Mitochondrial disruption in isolated human monocytes: An underlying mechanism for cadmium-induced immunotoxicity

Ulfat M. Omar, Ekramy M. Elmorsy, and Ayat B. Al-Ghafari

ABSTRACT

Cadmium (Cd) is an immunotoxic metal frequently found in the environment. The in vitro study undertaken here evaluated the immunotoxic effects of Cd in isolated human peripheral blood monocytes (hPBM). The results of the studies of exposures to varying doses of Cd (0, 0.1, 1, 10, and 100 μM, as cadmium dichloride [CdCl₂]) for 3, 6, 12, 24, 48, and 72 hr showed the test agent was cytotoxic to the cells in time- and concentration-related manners. Thereafter, using only those doses found to not cause extreme cell lethality a 48-hr period, the impact of 0.1 or 1 μM CdCl₂ on the cells was evaluated. Functionally, CdCl₂ treatment led to time- and concentration-related decreases in hPBM phagocytic activities as well as in the ability of the cells to form/release cytokines (including tumor necrosis factor [TNF]-α and interleukin [IL]-6) and other pro-inflammatory and anti-inflammatory cytokines production by various immune cells (reviewed in Hossein-Khannazer et al. 2020). In addition, CdCl₂ treatment resulted in significant increases in mitochondrial membrane fluidity (MMF) and cell unsaturated fatty acid content. Based on the results here, one might conclude that some of the effects that arose during the CdCl₂-induced dysfunction of the isolated hPBM (i.e. changes phagocytic activity, cytokine formation/secretion) could have evolved secondary to CdCl₂-induced disruptions of hPBM cell bioenergetics – an effect that itself was a culmination of an overall toxicity from CdCl₂ upon the mitochondria within these cells.

Introduction

Certain heavy metals, such as cadmium (Cd), after host exposure via different routes (mainly oral, occupational inhalation and among cigarette smokers) are able to be deposited in tissues and cause a variety of toxicities which can damage lungs, kidneys, liver and bones with reported immunotoxicity and carcinogenic effects (Kabir et al. 2021). In some cases, the metals can interact with circulating proteins and subsequently affect a variety of cells that bind to/respond to these proteins during the course of normal cell function or activation (Barbier et al. 2005; Ali et al. 2020). The immune system is complex and its many cells and related components are designed to respond to various antigens, pathogens, etc. In some cases, as a result of metal inter-actions with immune response-related proteins, functions of various immune cells are positively or negatively influenced. It is thus not surprising that immunotoxicities like hyper-sensitivity and autoimmunity (among many immuno-pathologies) have been repeatedly observed in workers and animal models after exposure (both acute or chronic) to heavy metals (Marth et al. 2001; Ebrahimi et al. 2020; Pesce et al. 2021). In general, metal exposure more often than not occurs in a chronic manner and these repeated longer-term exposures often result in serious pathogenic immune responses (reviewed in Hossein-Khannazer et al. 2020).

With regard to Cd, it has long been known that exposure to Cd even at low levels in the ambient air could result in increased respiratory tract infections due to a great extent to decreased alveolar macrophage functions that ultimately manifest as reductions in bacterial clearance (Gardner et al. 1977). Exposure to Cd (or lead [Pb]) was also shown to induce inflammation in different experimental models with significant effect on the pro-inflammatory and anti-inflammatory cytokines production by immune cells (Pedro et al. 2019; Larson-Casey et al. 2020; Choudhury et al. 2021). In addition, Cd – primarily when presented as soluble Cd²⁺ ion – was shown to induce cytotoxicity, apoptosis, and functional derangement in vitro in cultures of murine macrophages as well as chicken and mouse peritoneal macrophages (see Goering et al. 2000; Ramirez and Gimenez 2002; Kim and Sharma 2006; Zhang et al. 2020).

Mitochondria play a key role in regulating monocyte/macrophage responses to pathogens and tissue injury (Ravi et al. 2014). Several environmental pollutant metals, including Cd, lead, and mercury are known to accumulate in the mitochondria of many types of cells at higher levels than in other organelles of
the same exposed cells (Alkharashi et al. 2017; Ommati et al. 2019; Branca et al. 2020). This apparent accumulation has been suggested to be secondary to entry of the free ion forms of these metals into the cells – via calcium transporters (i.e. molecular mimicry) or as a result of chemical/electrical gradients (i.e. mitochondrial pH and charge). The latter seems to be a result of the mitochondrial matrix having a slightly alkaline pH and a negative charge due to proton pumping during oxidative phosphorylation (Sjöholm et al. 2017). Hence, with increasing accumulation over time, it would seem that metals like Cd should likely induce mitochondrial disruption.

