Potential interaction of cadmium chloride with pancreatic mitochondria: Implications for pancreatic cancer

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Abstract. Pancreatic cancer (PC) is insidious with a high mortality rate due to the lack of symptomology prior to diagnosis. Mitochondrial involvement in PC development is becoming accepted, and exposure to cadmium (Cd) is suspected of being a risk factor for the development of PC; however, the mechanisms involved remain unclear. In this study, we examined the role of Cd as a mitochondrial toxicant and whether alterations in mitochondrial function may be an underlying cause for the development of PC. In this study, cadmium chloride ($CdCl_2$)-mediated toxicity in hTERT-HPNE and AsPC-1 pancreatic cell lines was determined by MTT assay. We also investigated the release of LDH and the generation of free radicals. Mitochondrial toxicity assays were performed in media containing glucose (25 mM) or galactose (10 mM) and following exposure to $CdCl_2$ (0-100 μM) followed by MTT assay. For the confirmation of mitochondrial toxicity, we measured the release of ATP following exposure to $CdCl_2$. Initial experiments confirmed that exposure to $CdCl_2$ did not reduce the viability of either cell line until a concentration of >10 μM was used. Non-linear analysis of the response curves revealed lethal concentration 50% (LC_{50}) values for $CdCl_2$ in the HPNE cells of 77 μM compared to 42 μM in the AsPC-1 cells (P<0.01). The $CdCl_2$-mediated mitochondrial toxic effects were greater in the HPNE cells, suggesting a heightened sensitivity to the effects of $CdCl_2$, not due to elevated oxidative stress. Increased mitochondrial toxic sensitivity was indicated by a 73.4% reduction in IC_{50} values in the HPNE cells cultured in galactose compared to culture in glucose media, whereas the AsPC-1 cells exhibited a 58.8% reduction in IC_{50} values. In addition, the higher concentration of $CdCl_2$ elicited a significant cell-dependent effect on ATP release in both cell lines, suggestive of $CdCl_2$ being a mitochondrial toxicant. Cell survival was unaffected following exposure to low concentrations of $CdCl_2$; however, exposure did alter mitochondrial function (control cells > tumor cells). Therefore, the findings of this study indicate that the mitochondria may be a site of action for cadmium in promoting tumor development.

Introduction

The effects of cadmium (Cd) on biological systems have been extensively studied; however, they remain relatively enigmatic. Cd has an exceptionally long half-life in biological systems (years) which may underlie the physiological changes associated with exposure to Cd. Cd is found widely throughout the environment and is one of the more abundant toxicants to which humans are exposed in both occupational and environmental settings (1-3). Cadmium exerts toxic effects on many organs and systems of organs (4,5). Similarly, its endocrine disruptive activities have been described (6-9), as well as its hormetic effects (10). Cadmium, as well as Cd-containing compounds, have been classified as carcinogenic by the International Agency for Research on Cancer (11,12) based on epidemiological studies showing a causal connection with the development of lung cancer. Epidemiological studies have also implicated exposure to Cd to be involved in the development of other types of cancer, such as kidney cancer (13-15), prostate cancer (14,16,17), cancer of the testis (17), bladder cancer (18), breast cancer (19) and overall cancer mortality (20).

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The latest report on the global burden of cancer worldwide using GLOBOCAN 2018 estimated pancreatic cancer (PC) as the seventh leading cause of cancer-related mortality among both males and females with the projection that in the 28 countries of the European Union, this type of cancer will surpass breast cancer as the third leading cause of cancer-related mortality in the future (21). The prognosis of this type of cancer is very poor, mainly due to late-stage diagnosis. Hence, the elucidation of the etiology of this type of cancer and the identification of prognostic factors for it are of utmost importance. The quest for a legitimate biomarker to identify PC in the early stages is ongoing (22,23). A recent review article suggested the possible role of exposure to Cd in the development of PC (24). Some of the mechanisms involved in the carcinogenetic effects of Cd on the pancreas may be the induction of oxidative stress and interaction with bioelements, which have been observed and discussed in a number of studies (25-32). The development of PC is likely to be associated with the silencing of certain tumor suppressor genes and/or the activation of oncogenes, although the underlying molecular mechanisms remain undefined (22,23,33,34). However, interactions between certain environmental chemicals, with Cd being one of them, and these genes can be expected. Furthermore, the micro-RNA-mediated regulation of pancreatic cell proliferation has been demonstrated by Basu *et al* (35). Subsequently, microRNAs (miRNAs or miRs) were found to be present specifically in the mitochondria, originating from mitochondrial DNA and regulating genes coding for mitochondrial proteins in a direct manner, and consequently mitochondrial function (36). Concerning the role of Cd as a mitochondrial toxin, it has recently been studied, but with mixed results, suggesting that Cd may exert direct and indirect effects on the mitochondria (37-40). This ability of Cd to exert toxic effects on the mitochondria could certainly be one of the mechanisms of its carcinogenic effects on cells.

