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To cite this article: Cristina Monteiro, José Miguel P. Ferreira de Oliveira, Francisco Pinho, Verónica Bastos, Helena Oliveira, Francisco Peixoto & Conceição Santos (2018): Biochemical and transcriptional analyses of cadmium-induced mitochondrial dysfunction and oxidative stress in human osteoblasts, Journal of Toxicology and Environmental Health, Part A, DOI: 10.1080/15287394.2018.1485122

To link to this article: https://doi.org/10.1080/15287394.2018.1485122

Published online: 18 Jun 2018.
Biochemical and transcriptional analyses of cadmium-induced mitochondrial dysfunction and oxidative stress in human osteoblasts

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ABSTRACT
Cadmium (Cd) accumulation is known to occur predominantly in kidney and liver; however, low-level long-term exposure to Cd may also result in bone damage. Few studies have addressed Cd-induced toxicity in osteoblasts, particularly upon cell mitochondrial energy processing and putative associations with oxidative stress in bone. To assess the influence of Cd treatment on mitochondrial function and oxidative status in osteoblast cells, human MG-63 cells were treated with Cd (up to 65 μM) for 24 or 48 h. Intracellular reactive oxygen species (ROS), lipid and protein oxidation and antioxidant defense mechanisms such as total antioxidant activity (TAA) and gene expression of antioxidant enzymes were analyzed. In addition, Cd-induced effects on mitochondrial function were assessed by analyzing the activity of enzymes involved in mitochondrial respiration, membrane potential (ΔΨm), mitochondrial morphology and adenylate energy charge.

Treatment with Cd increased oxidative stress, concomitantly with lipid and protein oxidation. Real-time polymerase chain reaction (qRT-PCR) analyses of antioxidant genes catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione S-reductase (GSR), and superoxide dismutase (SOD1 and SOD2) exhibited a trend toward decrease in transcripts in Cd-stressed cells, particularly a downregulation of GSR. Longer treatment with Cd (48 h) resulted in energy charge states significantly below those commonly observed in living cells. Mitochondrial function was affected by ΔΨm reduction. Inhibition of mitochondrial respiratory chain enzymes and citrate synthase also occurred following Cd treatment. In conclusion, Cd induced mitochondrial dysfunction which appeared to be associated with oxidative stress in human osteoblasts.

ARTICLE HISTORY
Received 17 January 2018
Revised 10 May 2018
Accepted 3 June 2018.

KEYWORDS
Cadmium; ROS; human osteoblasts; mitochondrial dysfunction; antioxidant activity

Introduction
Humans are exposed to cadmium (Cd) through multiple ways including ingestion, inhalation and dermal exposure since this heavy metal is present in food, cigarettes, coal, consumer products (batteries, pigments, coatings and plastings, stabilizers for plastics, nonferrous alloys, and photovoltaic devices) (Isermann et al. 2017; Mead 2010; Rani et al. 2014; Xu et al. 2013). Chronic exposure to Cd may lead to different pathologies, such as neurological diseases, testicular harm and infertility, diabetes, cancer, and severe renal disorders (Elbaghdady, Alwaili, and EL-Demerdash 2017; Nair et al. 2013; Noël, Guérin, and Kolf-Clauw 2004). Rodríguez and Mandalunis (2016) demonstrated that Cd administration decreased bone volume and increased tibial yellow bone marrow in Wistar rats. Bougammoura et al. (2017) observed a decrease in femur length, width and area, shortening of diaphysis, and a reduction in length and area of distal and proximal proliferative zones in fetuses obtained from Cd-exposed mothers. Duranova et al. (2014) found that subchronic peroral exposure to Cd lowered femoral weight and produced altered histological structure of compact bone, induced an early stage of osteoporosis and reduced bone vascularization in rats. Bhattacharyya (2009) noted that long-term dietary
Cd exposures in rats, at levels corresponding to environmental exposures in humans, increased skeletal fragility and decreased mineral density. Cd also induces genomic instability through multifactorial mechanisms and affects cell proliferation, differentiation and may promote apoptosis (Barbosa 2017; Rani et al. 2014). Although Cd exposure may increase oxidative stress (Menon, Chang, and Kim 2016; Sandbichler and Höckner 2016), the activity of antioxidant enzymes might also be inhibited by Cd (Dai et al. 2018; Patra, Rautray, and Swarup 2011). Dai et al. (2018) showed that Cd decreased antioxidant capacity, activities of catalase (CAT), superoxide dismutase (SOD) and xanthine oxidase (XOD) as well as elevated malondialdehyde (MDA) levels. Lin et al. (2017) found that Cd reduced the activity of antioxidant enzymes in the hepatopancreas of Eriocheir sinensis. Longer periods of exposure to Cd may result in enhanced activity as a consequence of homeostatic adjustments (Thévenod and Lee 2013).