Because monocytes/macrophages undergo many energy-expending processes during their differentiation, activation, as well as engulfment of antigens/pathogens, exposures to Cd (as well as the other above-cited metals) can be considered as presenting an important immunotoxicological risk to exposed hosts. This supposition has already found support in past studies that have shown that disruption of monocyte/macrophage mitochondria plays a role in the pathogenesis of certain systemic immune-based diseases, including atherosclerosis, chronic kidney disease (CKD), major depression (Hénaut et al. 2019; Orekhov et al. 2020; Simon et al. 2021), and other adverse health outcomes (Al-Ghafari et al. 2019; Al Doghaith et al. 2021). Even so, specific mechanisms for how Cd acts on the mitochondria in these cells to bring about immuno-dysfunction remain ill-defined.

Monocytes have a multitude of roles in the immune response apart from acting as primary phagocytic cells during non-specific immune responses to antigens and/or microbes. For example, macrophages are essential for the propagation of immune and inflammatory responses in that they produce various types of cytokines like interleukin (IL-10), IL-6, IL-8, and tumor necrosis factor (TNF)-α (Wong et al. 2011; Ravi et al. 2014). These cells also produce chemokines that help to guide inflammatory cell trafficking to – and maturation at – sites of inflammation and/or where pathogens are present (Oo et al. 2010).

Because of their key role in non-specific and later, specific immune responses, it would seem that establishing how toxic metals like Cd might affect macrophages – with a focus on the impact on their mitochondria – would help define more precisely the immunotoxic mechanisms of action for this metal. By discerning how potential changes in the functional status of mito-chondria might be related to Cd-induced changes in cell function (like cytokine secretion and phagocytic activity), this could open up new avenues for understanding how other known immunotoxic metals might act to bring about their known effects on host immunity.

**Materials and methods**

**Isolation and culture of peripheral blood mononuclear cells (PBMC)**

For the study, human peripheral blood (15 ml) was obtained from healthy participants by venipuncture under aseptic conditions at the University Medical Center. Informed consent was obtained from each participant prior to blood collection. Confidentiality was maintained at all the steps of the project. The study design was approved by the research committee at Dar Al-Hekma University, Jeddah, Kingdom of Saudi Arabia (RC/2021/002).

Peripheral blood mononuclear cells (hPBM) from each collected sample were isolated by centrifugation (400 × g, 30 min, 4 °C) using a Ficoll-Paque PLUS density gradient (Amersham Biosciences, Little Chalfont, UK) as described previously (Reis et al. 2007). Cells recovered at the buffy coat interface were washed with phosphate-buffered saline (PBS)-EDTA (1 mM) solution, counted using hemocytometer, and then re-suspended at 10⁶ cells/ml in RPMI 1640 culture medium supplemented with 200 mM L-glutamine, 100 U penicillin/ml, and 10 mg streptomycin/ml (all from In Vitrogen, Carlsbad, CA). The cells were then seeded into 150-cm² polystyrene flasks (T-25 flasks) and incubated for 90 min at 37 °C in a humidified atmosphere containing 5% CO₂. The media was then decanted and non-adherent cells removed by gentle washing with PBS. The remaining adherent cells were then detached using sterile cell scrapers and ice-cold cell culture medium. The cells from each flask were washed again by PBS and cell viability ascertained via Trypan blue exclusion (routinely, viability ≥95%). Thereafter, the cells were pooled for use in the assays outlined below. Pooled cells from different participants were used as the main scope of the study was to evaluate Cd-induced cytotoxicity and bioenergetic disruption rather than the inter-individual variation in their mitochondrial response to Cd immunotoxicity which may interfere with the collected data robustness.

**Effects of cadmium on monocyte viability**

Cadmium dichloride (CdCl₂) (Sigma, St Louis, MO, USA) was used in the studies here to test the effects of Cd ions on isolated immune cells. The choice to use CdCl₂ was based on the fact that it is a widely used agent to assess a variety of Cd toxicities, its quite soluble in water and other vehicles (thereby mitigating potential effects regarding particle uptake seen with most insoluble forms of Cd), and its well-documented immunotoxic effects (see citations in Introduction).

