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Cadmium-induced genotoxicity in human osteoblast-like cells

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ABSTRACT

Cadmium (Cd) is a widespread heavy metal used in numerous industrial processes. Cd exerts toxicological effects mostly in kidney and liver. Bone is also an important target of Cd, however, the cellular mechanisms of Cd toxicological effects in the bone cells are still poorly understood. Therefore, the present work, aimed to investigate the putative cytotoxic and genotoxic effects of Cd to human bone cells. For that, the osteoblast-like MG-63 cells were exposed to 20 and 50 μM Cd for 24 and 48 h. Results showed a dose-dependent increase in Cd accumulation in cells and a decrease in cell viability, especially after 48 h. Cell cycle analysis showed a delay at S phase concomitant with a decrease in cells at G0/G1 phase. After 24 h, Cd treatment downregulated the expression of CHEK1, CHEK2 and CDK2 genes and upregulated the expression of CCNE1 gene. After 48 h, the expression of ATM and CCNB1 genes were downregulated. Also, a 3.3 fold increase on the expression of gene CCNE1 was detected. Both Cd doses induced DNA fragmentation at 48 h, while an increase in micronuclei (MN) and nucleoplasmic bridges (NPBs) together with an increase in the percentage of apoptotic/necrotic cells was detected for both time periods. Overall, our results demonstrate the cytotoxicity and genotoxicity of Cd in human bone cells. Also, the cytokinesis-block micronucleus (CBMN) assay parameters (MN, NPBs and the percentage of cells under apoptosis or necrosis) together with the cell cycle appear as the most sensitive to Cd cyto- and genotoxicity, being early affected even with the lowest Cd dose. Therefore, these cyto-/genotoxic techniques may be selected for early detection of Cd-induced toxicity.

1. Introduction

Cadmium (Cd) is a widespread heavy metal used in several industrial processes as smelting, battery manufacturing, pigment and plastic production and also in alloys, solders and electroplating [1,2]. One of the main sources of exposure to Cd is tobacco smoking, mostly due to tobacco plant hyperaccumulating characteristics and contamination of the soils where tobacco plants grow [1]. Moreover apart from being a widespread metal, Cd presents a biological half-life of 20–40 years in humans [3]. Recently, Thévenod and Lee [4] reported that chronic exposure to low Cd^{2+} doses emerged as a "*previously underestimated significant health hazard for ~10% of the general population that increases morbidity and mortality*".

Cd absorbed in the body accumulates mainly in the liver and kidney and its toxicological effects are observed mostly in lungs, prostate and belly [5]. Also, the bone tissue was reported as an important target of Cd toxicity. Itai-itai disease was the most severe case of mass Cd intoxication documented and originated from industrial contamination in Jinzu river basin. Among other symptoms, this disease was characterized by osteomalacia and osteoporosis due to Cd accumulation in bone [6]. Cd appears to alter skeletal processes by decreasing the accumulation of bone mass during skeletal growth and influencing bone metabolism and maturity causing osteopenia in rats [7]. In mice, Cd has been shown to stimulate the formation and activity of osteoclasts, the cells responsible of bone resorption [8]. Therefore, Cd can stimulate bone resorption and inhibit bone formation leading to bone loss. Moreover, in animals exposed to Cd bone demineralization began shortly after the start of Cd exposure and before the beginning of kidney damage [9,10], indicating that Cd directly affects bone metabolism.

Abbreviations: *ATM*, ataxia telangiectasia mutated; CBMN, cytokinesis-block micronucleus; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; F-actin, filamentous actin; FBS, fetal bovine serum; FCM, flow cytometry; FS, forward scatter; ICP-OES, inductively coupled plasma optical emission spectrometry; LMPA, low melting point agarose; MMS, methyl methanesulfonate; MN, micronucleus/micronuclei; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDI, nuclear division index; NMPA, normal melting point agarose; NPBs, nucleoplasmic bridges; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PI, propidium iodide.

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Also, Ogoshi et al. [11] reported that Cd at 100 ng/g dry weight in bone decreased the strength of rat femurs. These studies indicate that Cd may affect bone metabolism directly, however, the mechanisms by which Cd disrupts bone function are not fully understood.

Cd is not a nutrient and therefore it is unlikely to have specific dedicated transport mechanisms for cellular uptake. Studies of Levesque et al. [12] in osteoblast-like cell line MG-63 showed that Cd may enter the cell *via* Ca and Mg channels to enter cells and suggest that the effect of Cd in bone metabolism may be enhanced under low Ca/Mg levels.