Mitochondria are key organelles in several cell pathways including oxidative stress, energy processing and apoptosis. Besides being the major cellular source of reactive oxygen species (ROS) production, mitochondria are also major targets for ROS, which may lead to mitochondrial dysfunction and energy decline. Mitochondria were identified as key targets of Cd-mediated toxicity in liver, brain and heart of guinea pigs (Wang et al. 2004), trout erythrocytes (Orlando et al. 2017), and neuronal cells (Xu et al. 2017). In vitro assays provide similar profiles with Cd inducing mitochondrial disorders in vero cells (Murugavel et al. 2007), rat hepatoma cells (Belyaeva et al. 2008), human hepatoma cells and rat glioma cells (Belyaeva et al. 2006; Yang, Yu, and Gupta 2014) and in isolated mitochondria (Dorta et al. 2003; Wang et al. 2004). Dai et al. (2018) detected mitochondrial swelling, vacuolation, and disruption of mitochondrial cristae in Cd-treated cells. Wang et al. (2013) in mice kidney also noted Cd induced mitochondrial membrane potential collapse.

Although Cd affects bone tissues, the influence of this metal on oxidative status in human cells is not completely elucidated. There are reports on oxidative stress initiated by Cd on Saos-2 cells (Smith et al. 2009) and a rat model (Brzóska, Rogalska, and Kupraszewicz 2011); however, more studies are needed to understand the influence of Cd on oxidative status on human cells. It was demonstrated that Cd exerts direct cytotoxic effects on rat and human osteoblasts, which involve caspase- and MAPK pathways in metal-induced apoptosis (Arbon et al. 2012; Hu et al. 2015; Papa et al. 2015; Zhao et al. 2015). The aim of the present study was to examine the effects of Cd treatment on oxidative stress and mitochondrial function response using a human osteoblast cell line MG-63. Intracellular ROS and antioxidant defense mechanisms [total antioxidant activity (TAA), total glutathione, and gene expression of antioxidant enzymes] as well as protein and lipid oxidation determined. Further the effects on mitochondrial functions and energy status were assessed by determining the activity of enzymes involved in mitochondrial respiration, $\Delta \Psi_m$, mitochondrial morphology and adenylate energy charge (AEC).

**Methods and materials**

**Cell culture**

Human osteoblast cell line MG-63 which is often used as an osteoblast-model cell line (Hu et al. 2015) was kindly provided by INEB, University of Porto, Poal. MG-63 cells were cultured in vitro in MEM-α medium without nucleosides, supplemented with 10% (v/v) FBS, 100 Units/mL penicillin/100 µg/mL streptomycin and 2.5 µg/mL fungizone (Life Technologies, Carlsbad, CA, USA) at 37°C, 5% CO$_2$, in humidified atmosphere (Oliveira et al. 2014). Cell confluence and morphology were observed daily using an inverted phase contrast microscope Nikon Eclipse TS100 (Japan). Cells were subcultured when confluence reached 80%, at a proportion of 1:9, using 0.25% trypsin/1-mM EDTA (Life Technologies, Carlsbad, CA, USA). For metal treatments, cells were left 24 h for adhesion. Subsequently, the medium was replaced by medium containing CdCl$_2$ (Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 20, 50, or 65 µM. Culture media without CdCl$_2$ served as control in each experiment. MG-63 cells were cultured in the referred conditions for 24 and 48 h. The concentrations selected were
based upon previous findings (Oliveira et al. 2014) where two concentrations are under IC$_{30}$ and one over IC$_{30}$, since the IC$_{30}$ and IC$_{50}$ were 68 μM, and 91 μM for 24 h and 54, and 91 μM for 48 h, respectively.