The nature of Cd-related mitochondrial toxicity has led to speculation that the toxicity observed can be mediated either by direct or indirect mechanisms. The aim of this study was to enhance our understanding of the effects of Cd on the mitochondria and to determine whether Cd is a direct or an indirect mitochondrial toxin. Current literature has been unclear and at times, conflicting (37). One concept that is becoming more accepted is that there is an organ-specificity regarding Cd toxicity. There is overlap between some of the major organs examined, although there are also differences in Cd-mediated responses. In the kidneys, there is speculation that Cd interferes with normal mitochondrial function, leading to increased oxidative stress and apoptosis (40), which is further supported by *in vitro* studies utilizing human kidney cell lines showing that Cd directly alters mitochondrial membrane permeability and potential (39). Li *et al* extended these findings to isolated and purified mitochondria (38). In hepatic cells, exposure to Cd has been shown to decrease mitochondrial membrane permeability, but also to directly alter apoptotic pathways by increasing the activity of both caspase-3 and caspase-9, as well as increasing the activity of p53 (41). Recently, the effects of $CdCl_2$ on the pancreas and pancreatic cells have been further described (24). In pancreatic β-cells (RIN-m5F cells), exposure to $CdCl_2$ has been shown to result in the death of β-cells following increased oxidative stress followed by a downstream-mediated activation of apoptotic pathways regulated by the mitochondria (42). Some

researchers have postulated that environmental or occupational exposure to Cd, and the resulting Cd-mediated death or damage to β-cells is an underlying cause of diabetes and obesity (43).

In this study, we examined the effects of cadmium chloride ($CdCl_2$) on mitochondrial function due to the involvement of mitochondria in the 'Warburg effect'. The Warburg effect describes how tumor cells bypass oxidative phosphorylation [responsible for the generation of 38 adenosine triphosphates (ATPs)] and instead utilize aerobic glycolysis [generating only 2 ATPs, but other metabolites which could be useful in cellular proliferation (44)]. An advancement in our understanding of the peculiar phenomenon that was identified by Otto Warburg almost 100 years ago has progressed very little until the last 5-10 years when a significant surge in interest has taken place (45,46). Although one single theory has not come to the forefront, numerous hypotheses have been presented, suggesting that the switch from oxidative phosphorylation to aerobic glycolysis signals the transition from a normal cell to a tumor cell (46). Another hypothesis is that switching to aerobic glycolysis can protect the tumor cell from sugar-induced death when glucose uptake is high, and by changing to a high production of lactate, pro-proliferative intermediates can be produced (47). Compounds, such as Cd, that damage the mitochondria promote a shift towards aerobic glycolysis, thus leading to tumor formation. Researchers that have examined toxicant [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] damage to the mitochondria have reported that oxidative phosphorylation is decreased, that apoptotic pathways are altered and there is a bioenergetic shift in glucose utilization (48). Indirect genetic damage to the mitochondria that will result in epigenetic alterations has also been demonstrated in tumor cells, as well as inherited genetic abnormalities that predispose or facilitate the progression of the Warburg effect in tumor cells (49). The apparent importance of the mitochondria and the Warburg effect has led researchers to postulate that either 'turning' oxidative phosphorylation 'back on' or shunting away from lactate production/utilization may be an effective means of controlling tumor growth (45,50).

Recently, we performed a literature search for 'cadmium', 'Warburg Effect', or 'Crabtree Effect' and the references that we obtained were more specific for the effects of Cd on sugar utilization in yeast, which is what the Crabtree effect initially described (51,52). The effect of Cd on cellular energy utilization is an area of research that needs to be addressed for a better understanding of the effects of Cd on the pancreas and on the development of PC, diabetes, obesity and other pancreatic dysfunctions. In this study, we designed a series of assays that would systematically address the Cd-mediated effects on pancreatic cells using a control epithelial cell line (HPNE) and the pancreatic tumor cell line, AsPC-1. The goals of these experiments were to first determine the level of toxicity following the exposure of both cell lines to Cd, and to then use subtoxic concentrations to determine mitochondrial toxicity. Our initial concentrations were determined using Cd concentrations obtained from pancreatic tissue, from a pancreatic tumor, the area surrounding the tumor and from 'healthy' pancreatic tissue (53). After determining the threshold for Cd-mediated toxicity, we examined the production of free radicals following exposure to Cd and then used the glucose-galactose assay to assess the toxic potential of Cd on the mitochondria by

measuring the shift in the toxicity curve and change in IC_{50} values comparing the glucose curve to the galactose curve. From these experiments, we aim to improve our understanding of Cd-mediated intracellular changes in pancreatic cells.