Overall toxicity of the test Cd compound on the isolated PBMC was evaluated using both MTT (3-[4,5-dimethylthiazol, 2-yl]-2,5-diphenyl tetrazolium bromide) and LDH (lactate dehydrogenase) assays. For both assays, pooled cells were seeded into 96-well plates (at 5 × 10⁴ cells/0.1 ml/well) and incubated overnight at 37 °C. Thereafter, medium was removed from each well and cells were treated with the CdCl₂. For each experiment, CdCl₂ stock solutions were freshly prepared in double-distilled water; for treatments, stocks were diluted with culture medium to obtain different test concentrations (0.1, 1, 10, 100, and 1000 μM) for use in dedicated wells. After receiving the CdCl₂ (or only medium as control), the plates were incubated at 37 °C for 3, 6, 12, 24, 48, and 72 hr. Dedicated plates were generated for each timepoint and for each assay to be performed.

At each timepoint, an MTT assay was conducted as outlined in Elmorsy et al. (2014). An LDH assay in parallel time-matched plates was conducted using a commercial kit (Clontech, Mountain View, CA), following manufacturer protocols. All OD values for the MTT and LDH assays in the wells (at 590 and 450 nm, respectively) were measured in an MRX Microplate Reader (Dyne Technologies, Richmond, Virginia). Wells containing 2% Triton X-100 in culture medium were used as positive controls for the LDH assay. For the MTT assays, untreated cells were used as positive control wells (100% viability). In all assays, blank wells (without cells) were subtracted from all well readings before further analysis. In each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.
**Determination of cytokine secretion**

Spontaneous (i.e., not induced by mitogen) formation/release of specific cytokines (e.g., IL-6, IL-8, TNFα) by the PBMC was evaluated using commercial ELISA kits (Abcam, Cambridge, MA, USA). In brief, pooled monocytes were seeded at 10^6 cells/1 ml/well into 6-well plates and then exposed for 6, 18, 24, and 48 hr (dedicated plates for each timepoint) to 0, 0.1, or 1 μM CdCl₂. At the end of each time period, cell-free supernatants were generated in the wells by successive centrifugation at 2000, 7000, and 13,000 rpm for 10 min each; generated well supernatants were then collected and stored at −20°C until all samples could be batch-analyzed. All analyses were performed in triplicate. Concentrations of each cytokine in a test supernatant were extrapolated from a standard curve generated in parallel using kit-provided standards. All cytokine concentrations were normalized to the total number of remaining attached cells present in a well at the end of the given exposure to the CdCl₂. All experiments were conducted in triplicate. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Cellular phagocytic ability assay**

The effect of CdCl₂ on isolated cell ability to phagocytize 1-μm latex beads (fluorescent yellow-green carboxylate-modified polystyrene beads; Sigma) was evaluated using the method of Schroeder and Kinden (1983). In brief, the pooled monocytes were seeded at 10^6 cells/1 ml/well in 12-well plates and then exposed to 0, 0.1, or 1 μM CdCl₂ for 6, 12, 24, and 48 hr. At the end of each timeframe, phagocytic activity of the treated cells was evaluated. For this, the cells were washed twice with PBS and then fresh culture medium containing beads (at a 10:1 bead:cell ratio) was added to each well. After 1 hr to allow the cells to phagocytize the beads (at 37°C), the plates were gently centrifuged (225 × g, 5 min) to help separate the cells from any non-phagocytosed beads (which remain in medium supernatant). After decanting the non-ingested materials, cells were washed twice and bead-free medium was added to each well, and fluorescence in each well was measured at excitation and emission wavelengths of 440 and 485 nm (respectively) in a fluorescence microplate reader ‘TopCount’ (Perkin Elmer, Ueberlingen, Germany). All estimates for the phagocytic endpoints assessed here were normalized to the total number of remaining attached cells present in a well at the end of the given exposure to the CdCl₂. All experiments were conducted in triplicate. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Assessment of intracellular ATP content**

To ascertain the effect of the CdCl₂ on intracellular ATP content, the pooled cells were seeded at 10^6/well (100 μl/well) in 96-well plates and cultured overnight at 37°C. After this, the culture medium was removed and dedicated wells then received CdCl₂ at 0, 0.1, 1, 10, or 100 μM and the plates were incubated at 37°C for 6, 12, 24, and 48 hr. At the end of each timeframe, intracellular ATP content was assessed using a commercial kit (Abcam, Cambridge, MA), following manufacturer protocols. Sample luminescence in each well was measured by single photon counting using a TopCount plate reader (Perkin Elmer). Basal values of medium luminescence were subtracted from each well-estimated value. Cell ATP content in each treated well was presented as a percentage of that seen in time-matched untreated control wells (after normalizing values back to the total number of remaining attached cells present in a well at the end of the given exposure to the CdCl₂). All experiments were conducted in triplicate for each timepoint. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Determination of mitochondrial complex activity**

The effects of the CdCl₂ on mitochondrial Complexes I and III in the isolated cells were assessed. Unlike the effects on total cell ATP which evaluated an expansive range of CdCl₂ levels, these protocols only utilized the 0.1 and 1 μM concentrations so as to allow for potential aligning of outcomes with the other endpoints evaluated above.