**Intracellular adenine nucleotides**

Quantification of adenine nucleotides was used to assess cellular energy status indicated by the equation of AEC (EC) = ([ATP] + \(\frac{1}{2}\) [ADP])/([ATP] + [ADP] + [AMP]) (Atkinson 1968). To quantify adenine nucleotides, 1.5 × 10$^6$ cells were plated and treated with Cd for 24 or 48 h. After Cd treatment, cells were washed and scraped in cold PBS pH 7.2. Cell suspension was centrifuged at 1000×g for 10 min at 4°C. The pellet was then resuspended in 350-µL cold PBS pH 7.2. The extraction procedure was performed according to Ryll and Wagner (1991). Adenine nucleotides were identified and quantified by HPLC following the method described by Stocchi et al. (1985). An UltiMate 3000 Column Compartment Dionex (Model TCC-3200) equipped with a PDA 100 Dionex, and an ACE 5 C18 reversed phase column (250 mm × 4.6 mm × 5 μm) with a precolumn were used at 25°C. The flow rate was 0.7 mL/min and elution solvent system comprised solvent A (1.2% methanol in KH$_2$PO$_4$ 0.1 M, pH 6.5) and solvent B (20% methanol in KH$_2$PO$_4$ 0.1 M, pH 6.5). The detector was set at 260 nm to detect adenine nucleotides. The compounds were identified based upon retention time of commercial standards of ATP (>99% HPLC, Sigma-Aldrich, St. Louis, MO, USA), ADP (98% HPLC, Sigma-Aldrich, St. Louis, MO, USA) and AMP (>99% HPLC, Sigma-Aldrich, St. Louis, MO, USA) and comparison with the UV spectrum.

**Mitochondria isolation**

Mitochondria were isolated from cultures obtained by growing cells in 150-mm Petri dishes at an initial density of approximately 1 × 10$^7$ cells and treated with Cd for 24 or 48 h. Cells were washed and scraped in cold PBS pH 7.2. Cell pellets were washed twice with ice-cold PBS, subsequently resuspended in homogenization buffer (210-mM mannitol, 70-mM sucrose, 10-mM HEPES, 1-mM EDTA, 10-mM KCl, 1.5-mM MgCl$_2$, 1-mM dithiothreitol, and 0.1-mM phenylmethylsulfonyl fluoride at pH 7.4), homogenized with a 5-mL syringe fitted with 25 G and centrifuged according to Xiao et al. (2012). Cells breakage was checked by phase-contrast microscopy. The homogenates were centrifuged twice at 800× g for 10 min at 4°C. Supernatants were then centrifuged at 14,000× g for 10 min at 4°C, the resulting mitochondrial fraction were resuspended in a minimum volume of this homogenization buffer and frozen at −80°C. Protein content in the mitochondrial suspension was determined by Bradford method (1976).

**Mitochondrial respiratory chain enzymes and citrate synthase activities**

Mitochondrial fraction was frozen and thawed three times prior to assessment of enzymatic activities to make accessible the active sites of these enzymes. Complex I (NADH-ubiquinone oxidoreductase) activity was determined by measuring the absorbance fall at 340 nm resulting from NADH concentration decrease at 37°C as previously described by Kiebish et al. (2013), with minor modifications. The assay was performed in buffer containing 50 mM K$_2$HPO$_4$ (pH 7.4), 2-mM KCN, 5-mM MgCl$_2$, 2.5-mg/mL BSA, 2-µM antimycin, 100-µM decylubiquinone, and 0.3-mM NADH (ε = 6.22 mM$^{-1}$ cm$^{-1}$). The reaction was initiated by adding purified mitochondria (50-µg total protein). Enzyme activity was measured for 5 min and values recorded 30 s after the initiation of the reaction and expressed as mmol/min/mg. The specific activity was determined by the slope of the reaction in the linear range in the presence or absence of 1-µM rotenone (Complex I inhibitor). The enzymatic activity was calculated based on the molar extinction coefficient of NADH (6.2 mM$^{-1}$ cm$^{-1}$).

Complex IV (cytochrome c oxidase) specific activity was determined by measuring the oxidation of reduced cytochrome c at 550 nm (ε = 21.84 mM$^{-1}$ cm$^{-1}$) in the presence or absence of 2.75-mM KCN. The assay was performed at 30 °C in 50-mM K$_2$HPO$_4$ (pH 7.4), containing 0.2-µM rotenone, 0.2-µM antimycin. After 2 min the reaction was initiated by adding 11 µM reduced cytochrome c and enzyme activity measured for
3 min. Specific activity was calculated based on the molar absorptivity coefficient of cytochrome c (18.5 mM$^{-1}$ cm$^{-1}$).

