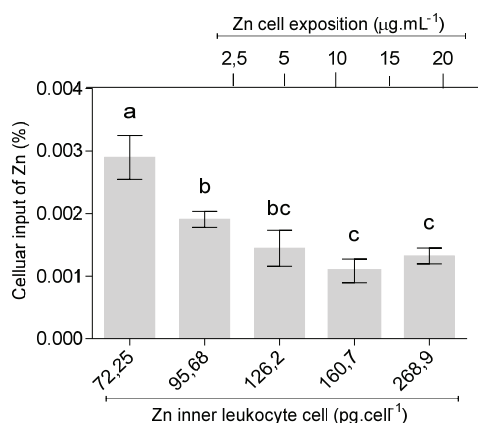


Figure 2. Percentage of cellular input of Zn in human leukocyte. Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons and expressed average \pm SEM, with $p < 0.05$. a, b, c – the superscript letters indicate statistical difference between groups; NC = negative control, PC = positive control.

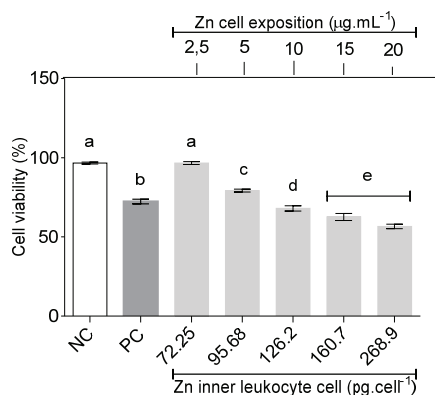


Figure 3. Percentage of cells in cell viability assay in human leukocytes exposed to different Zn concentrations. Data were analyzed by one-way ANOVA followed by Tukey test for multiple comparisons and expressed average \pm SEM, with $p < 0.001$; a, b, c, d, e – the superscript letters indicate statistical difference between groups; NC = negative control, PC = positive control.

The cytotoxicity can be induced through several mechanisms. In the case of Zn exposure, the literature indicates the involvement of oxidative stress generation induced by glutathione (GSH) depletion, extrapolation of metalloproteins binding capacity, such as metallothionein, and the interference with the redox state of macromolecules (KIM et al., 2003; SLIWINSKI et al., 2009). Another mechanism involves an imbalance in the

concentrations of antioxidant enzymes, in particular, glutathione peroxidase, superoxide dismutase and catalase (DINELEY et al., 2003; SLIWINSKI et al., 2009).

In fact, it is known that 100 μM ($\sim 21\mu\text{g mL}^{-1}$) of Zn is able to induce cell death and decrease the viability of different cell lines by activation of ERK1/2, p38MAPK, AKT (FREDERICKSON et al., 2005), Ras/Raf/MEK/ERK (SEO et al., 2001) and modulation of MAPK and PI3K/AKT pathways (AN et al., 2005), where the generation of reactive oxygen species seem to be present, at least in part, in the mechanisms of these pathways.

Genotoxicity profile

Regarding the damage evaluation to the leukocyte DNA after ZnCl_2 exposure, the comet assay demonstrated that exposure concentrations of 2.5 and 5.0 $\mu\text{g mL}^{-1}$, where the intracellular contents were, respectively, 72.25 and 95.68 $\text{pg leukocyte cell}^{-1}$, did not cause significant damage to cellular DNA, since their Zn levels were similar to the level of the negative control ($p < 0.001$), as can be seen in Figure 4. In contrast, in the exposure concentration range from 10 to 20 $\mu\text{g mL}^{-1}$, which is equivalent to intracellular levels from 126.2 to 268.9 $\text{pg leukocyte cell}^{-1}$, it is possible to see significantly higher damage levels, although lower than observed for the positive control ($p < 0.001$).

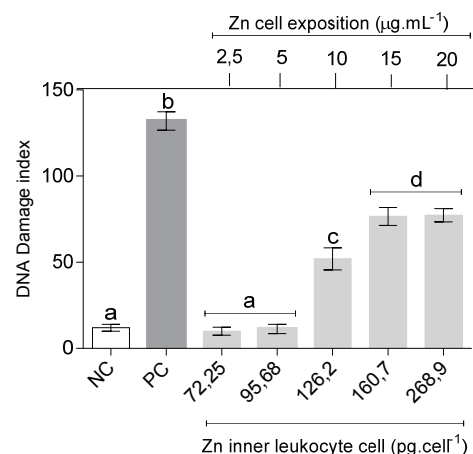


Figure 4. Rate of DNA damage from human leukocytes exposed to different ZnCl_2 concentrations and its respective intracellular levels following exposure. Higher 'X' axis places concentrations of ZnCl_2 exposure and lower 'X' axis places Zn intracellular levels after exposure. Data were analyzed by one-way ANOVA followed by Tukey test for multiple comparisons and expressed average \pm SEM, with $p < 0.001$. a, b, c, d, e – the superscript letters indicate statistical difference between groups; NC = negative control, PC = positive control.

Zn is a structural component of DNA and RNA polymerases, for over 3,000 transcription factors,

including zinc finger proteins, and has a modulatory role in cell growth, both in normal and cancer cells (SINGH; GARG, 1998). However, at high concentrations it alters its cellular effects, from protector to inducing oxidative stress (GUAN et al., 2012). In this regard, reactive oxygen species can oxidize nucleobases in DNA, especially guanine, forming 8-hydroxy-2-deoxyguanosine, which ultimately leads to mutagenic or carcinogenic cell formation (SINGH; GARG, 1998).

Additionally, it is well known that the Zn concentration leads to increased expression of p53 mitogen, MAPK and PI3K/AKT families, and antiapoptotic proteins Bcl-2 and Bcl-XL (WILLIAMS et al., 2004; MIN, et al., 2003; SLIWINSKI et al., 2009). These events are, according to the authors, linked to oxidative damage and breakage of DNA strands.

Within this context, the data in the literature suggest that Zn can induce damage in different cell lines; however, such damages are dependent on the duration of exposure, concentration and the molecule of which the metal is a part. Actually, Sharif et al. (2012) found genotoxicity in keratinocytes exposed to 32 μM , about 9 $\mu\text{M mL}^{-1}$ of Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), a concentration about three times smaller than tested by Sliwinski et al. (2009) (100 μM) in leukocytes.

Additionally, Guan et al. (2012) showed that a ZnO concentration of 25 $\mu\text{g mL}^{-1}$ is able to induce cytotoxicity and genotoxicity in hepatocytes and embryonic kidney cells. In contrast, Yang et al. (2013) found damage to thalamic and hippocampal cells - CA3 region of mice - only when they were exposed to a much higher concentration of 265 $\mu\text{g mL}^{-1}$ ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$).

Altogether, our results provide new insights regarding the concentration, exposure length and Zn molecule (ZnCl_2) capable of inducing cell cytotoxicity and genotoxicity, particularly, in human leukocytes.

Conclusion

This study provided a better understanding about the *in vitro* ZnCl_2 cytotoxicity and genotoxicity in human leukocytes. ZnCl_2 as source shows cytotoxicity and genotoxicity at exposure concentrations of 5 and 10 $\mu\text{g mL}^{-1}$, with intracellular levels of 95.68 and 126.2 $\mu\text{g cell}^{-1}$, respectively. In an unprecedented strategy, the intracellular Zn level was correlated with the different exposure concentrations and, from this, we could induce that the Zn inflow occurs under zero-order kinetics.

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