In brief, pooled cells were seeded at 10^6 cells/1 ml/well in 12-well plates, placed overnight at 37°C, and then exposed to CdCl₂ at 0.1 or 1 μM for 6, 12, 24, and 48 hr. For the Complex I assay, a monocyte mitochondrial-enriched fraction was prepared following the method of Spinazzi et al. (2012). In brief, remaining adherent cells were harvested from the wells, washed three times with PBS by centrifugation, counted, and the final pellet then snap-frozen in liquid N₂. The cells were then thawed and re-suspended in 1 ml (10 mM) ice-cold, hypotonic Tris buffer (pH 7.6) and homogenized with a glass/Teflon tissue grinder. To the disrupted materials, 200 μl of a 1.5 M sucrose solution was added and the homogenate was then centrifuged (600 × g, 10 min, 2°C). The resulting supernatant was collected and centrifuged again (14,000 × g, 10 min, 2°C). After discarding the new supernatant, the mitochondrial pellet was re-suspended in 0.5 ml ice-cold hypotonic 10 mM Tris buffer (pH 7.6). This mitochondrial solution underwent three cycles of liquid N₂ freeze-thawing to disrupt mitochondrial membranes just before they were to be assayed. Levels of proteins liberated into the buffer were then quantified using a Bradford assay.

For the Complex III assay, cell lysate was prepared according to Spinazzi et al. (2012). In brief, the CdCl₂-treated/control cells were harvested as above, washed with PBS, suspended in 0.4 ml of hypotonic 20 mM potassium phosphate buffer (pH 7.5), and then homogenized using a 50-μl Hamilton syringe. The resulting cell lysate then underwent three freeze-thawing cycles; the final materials were placed on ice until use in the assay. An aliquot was removed for measuring its protein content using a Bradford assay.

For the Complex I assay, NADH (100 μM) and ubiquinone (60 μM) were used as terminal electron acceptors; for the Complex III assay, decylubiquinone (an analog of ubiqui-none co-enzyme Q10; 100 μM) and cytochrome c (75 μM) were the electron acceptors. Absorbance values at 340 and 550 nm wavelength were employed in the Complexes I and III assay measures, respectively. Rotenone (10 μM) and potassium cyanide (300 μM) were used as specific inhibitors for calibration of the activity of the Complexes I and III, respectively. Specific complex enzyme activity (in nmol/min/mg) was calculated using the equation: 

\[ \text{activity} = \frac{(1000 \times \Delta \text{ absorbance/min})/([\text{extinction coefficient}] \times \text{volume of sample used [in ml]} \times (\text{sample protein concentration [in mg/ml]}))}{1} \]

The extinction coefficient constants used were 6.81 mM⁻¹·cm⁻¹ for NADH (Complex I activity) and 18.5 mM⁻¹·cm⁻¹ for reduced cytochrome c (Complex III activity) (Hourled et al. 2012). Experiments were repeated at least five times for data robustness. All data were normalizing values back to the original total number of remaining attached cells present.
in a well at the end of the given exposure to the CdCl₂ as this impacted on the total protein content level in the original isolates obtained.

**Lactate production**

Cells were seeded in 24-well plates (5 × 10⁴ cells per well in/1 ml/well) and treated with Cd (0.1 and 1 µM concentrations) for 6, 12, 24 and 48 hr. At each timepoint, medium was collected from each well and the cells then trypsinized and counted. Lactate levels in the isolated media were measured using a commercial lactate assay kit (Biovision, Mountain View, CA, USA) according to manufacturer protocols. Lactate production levels were normalized to total live cell numbers. All experiments were performed in triplicate for each timepoint. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Evaluation of oxygen consumption rate**

The oxygen consumption rate (OCR) of the isolated cells was evaluated polargraphically using Clark oxygen electrodes (Rank Brothers, Bottisham, UK). To study the early effects of the CdCl₂ on OCR, pooled 'naïve' cells were suspended in modified Hank’s balanced salt solution (HBSS; containing 5.6 mM KCl, 140 mM NaCl, 4.2 mM NaHCO₃, 1.2 mM NaH₂PO₄, 2.6 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES [pH 7.4]) supplemented with 0.1% (w/v) bovine serum albumin (Sigma). For the experiment, 1000 µL HBSS containing a fixed density of cells was added to the chamber of each electrode. Every 10 min thereafter, 1 µL of CdCl₂ solution was added to the suspension such that subsequent increases in CdCl₂ concentrations in the chambers (to final concentrations of 0.1 or 1 µM) were attained. Within 10 min of the final CdCl₂ addition, 2 µL of 6 mM sodium azide solution was added to each chamber (to confirm cell viability was maintained through the course of experiment and any potential effect from chamber air leak was overcome). The O₂ consumption was then measured with the electrodes. From the data gathered over 300 sec, the OCR was calculated from changes in pO₂ levels in the recorded curves using WinDaq/Lite (data acquisition software version V.1; DATAQ Instruments, Akron, OH). The effect of azide was measured 60 sec after its addition.