Recently, Thévenod and Lee [4] reviewed the cellular signaling cascades elicited by Cd and describe that after acute Cd exposure, the levels of Ca and ROS increase, acting as second messenger systems in the cell. Other cell signaling mechanisms (including upstream signaling pathways for apoptosis *vs* upstream signaling pathways for cell survival) are also described [4].

Cd does not catalyze Fenton-type reactions, however it is known to induce oxidative stress indirectly through interference in antioxidative balance by inhibition of antioxidative enzymes activity and depletion of enzymatic/non-enzymatic antioxidants such as reduced glutathione (GSH) [13–17]. For what is known in bone cells, Brzóska et al. [18] showed that Cd disturbs oxidative status of rat bone, contributing to damage of this tissue. It has been also hypothesized that Cd might induce oxidative stress damage in osteoblasts through the decrease of the expression of RUNX2 leading to apoptosis [16]. There are also evidences that Cd does not interact directly with DNA [19], moreover some authors consider it as "*weakly genotoxic*" [[20], for a review]. However, the oxidative stress generated in Cd-exposed cells may originate DNA strand breaks, chromosomal aberration, micronuclei occurrence and generation of 8-OHdG (8-oxo-2′-deoxyguanosine) in several types of mammalian cells [15,21–23]. Wang et al. [24] found that rats exposed to chronic low doses of Cd showed persistent damage in kidney with increased cell proliferation and decrease in global DNA methylation. Also, Cd exposure increased the occurrence of MN in polychromatic erythrocytes in rats [25] and in fish models (zebrafish) [26]. Finally, humans occupationally exposed to Cd showed increased levels of chromosomal aberrations and sister chromatid exchanges [27]. These authors highlighted the reliability cytogenetic techniques in the detection of Cd-induced mutagenicity [27]. Cd has also been shown to interfere with genomic stability by inhibiting a number of DNA repair enzymes. In fact, Cd inhibits several systems such as nucleotide excision repair (NER), base excision repair (BER) [28], mismatch repair (MMR) and the non-homologous end-joining (NHEJ), the major double strand breaks repair pathway [29]. Also, our previous work has proved that Cd exposure induces microsatellite instability in mice testis [30].

In this context, the aim of the present study was to: (a) give an updated overview of the genotoxic and cytotoxic effects of Cd in human osteoblast-like MG-63 cells using several biomarkers; (b) select the most sensitive and reliable biomarkers considering the importance of using cytogenetic techniques as a sensitive and effective means for detection of Cd-induced genotoxicity/mutagenicity.

2. Materials and methods

2.1. Cell culture

Human osteoblast-like cell line MG-63 was kindly provided by INEB, University of Porto, Portugal. MG-63 cell line was cultured *in vitro* cultured in minimal essential medium with α modification (MEM-α) without nucleosides, supplemented with 10% (v/v) FBS, 100 Units mL^{-1} penicillin/100 μg mL^{-1} streptomycin and 2.5 μg mL^{-1} fungizone, (all medium components from Life Technologies, Carlsbad, CA, USA) at 37 °C, 5% CO_2, in a humidified atmosphere. Cell confluence and morphology were daily observed under an inverted phase contrast microscope Nikon Eclipse TS100 (Japan). Cells were subcultured when confluence reached 80% using 0.25% trypsin/1 mM EDTA (Life Technologies, Carlsbad, CA, USA). For metal exposure, cells were left 24 h for adhesion and after that medium was replaced with fresh medium containing $CdCl_2$ (Sigma–Aldrich, St. Louis, MO, USA) at concentrations of 20 and 50 μM (selected based on MTT results). Culture medium without $CdCl_2$ served as control in each experiment. MG-63 cells were cultured in the referred conditions for 24 h and 48 h.

2.2. Cell viability

Cell viability was performed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to Twentyman and Luscombe [31] with slight modifications. Briefly, cells were seeded in a 96-well plate at 1×10^3 cells/well and, after cell adhesion, they were exposed to Cd from 5 to 150 μM. At the end of each exposure time, 50 μL of MTT (Sigma–Aldrich, St. Louis, MO) solution (1 mg mL^{-1} in PBS pH 7.2) were added to the medium and cells were incubated for 4 h at 37 °C, 5% CO_2, in darkness. The formed formazan crystals were solubilized with DMSO (Sigma–Aldrich, St. Louis, MO) and left on a shaking plate for 2 h at RT protected from light. The absorbance was read at 570 nm using a microplate reader Synergy™ HT Multi-Mode (BioTeK®, Winooski, VT, USA). The IC_{30} and IC_{50} of Cd for cell viability of MG-63 cell line under the conditions tested was calculated using Sigma Plot 11.0 software (Systat Software Inc., Germany) with the four-parameter logistic function standard curve analysis for dose-response.