Citrate synthase specific activity was spectrophotometrically measured according to Srere (1969), with minor modifications. The assay was performed at 30°C in a buffer, 200-mM Tris-HCl (pH 8), 2 μL 10-μM DTNB, 25 μL 1-mM oxaloacetate, and 50-μg mitochondrial. The reaction was started by the addition of 92.5 μL of 0.37-mM acetyl-CoA and monitored at 412 nm for 3 min. Citrate synthase specific activity was determined by measuring the decrease of DTNB concentration at 412 nm ($\varepsilon = 13.6$ mM$^{-1}$ cm$^{-1}$) (Synergy™ HT Multi-Mode, BioTek), and values expressed as nmol/min/mg protein.

**Mitochondrial membrane potential ($\Delta\Psi_m$)**

$\Delta\Psi_m$ was assessed by flow cytometry following the method described by Barros et al. (2013). Briefly, 1.5 × 10$^5$ cells were seeded in a 6-well plate and, after cell adhesion, they were treated with Cd as described above. At the end of the respective treatment time, cells were washed with PBS pH 7.2 and incubated with 5-µg/mL Rho123 (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, at 37°C. The cells were washed with PBS pH 7.2, trypsinized, and centrifuged at 800× g for 5 min, at 4°C. After centrifugation, the cell pellet was washed with 1-mL PBS pH 7.2, 1% BSA, and resuspended in 500-µL PBS pH 7.2 with 1% BSA and 5 μg/mL PI (Sigma-Aldrich, St. Louis, MO, USA). The samples were then analyzed by flow cytometry using a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL, USA) and at least 10000 events were analyzed. Data were acquired using the SYSTEM II (v. 2.5) software. The fluorescent intensity correlating with $\Delta\Psi_m$ as a function of mitochondria was recorded at 488-nm excitation and 525-nm emission wavelengths. Flow-cytometry data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA). The loss of $\Delta\Psi_m$ was visualized as indicated by the reduction of Rho123 fluorescence intensity.

**Intracellular ROS**

Intracellular ROS were measured using the fluorogenic probe DCFH$_2$-DA (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated in 6-well plates at a density of 150 × 10$^3$ cells/well. After 24 or 48 h Cd treatments, cells were washed with PBS pH 7.2 (Gibco by Life Technologies) and incubated 30 min at 37°C with serum-free MEM-α containing 10 μM DCFH$_2$-DA. Cells were trypsinized and collected in cold MEM-α and analyzed in a flow cytometer (Coulter Electronics, Hialeah, FL, USA). DCF was analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). For each sample, the number of events reached at least 10000. Hydrogen peroxide was used as positive control (data not shown).
Total antioxidant activity

TAA was determined with Antioxidant Assay Kit (MAK187, Sigma-Aldrich, St. Louis, MO, USA). Cells were plated in 100-mm Petri dishes at a density of $1.5 \times 10^6$ cells. Cells were washed with PBS pH 7.2 and used for subsequent TAA analysis. The absorbance was read at 405 nm in a microplate reader (Synergy™ HT Multi-Mode, BioTeK). The absorbance decrease reflects the increase of antioxidant activity (Trolox, a water-soluble vitamin E analog, was used as a standard or control antioxidant). The results were normalized by protein concentration. To calculate the TAA/ROS ratio the results obtained of TAA and divided by the values obtained of ROS production.

Protein quantification, protein oxidation, and lipid peroxidation

Total protein was quantified by the method of Bradford Assay (B6916, Sigma-Aldrich, St. Louis, MO, USA). The absorbance was read at 595 nm and a standard curve of BSA (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate the unknown concentrations. Protein oxidation was measured using Protein Carbonyl Colorimetric Assay Kit (Ref 10005020 Cayman Chemical Company, USA) following the manufacturer’s instructions. Carbonyl content was expressed in nmol/protein. Lipid peroxidation was measured by fluorimetric thiobarbituric acid reactive substances (TBARS) assay (Ahmad et al. 2012). Fluorescence was read at an excitation $\lambda_{528}$ nm and an emission $\lambda_{550}$ nm, using a microplate reader (Synergy™ HT Multi-Mode, BioTeK). TBARS concentration was normalized to protein concentration.