To assess the late effect of CdCl₂ on OCR, naïve cells were harvested and then incubated for 24 hr with medium containing a final CdCl₂ concentration of 0, 0.1, or 1 µM. At the end of the incubation, the cells were trypsinized, centrifuged, re-suspended in HBSS, and counted by hemocytometer. The OCR was then assessed as above, again using the WinDaq/Lite software. In each case, measurements were terminated by addition of 2 µl azide solution to each cell suspension.

**Evaluation of mitochondrial membrane potential**

The effect of CdCl₂ on isolated hPBM mitochondrial membrane potential (MMP) was evaluated using MitoTracker Green staining. In brief, cells were seeded into 24-well plates (5 × 10⁴ cells/0.5 ml/well) and then incubated at 37°C for 24 hr. At that point, the cells were treated with at 0, 0.1, or 1 µM CdCl₂ and cultured a further 6, 12, 24, and 48 hr. Culture medium was then removed and the cells washed with PBS; unattached cells were counted to calculate total cells remaining in each well. Thereafter, MitoTracker Green solution (50 nM; Invitrogen, Eugene, OR) was added to each well, the plate was incubated 30 min at 37°C, and then well fluorescence well was assessed using the TopCount plate reader (excitation and emission wave-lengths of 490 and 516 nm, respectively). Wells that received the uncoupling agent carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP, 100 nM) served as positive controls. Final fluorescence values were all normalized to the total number of remaining attached cells in a well at the end of the given exposure to the CdCl₂. All experiments were conducted in triplicate. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Evaluation of mitochondrial membrane fluidity**

A trimethylammonium-diphenylhexatriene (TMA-DPH) fluorescent probe (Sigma) was used to evaluate the effects of CdCl₂ on mitochondrial membrane fluidity (MMF) according to the method of Pérez-Hernández et al. (2017). In this analysis, pooled cells were seeded at 10⁶ cells/ml/well in 12-well plates and incubated overnight at 37°C. The cells were then exposed for 24 hr to 0, 0.1, or 1 µM CdCl₂. At the end of the exposure, remaining adherent cells were harvested, counted, and their mitochondrial-enriched fraction then prepared (as previously outlined for Complex I assay). For the assay, 250 µl of 1 mM TMA-DPH probe solution was added to 750 µl of mitochondrial suspension (present in tube at 0.5 mg protein/ml) and the mixture then incubated in the dark for 30 min with continuous stirring. Fluorescence polarization was then measured using a fluorescence spectrometer (Perkin Elmer) at excitation and emission wavelengths of 365 and 425 nm, respectively. Fluorescence was normalized to the total number of remaining attached cells found in the well at the end of the given exposure to CdCl₂. All experiments were conducted in triplicates. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

**Determination of mitochondrial membrane fatty acid composition**

In this assay, naïve pooled cells were seeded at 10⁶ cells/ml/well in 12-well plates. Cells were kept overnight at 37°C before being exposed to 0, 0.1, or 1 µM CdCl₂ for 24 hr. The remaining adherent cells were then harvested, counted, and mitochondria isolated as above (method of Spinazzi et al. 2012). Mitochondrial lipids were extracted following the protocol of Oemer et al. (2018) and then trans-esterified using boron trifluoride (14% [w/v] in methanol) (Morrison and Smith 1964). Membrane fatty acid (MFA) composition was analyzed by passing the materials through a 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA) linked in line to a flame ionization detector (FID). Ultra-high purity N₂ gas was used as the carrier; flow-rate was maintained at rate 14 ml/min. Both the injector and detector temperatures were kept at 250°C; column temperature was modified to 180°C for 5 min followed by a gradual increase (5°C/min) until a final level of 240°C was attained. Based on retention times and peak area for standards, MFA compositions for each sample were determined. Levels for each component were reported in terms of mol%.
Effect of co-enzyme Q10 on mitochondrial bioenergetics