For 24 h of exposure, the IC_{30} and IC_{50} of Cd for cell viability of MG-63 cells are 68 μM, and 91 μM, respectively. For 48 h of exposure, the IC_{30} and IC_{50} are 54, and 91 μM, respectively. The IC_{30} has been indicated by Tice et al. [32] as a threshold concentration of cytotoxic effects to further analyze genotoxicity. Therefore, concentrations below that value should be used. Based on the results, two doses under IC_{30} were chosen for further assays: 20 and 50 μM Cd.

2.3. Cadmium quantification

Cells (1.5×10^6) were cultured in 100 mm dishes and exposed to Cd as described above. After Cd treatment, cells were washed and scraped in cold PBS at pH 7.2. Then, cell suspensions were harvested and centrifuged at 1000 × *g* for 10 min at 4 °C and the pellets were resuspended in 300 μL of cold ultrapure water. Then, samples were homogenized by sonication for 30 s, intermittently. From these samples, 50 μL were retrieved for total protein quantification by the Bradford method [33]. For Cd quantification, 200 μL of concentrated HNO_3 (65% (v/v)) (Panreac, Barcelona, Spain) were added to 200 μL of remaining sample. Each sample was vortexed and placed in a water bath for 2 h at 80 °C and then transferred to 60 °C overnight. Finally, the three independent biological replicates per Cd treatment samples were diluted with ultra pure water to yield 4% HNO_3 final concentration and stored at RT until Cd analysis by inductively coupled plasma optical emission spectrometry (ICP-OES) in a Jobin Yvon Activa M. Spectrometer (Horiba scientific Inc, NJ, USA). The detection limit was 2 μg L^{-1}. Cd contents were expressed as μg mg^{-1} protein.

2.4. Cell cycle analysis

Cell cycle analysis was performed according to the method described by Almeida et al. [34] with slight modifications. Cells were seeded in 6-well plates with an initial density of 5×10^5 cells. After Cd treatment cells were trypsinized and centrifuged at 600 × *g* for 10 min at 4 °C. Then, cells were washed with PBS pH 7.2, and resuspended in cold 85% ethanol for fixation and stored at −20 °C.

For cell cycle analysis, fixed cells were washed in PBS pH 7.2, and filtered through a 55 μm nylon mesh to eliminate large clusters. Then, for each sample 50 μg mL^{-1} RNase and 50 μg mL^{-1} propidium iodide (PI) (both from Sigma–Aldrich, St. Louis, MO, USA) were added. Samples were then incubated for 20 min at RT in the obscurity until analysis. The relative light scatter properties, side scatter (SS) and forward scatter (FS), as well as the relative fluorescence intensity of PI-stained nuclei were measured with a Beckman Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL, USA). The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Acquisitions were made using SYSTEM II™ software (v. 3.0, Beckman Coulter, USA). The amplification was adjusted so that the peak corresponding to cells in G0/G1 phase of cell cycle was positioned at channel 200. For each sample, a minimum of 5000 events were recorded. Cell cycle analysis was performed using the FlowJo software (Tree Star Inc., Ashland, OR, USA). Results were expressed as percentage of nuclei in G0/G1, S and G2 phase of the cell cycle.

2.5. Gene expression of cell cycle related proteins and DNA damage checkpoints

The web interface Primer3 [35] was used for the design of gene-specific primer pairs (Table 1), which were confirmed for genome single hits by the UCSC In-Silico PCR Genome Browser (http://genome.ucsc.edu/cgi-bin/hgPcr). RNA was extracted using the Trizol method from MG-63 control and exposed to 50 μM Cd cells for 24 and 48 h. Phase-Lock Gel Heavy tubes (5 Prime 3 Prime, Inc., Boulder, CO, USA) were used for phase separation. The aqueous phase was mixed with 1 vol 70% ethanol and RNA was purified using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). For cDNA synthesis, 2 μg total RNA were pre-incubated with DNase I (Sigma–Aldrich, St. Louis, MO, USA) and reverse-transcribed with 1 mM Oligo dT18, using the Omniscript RT Kit (Qiagen, Hilden, Germany). The cDNA samples were prediluted in ultrapure MilliQ water (1:20). The final individual qPCR reactions contained iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), 1.5 μM each gene-specific primer and

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