Gene expression of antioxidant enzymes

RNA extraction and qPCR-gene expression was analyzed. The forward and reverse primers for the selected genes were designed using Primer3 (Rozen and Skaletsky 2000) and are listed in Table 1. Primer specificity was confirmed using the In-Silico PCR UCSC Genome Browser (Kent et al. 2002). Cells ($1.5 \times 10^6$) were plated in 100 mm dishes and treated with 50-µM Cd for 24 h. Then cells were washed in PBS pH 7.2, and 1-mL TRIzol® reagent (Life Technologies, Saint Louis, MO, USA) was added for cell lysis, and RNA extraction and preservation (Ferreira De Oliveira et al. 2014). cDNA synthesis was carried out after incubation of 2-µg total RNA with DNase I (Sigma-Aldrich, St. Louis, MO, USA). RNA was reverse-transcribed using the Omniscript RT Kit (Ref 205110, Qiagen, Hilden, Germany) and final qPCR reactions took place according to Ferreira De Oliveira et al. (2014). Gene expression relative to controls, and normalized with the GAPDH reference gene, was calculated (Pfaffl 2001).

Statistical analysis

At least three independent assays were performed for each analysis. The qPCR data were expressed as mean ± SE, while remaining data were expressed as mean ± SD. The qPCR data were analyzed by one-way ANOVA, followed by a Holm–Sidak test, while remaining data were analyzed by two-way ANOVA to evaluate the significance of differences in the parameters. When necessary, data were transformed to achieve normality and equality of variances. When justified, Pearson’s correlation was performed and the respective correlation coefficient was presented as $r$. The level of statistical significance was set at $p < 0.05$. All the statistical analyses were performed with SigmaPlot Version 11.0 for Windows.

Results

Cell energy status and activity of mitochondrial respiratory chain enzymes and citrate synthase

Cd did not markedly affect the energy status of cells treated with Cd for 24 h but after 48 h all Cd concentrations lowered AEC in MG-63 cells.

Table 1. Primer sequences used for qPCR obtained from Ferreira De Oliveira et al. (2014).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>TGAACCTGCTCCATCGGTGC</td>
<td>TATGGATGCTGCTCCAG</td>
</tr>
<tr>
<td>GPX1</td>
<td>CCGGACTACACCCAGAGTAA</td>
<td>TCTCTTCGTTCTTGGCTTC</td>
</tr>
<tr>
<td>GSR</td>
<td>GATCCAAGCGCCTCGATA</td>
<td>TCGCTGGTTATTCCTAAGCTG</td>
</tr>
<tr>
<td>SOD1</td>
<td>GGTTGGCCAGATGTTGCTAT</td>
<td>TCACAGGCTTTCTCTGCTT</td>
</tr>
<tr>
<td>SOD2</td>
<td>CCCTGGAACCTCACATCAAC</td>
<td>CTGAAGAGCTATCGGCGTGA</td>
</tr>
</tbody>
</table>
Concerning enzymes involved in the TCA cycle, Figure 1B shows that Cd reduced the activity of citrate synthase in cells treated with 50 and 65 µM. Regarding the mitochondrial respiratory chain, Complex I activity of MG-63 cells was significantly inhibited with 65 µM Cd after 24 h compared to control (Figure 1C). After 48 h, Complex I activity of Cd-treated cells was low exhibiting values similar to those of controls. Further, Complex IV activity (Figure 1D) displayed a concentration- and time-dependent inhibition with Cd.

δψm and mitochondrial morphology

The cytograms from control and treated cells after Rho123 incubation indicated that the profile of the cell population positive for Rho123 was changed with Cd exposure. Figure 2 illustrates that Cd decreased ΔΨm in cells subjected to...
65 µM after both treatment times. **Figure 3** shows the evaluation of mitochondria morphology that was conducted in order to compare the cytological phenomena/damage in the cell after Cd incubation. After 24 h, although control cells presented variability in mitochondrial morphology, showing filamentous, as well as fragmented and intermediate mitochondria, it was observed that at ≥50 µM there was a rise in number and prevalence of fragmented mitochondria. After 48 h, mitochondria morphology of Cd-treated cells was similar to those of controls although mitochondrial swelling was noted in cells treated with 50 and 65 µM Cd.