To test if the presence of co-enzyme Q10 (Co-Q10) that was expected to enhance mitochondrial bioenergetic parameters (Bergamini et al. 2012) could provide similar protections to the treated cells here, pooled naïve cells were seeded as in the MTT and phagocytosis assays (see above) and then received a medium containing 1 mM water-soluble Co-Q10 formulation (Q-ter; Scharper Therapeutics, Milan, Italy). After incubation at 37°C for 24 hr, the wells then directly received CdCl2 at 0, 0.1, or 1 μM (i.e. Co-Q10 co-present during metal exposure) and the plate was incubated a further 24 hr. In some cases, the Co-Q10 was removed at the time Cd was introduced. Under each scenario, after the final 24-hr period, both MTT and phagocytosis assays were performed as described above. Again, all data were normalized to the total number of remaining attached cells in a well at the end of the given CdCl2 exposure. All experiments were conducted in triplicates. Within each experiment, each studied treatment concentration at any timepoint was evaluated in triplicate wells in each plate.

Statistical analysis

Effective concentration-50 values (EC50) were defined using non-linear regression best non-line curve-fitting statistics employing response vs. log concentration variable slope response models. One-/two-way analysis of variance (ANOVA) tests, with a Dunn’s or Bonferroni multiple comparison post-hoc test, were used as appropriate. A one-way ANOVA was used to compare between the means of three or more independent (unrelated) groups in relation to one variable (i.e. exposure time or concentration) as in the studies of oxygen consumption rates, MMF, and MFA composition assays. The two-way ANOVA was used to assess variable changes based on the levels of two categorical variables (i.e. exposure time and concentrations) as was the case for data pertaining to cytokine levels, phagocytosis, mitochondrial complexes, lactate and mitotracker green assays. An unpaired Student’s t-test was used for comparisons of data from two different groups in the experiments that tested the protective effects of Co-Q10. A Kruskal–Wallis test was used to compare the ratios of unsaturated:saturated fatty acids in the samples. All statistical analyses were performed using Prism 5 (GraphPad, San Diego, CA, USA). Statistical significance was defined as p < 0.05.

Results

The current work was conducted to study the cytotoxic effect of CdCl2 on isolated human peripheral blood monocytes (hPBM). In addition, potential effects of the CdCl2 on mitochondrial bioenergetics in these cells were also evaluated. Data from the MTT and LDH assays showed that CdCl2 was cytotoxic to the isolated cells in both a concentration- and duration of exposure-related manner. Both assays showed CdCl2 caused significant cytotoxicity at concentration of ≥1 μM after just 24 hr of exposure (Figure 1).

Effects of CdCl2 on a variety of isolated PBMC functions were also studied; however, these were only done using doses of the metal agent that had not caused extreme levels of cell death within a 48-period of incubation. With regard to impact on secretion of select pro-inflam-matory cytokines (e.g. TNFa, IL-6, and IL-8), the data revealed that both 0.1 and 1 μM CdCl2 induced significant concentration- and exposure length-related effects on the spontaneous (non-mitogen-induced) release of all three (Figure 2(A–C); Table S1). The studies also indicated that treatment with these low doses of CdCl2 led to significant concentration- and exposure length-related decreases in cell phagocytic activity, i.e. ability to ingest test particles (Figure 2(D)).
The 12-hr post exposure data revealed that the percentages of phagocytic monocytes were 50.3, 45.0, and 39.3 among the control, 0.1 \( \mu \text{M} \) CdCl\(_2\), and 1 \( \mu \text{M} \) CdCl\(_2\) cells, respectively. Regarding the effect of CdCl\(_2\) on cell bioenergetics, the results of the ATP assays indicated that intracellular ATP production was reduced by the CdCl\(_2\) in a concentration- and exposure...
length-related manner (Figure 3). The results of the Complex assays showed that only at a level of 1 mM did CdCl₂ significantly inhibited both Complexes I and III, and this effect only became apparent after 24 hr of exposure (values fell to 90.2 ± 3.0% and 88.0 ± 3.4% of the corresponding control samples complexes activities for Complexes I and III, respectively) (Figure 3). Interestingly, the lactate production assays showed that CdCl₂ (at 1 mM) significantly increased lactate production by the isolated hPBM to around 1.2- and 1.3-fold that of control values at 24 and 48 hr post-exposure, respectively (Figure 3 and Table S2), but not earlier.

To ascertain if CdCl₂ was affecting isolated hPBM mitochondrial membrane potentials (MMP). CdCl₂ (at 1 mM) significantly decreased MMP of the isolated hPBM to around 90.2 ± 2.1% and 81.2 ± 3.3% of control values at 24 and 48 hr post-exposure, respectively (Figure 4(A) and Table S2). Again, significant effects were not seen at the earlier timepoints. The OCR studies of potential early and/or late effects from the CdCl₂ showed that only the 1 mM level of test agent caused significant reductions in OCR in either timeframe (within 10 min or after 24 hr, respectively) (Figure 4(B,C)). With 1 mM CdCl₂ treatments, the OCR was significantly decreased to 65 and 58% of that in nontreated cells in the early and late effect experiments, respectively.

Because mitochondrial enzymes are mainly located in the mitochondrial membrane inner layer, to potentially explain some of the above-noted outcomes, the effect of CdCl₂ on cell mitochondrial membrane fluidity (MMF) was evaluated. The results showed that 1 mM Cd significantly increased the MMF of the treated cells (Figures 4(D)). Specifically, the results showed that fluidity was increased 80% with the 24-hr 1 mM CdCl₂ treatment in comparison to values seen in control samples. The 0.1 mM level of the test agent imparted no significant effect in this same timeframe.

Compositional analyses of mitochondrial membranes in these cells indicated that there was a major shift in presence of select lipids caused by the 1 mM CdCl₂. After 24 hr of treatment, 1 mM CdCl₂ caused significant elevations in levels of oleic, linoleic, and docosahexaenoic acids and concurrent significant decreases in the levels of palmitic, steric, and arachidonic acids (Figure 5(A–G)). The levels of change (increase) from control cell values were to 110.3 ± 10.1%, 114.4 ± 7.2%, and 114.1 ± 8.5% of oleic, linoleic, and docosahexaenoic acid levels in control cells. The levels of change (decrease) from control cell values were to 84.0 ± 10.7%, 90.3 ± 6.4%, and 84.1 ± 8.8%, respectively, for palmitic, steric, and arachidonic acids. Only levels of palmitoleic acid were not significantly impacted by the Cd treatments (either level). As above, the 0.1 mM CdCl₂ imparted no significant effect on the lipid profiles in this same timeframe. The net result of all these changes was a significant increase in the unsaturated/saturated fatty acid ratio within the mitochondria of the cells (i.e. shift
from 0.40 ± 0.02 for control cells to 0.50 ± 0.01 for the 1 mM CdCl₂-treated cells; Figure 5(H)). Interestingly, the analyses of the effects of Co-Q10 against Cd-induced cytotoxicity and functional disruption of cell phagocytic abilities found that Co-Q10 could alleviate the cytotoxic effect and the functional alterations (Figure 6).

**Discussion**

This study was conducted to evaluate the effects of the widely-encountered environmental toxicant metal cadmium (Cd) on the bioenergetics of isolated human monocytes and the potential protective effect of mitochondrial enhancers such as Co-Q10 to mitigate Cd-induced immunotoxicities.

Effects from Cd on isolated human monocyte bioenergetics were investigated using Cd ion at levels that spanned reported blood Cd levels (i.e. 0.1 and 1 μM). Specifically, mean blood Cd levels were reported as 11.63 ± 1.73 μg/dl (≈ 1 μM) in occupationally-exposed subjects, while values have been seen to be 2.03 ± 0.55 μg/dl (≈ 0.18 μM) in non-exposed subjects (Chakraborty et al. 2013, Alli, 2015). In those studies, effects of Cd on ATP production were assayed using a fairly wide range of concentrations (from 0.1 to 100 μM) to estimate EC₅₀. Because Cd is known to be accumulate in tissues, the higher concentrations used then were intended to mimic the effect of a chronic prolonged exposure within the limited timeframe of the in vitro studies. Those studies found that Cd could lead to a significant decrease in monocyte cell ATP production via an inhibition of mitochondrial complex activities. This effect was associated with marked decrease in the mitochondrial membrane potentials (MMP) and oxygen consumption rates (OCR) of the exposed cells relative to the control samples. In parallel, lactate production was increased. This increase is most likely due to increased anaerobic glycolysis to compensate the inhibited aerobic oxidative phosphorylation pathway. In addition, Cd was shown to impact cell ATP production via an inhibition of mitochondrial complex activities.