**Oxidative status, antioxidant battery and transcriptional analyses**

Cd led to an accumulation of ROS in cells treated 24 or 48 h with 65 µM metal (**Figure 4A**). In **Figure 4B**, it was not possible to establish a cause–effect correlation, but it seems that TAA tended to fall with 50 µM Cd after 24 h. In **Figure 4C**, the antioxidant capacity of cells vs. ROS formation is presented. Considering the behavior as function of Cd concentrations, after 24 h, cells exhibited a reduction in the ratio TAA/ROS in a concentration-dependent manner. After 48 h, cells showed different behavior: those treated with 20 µM Cd displayed higher ratio, while cells treated with higher concentrations had lower TAA/ROS ratios. Considering the responses regarding the period of treatment, the TAA/ROS for 48 h was higher than for 24 h for both 20 and 50 µM. In contrast, cells at 65 µM Cd showed a decrease of this ratio with lower values after 24 or 48 h.

Cd did not induce protein oxidation at 24 h; however, the metal produced a concentration-dependent increase of protein carbonyl levels after 48 h (**Figure 5A**). After 24 h, TBARS content fell in a concentration-dependent manner and significantly for concentrations higher than 20 µM (**Figure 5B**). After 48 h, the same pattern was observed, but at the highest concentration of 65 µM Cd the greatest elevation in lipid peroxidation was detected.
Among the analyzed genes of antioxidant enzymes, after 24 h and despite ROS accumulation, no significant change was found in the transcript levels of most antioxidant enzyme genes analyzed. In the case of the GSR gene, expression was decreased to approximately 25% in cells treated with 50 µM for 24 h, compared to control (Figure 6).

**Discussion**

Data demonstrated that with time Cd decreased the capacity of the mitochondria to maintain a balanced ratio \((\text{ATP} + \text{ADP}/2)/(\text{AMP} + \text{ADP} + \text{ATP})\) suggesting a dysfunction in the AEC particularly after 48 h which occurred in conjunction with other changes such as Complex I activity, protein carbonyl and TBARS at the higher concentrations.
concentration. The effects on AEC data are in agreement with results in other species. In oyster, Cd accumulated in mitochondria and lowered ADP-stimulated respiration (Sokolova, Sokolov, and Ponnappa 2005). These results indicate impairment of mitochondrial capacity for ATP production as result of Cd treatment. AEC was also found to be a highly sensitive endpoint rather than the amount of ATP as a marker of changes in energy metabolism in cells and for determination of adverse effects attributed to stress. Although rarely used in toxicological assays, AEC use as an endpoint needs to be strengthened. This is particularly relevant as mitochondrial energy metabolism may be intimately related with oxidative status of the cell, and its analysis may complement the routinely used antioxidant biomarkers. Guida, Walker, and Reina (2016) indicated AEC as a new and useful indicator of capture stress in chondrichthyan. Baranowska-Bosiacka et al. (2011) used this technique to demonstrate that Pb affected the energy status of rats cultured primary cerebellar granule neurons. In the freshwater crab Sinopotamon henanense hepatopancreas cells elevation in Cd concentrations and time of treatments, decreased the ratios of ATP/ADP, lowering energy production (Yang et al. 2015).

Control cells for the 48-h treatment (having the same culture medium during 48 h) exhibited a diminished glucose intake compared to controls incubated for the 24 h. The decrease of glucose supply may reduce the activity of Complex I (Cannino et al. 2012). Our data also showed that Complexes I and IV of the electron transport chain are sensitive to increasing Cd concentrations supporting observations for other cell/tissues that also demonstrated changes in Complexes I–IV, e.g., in rainbow trout (Adiele, Stevens, and Kamunde 2012). The marked impairment in this chain function correlates with the decrease in the AEC. These combined losses (Complexes I and IV, and AEC) suggest that Cd impairs the mitochondrial electron chain leading to biochemical disorders that may influence ATP formation.

Together with the negative effects on the electron chain and AEC, the impact of Cd on the activity of enzymes involved in mitochondrial respiration, particularly citrate synthase (the first step in the TCA cycle, which regulates energy generation in mitochondrial respiration) was significantly reduced by Cd in osteoblast-like cells, supporting that the TCA cycle is impaired by this metal.

These data were associated with the observed adverse effects of Cd on ΔΨm. This parameter indicates that Cd may induce mitochondria dysfunction, and probably this may be related to an increase in depolarization of the transmembrane potential. Our data are in agreement with the findings of Ly, Grubb, and Lawen (2003); Wang et al. (2013); (2014); Dorta et al. (2003), that Cd may lead to opening mitochondrial permeability transition pores which may contribute to depolarization of the transmembrane potential (ΔΨm), and often associated with apoptotic processes. Recently Oliveira et al. (2014) found that Cd also induced apoptosis in MG-63 cells.