**Figure 4.** Effect of CdCl₂ on human peripheral blood monocyte (hPBM) bioenergetics/mitochondrial parameters (A) Mitochondrial membrane potential (MMP). (B,C) Oxygen consumption rates (OCR). (D) Mitochondrial membrane fluidity (MMF). MG assays were conducted in triplicate; OCR and MMF experiments were repeated ≥5 times for each sample. A two-way ANOVA with a Bonferroni multiple comparison post-hoc test was used to study the MMP data to analyze the concurrent effects of exposure duration and CdCl₂ concentrations. A one-way ANOVA with a Dunn’s multiple comparison post-hoc test was used to evaluate the OCR and MMF data. *p < 0.05; **p < 0.01 vs. control non-treated samples at same timepoint. Two way ANOVA was used to study OCR and MMF data. All data were normalized to total number of remaining attached cells in a well at the end of the given exposure to the CdCl₂.
cause a significant increase in mitochondrial membrane fluidity (MMF) - with a marked alteration in the mitochondrial membrane fatty acid composition. The observed effects of CdCl₂ on the isolated hPBM bioenergetics here were in accord-ance with results from other previous studies. There have been reports that in embryonic kidney cells,
Cd exposure can cause increases in mitochondrial membrane permeability (Mao et al. 2011). Moreover, Al-Ghafari et al. (2019) reported that Cd and Pb were both inhibitors of mitochondrial Complexes I and III in human osteoblasts. In addition, Cd-induced mitochondrial dysfunction was found to play a major role in a variety of Cd-triggered neurotoxicities and neurodegenerative disorders (Branca et al. 2018).

As the mitochondrial membranes are the main sites of the electron transport chain and the other bioenergetics functional proteins, MMF controls the membranes' molecular properties and the rate of motion and functionality of the membrane proteins and enzymes. The current study showed that Cd significantly increased the human PBMC MMF. The effect of MMF on activity of the bioenergetics molecules is not clearly understood; however, Cid-Hernández et al. (2015) reported that an increase in MMF was associated with increased ATPase enzyme activity and accordingly an expected decrease in cell ATP molecule levels.

As phospholipids are the main components of the mitochondrial membranes, the effect of Cd on the membranes' fatty acids composition was investigated by gas chromatography. Data showed that Cd significantly increased the polyunsaturated fatty acids in comparison with the saturated fatty acids with expected decrease in the membranes' fatty acids melting point and increased MMF. This agrees with previously published data. Cd was reported to increase MMF of isolated mitochondria of rats' liver in 10 and 20 μM concentrations with reported decreased fluidity in higher concentrations, that is, 30–100 μM (Zhang et al. 2011). In addition, structural effects of Cd on mitochondria were reported in other studies. For example, Cd was reported to induce mitochondrial swelling and altered morphology and to disrupt mitochondrial membrane permeability to potassium and hydrogen ions in different in vivo and in vitro studies (Mao et al. 2011; Belyaeva et al. 2012; Kahrizi et al. 2016).

The current data highlight the hazardous effects of Cd ions as immunotoxic agents. In addition, the present study showed that Cd at relatively low non-cytotoxic concentrations could disrupt mitochondrial bioenergetics via different structural and functional alterations, leading to significant decreases in cell ATP production that would impact on the functions of the monocytes. In parallel, the mitochondrial impairment induced by CdCl₂ here was expected to stimulate signaling pathways that trigger oxidative damage and apoptosis, further affecting overall innate immunity. Thus, the current findings highlight the importance of evaluating bioenergetics to assess potential harmful effects of environmental pollutants like Cd.

In addition, the current data illustrate that CdCl₂ can induce monocyte mitochondrial dysfunction. This is in keeping with some other studies wherein those authors linked monocyte mitochondrial dysfunction to other Cd-associated pathologies, including atherosclerosis, kidney disease, and neuro-depression (Hénaut et al. 2019; Orekhov et al. 2020; Simon et al. 2021). On the other hand, in many other studies, the same clinical disorders were only associated with higher Cd blood levels (Satarug et al. 2017; Diaz et al. 2021; Hwang and Ahn 2021). It is plausible the effects seen here with 'relatively' lower levels of Cd ion (vs. the high blood levels in the cited studies) could merely reflect the in vitro conditions employed, i.e. there is little interference from binding proteins/other sites in blood/tissues by which Cd might interact before reaching immune cells per se.

This latter point illustrates the key limitations of in vitro studies of the type performed here. Specifically, it shows that there are many difficulties to potentially translate the actual response of mitochondria when cells are exposed to the Cd ions in a normal in vivo scenario. Clearly, further in vivo data is required to evaluate the robustness of the current work results and its potential translatable to mechanisms of immunotoxicity in human populations exposed to Cd in the environment or on the job.

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**ORCID**

Ulufat M. Omar (http://orcid.org/0000-0002-9751-8476)

Ekramy M. Elmorsy (http://orcid.org/0000-0002-7444-2499)
The data that support the findings of this study are available from the corresponding author [EME] upon reasonable request.

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