Our findings demonstrate that Cd increased morphological damage, as evidenced by rise in number of fragmented mitochondria supporting severe dysfunction observed in osteoblast mitochondria and energy processing. It should be noted that some recovery was detected after 48 h for lower Cd concentrations suggesting a reversible process occurred, while at higher Cd concentrations increased the presence of swelling, correlated with the increased apoptosis (Oliveira et al. 2014). The pathways regulating such responses in osteoblasts remain unknown. Arbon
et al. (2012) using Saos-2 cells, reported that Cd inhibited osteoblasts proliferation via ERK signaling pathway and identified sclerostin (SOST), whose main function is to inhibit bone formation, as a target for metal-induced osteotoxicity. Ha et al. (2016), using Saos-2 and MG-63 cells found that the calmodulin-dependent phosphodiesterase pathway facilitated Cd-induced sustained ERK activation leading to apoptosis. Messner et al. (2016) noted that Cd-induced death signaling starts with the causation of DNA damage and a cytosolic calcium flux, and that these two events lead to an apoptosis signaling-related mitochondrial membrane depolarization and classical DNA damage response.

The gene expression of the different transcripts of antioxidant enzymes demonstrated no marked alterations despite ROS accumulation with significant decreases of GSR gene expression. In contrast in liver cells these transcripts exhibited a different profile following Cd exposure. In male mice, hepatic activities of GPX, CAT and glutathione S-transferase increased in response to Cd. The transcription status of hepatic SOD1, SOD2, CAT, GPX, GSTα1, and GR were also elevated by Cd (Liu et al. 2015). In liver of pufferfish treated with Cd, hepatic tissue displayed upregulation of GR CAT, GPx1, and Cu/Zn-SOD (Kim et al. 2010). In zebrafish SOD1 and SOD2 mRNA levels were up-regulated by non-lethal concentration Cd concentrations (Yin et al. 2018). The impairment of these enzymes transcripts (particularly GR), may exert consequences on the antioxidant cascade of events, and/or be affected at conditions different from those tested here. Pérez-Díaz et al. (2013) noted that in male Wistar rats treated with 100 ppm Cd increased CAT, GPX, and SOD mRNA expressions and CAT, GPX protein levels occurred.

Deregulated oxidative stress often leads to oxidation of macromolecules, with particular evidence in lipids (assessed by MDA/TBARS), proteins (often assessed by the formation of carbonyls) and/or nucleic acids (often associated with DNA mutations). It was evident that the concentrations of Cd studied produced (mostly after 48 h) enhanced oxidation of proteins and lipids in MG-63 cells. Considering that lipids and proteins are major constituents of membranes, it might be postulated that membranes may ultimately be affected with loss of permeability as measured by flow cytometry enabling propidium iodide entry. A correlation between increased MDA/oxidative stress and reduced ATP synthesis in Complex I substrates-energized mitochondria was reported in trout by Adiele, Stevens, and Kamunde (2012). Previously (Oliveira et al. 2014) demonstrated that these Cd-exposed cells exhibited apoptosis. The link existed between this process and Cd-induced oxidative and loss of mitochondrial function needs to be further examined.

Conclusions

This is the first study to examine the effects of Cd on osteoblast-like cells oxidative stress and mitochondrial function. Cd severely impaired electron flux transport and in TCA cycle produced a deficient AEC, together with a decrease in mitochondrial ΔΨm and increased quantity and severity of morphological abnormalities. The Cd-mediated induced oxidative stress occurred in conjunction with fall in GR transcript and elevation in lipid/protein oxidation.

Conflicting interests

The authors declare that there is no conflict of interest.

Funding

This work was developed in the scope of the projects POCI/01/0145/FEDER/007265 (ref. FCT UID/QUI/50006/2013), POCI/01/0145/FEDER/007728 (ref. FCT UID/MULTI/04378/2013), and CESAM and POCI-01-0145-FEDER-007638 (ref. FCT UID/AMB/50017), financed by national funds through the FCT/MEC and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. FCT-awarded grants to JMP Ferreira de Oliveira (SFRH/BPD/74868/2010) and H Oliveira (SFRH/BPD/111736/2015) are acknowledged.

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