Materials and methods

Cell lines and cell culture maintenance. All cell culture experiments were performed using standard Biosafety Level 2 (BSL-2) guidelines. The study was approved by the Oklahoma State University Center for Health Science Institutional Biosafety committee in compliance with all National Institutes of Health regulations. Cell lines purchased from the American Type Culture Collection (ATCC). Pancreatic hTERT-HPNE ('human pancreatic Nestin-expressing' cells; ATCC® CRL-4023™, immortalized pancreatic control cells, referred to as 'HPNE') and AsPC-1 (ATCC® CRL-1682™, pancreatic tumor cells) cells were grown and maintained as described in the ATCC-suggested protocols. HPNE cell growth media consisted of DMEM base media with 2 mM glutamine, and 1.5 g/l sodium bicarbonate supplemented with M3 Base media supplement (M300F-500; Incell Corp.) at a ratio of 3:1. To produce complete growth media, 5% fetal bovine serum, 10 ng/ml human epidermal growth factor, 1 mg/ml glucose and 750 ng/ml Puromycin (Sigma-Aldrich and Corning) were added to the DMEM:M3 base. Base AsPC-1 media was RPMI-1640 with 2 mM glutamine, and complete growth media consisted of supplementation with 10% fetal bovine serum, and 1% penicillin/streptomycin (Sigma-Aldrich and Corning). Assay media used in the cytotoxicity assays was a base MEM supplemented with 2 mM glutamine and 1% fetal bovine serum. Assay media was phenol-free and low serum to minimize the confounding factors associated with the influence of protective growth factors in serum. Cells were allowed to adhere for at least 24 h before exposure. Following adherence, growth media were removed and replaced with assay media containing the appropriate treatment(s).

Cell viability and cell growth assays. All lactate dehydrogenase (LDH) assays (Cytotox-ONE™ kit; G7891, Promega) were performed essentially as previously described (54). The HPNE and AsPC-1 cell lines (20-50,000 cells/well) were plated and allowed to adhere for at least 24 h prior to the assay. LDH assays determined i) the number of viable cells within the culture; ii) the percentage of the total number of cells that were viable. Initial serum-comparison assays experiments examined the effects of various serum concentrations on cell growth and viability. Following the removal of the growth media, MEM supplemented with 0% (control), or 1, 2.5, 5 and 10% fetal bovine serum was added to appropriate wells followed by incubation at 37°C in 5% CO_2 for 48 h. Following exposure, the LDH assay was carried out according to the manufacturer's instructions. The ability to quantify dead and live cells is described in the following formula: {[(total RFU) - (Media RFU)] ÷ (total RFU)} x100. The amount of fluorescence produced was quantified using a Biotek plate reader (Biotek Instruments) set to an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Once we determined whether or not changing the serum concentration would affect cell viability and cell number, we exposed both the HPNE and AsPC-1 cells to 2 ppm (18 μM),

6 ppm (54 μM) and 14 ppm (126 μM) $CdCl_2$ for 48 h. We have previously analyzed human pancreatic tissue and measured the content of Cd (53). The highest concentration was found within the tumor, the intermediate concentration was in the region immediately surrounding the tumor zone, and the lowest concentration was found at a site distant from the tumor. We used the molecular contribution of Cd to $CdCl_2$ and calculated the concentration of Cd alone. This assay was performed to aid in the identification of the threshold of toxicity associated with exposure to $CdCl_2$. Following exposure for 48 h, both cell viability and the total number of cells were determined as described in the serum-comparison assays described above. The rationale for using $CdCl_2$ as our form of Cd is based on existing literature and published studies using $CdCl_2$ both in *in vitro* and *in vivo* studies (24,25,27,31,53). The use of $CdCl_2$ will strengthen conclusions comparing our findings to the existing literature using $CdCl_2$.

Measurement of oxidative stress. Both cell lines were grown in 25 cm^2 flasks and seeded into a 96-well (black/clear bottom) plate at an initial density of 10^{-5} cells/ml (or 10^{-4} cells/well). The cells were allowed to adhere for 24 h and to initiate the assay, the growth media was removed followed by washing the cells (twice) with warmed PBS. The cells were then loaded with 100 μM DCFH-diacetate (DCFH-DA) for 30 min at 37°C in 5% CO_2, while being protected from light. Following the removal and washing with PBS, the cells were exposed to one of three treatments; control (PBS only; 0 μM $CdCl_2$), 50 μM $CdCl_2$ or 100 μM $CdCl_2$. Baseline fluorescence was determined in the PBS-only group prior to the addition of $CdCl_2$. The cells were returned to the incubator to allow for the continuation of the reaction. The plates were removed after 30, 60, 90 and 120 min and the fluorescence resulting from the conversion of dichlorofluorescin (DCFH) to dichlorofluorescein (DCF) was measured using a Biotek plate reader set to an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The amount of fluorescence detected is directly correlated to the amount of oxidative stress occurring within the cell. Data were then expressed as the means ± SEM of n=8 assayed in duplicate.

Determination of lethal concentration 50% (LC_{50}) values for $CdCl_2$. The HPNE and AsPC-1 cell lines were maintained as described above. The cells were plated at a final density of approximately 10^5 cells/ml in clear 96-well plates. The cells were allowed to adhere for at least 24 h and the media were carefully removed and assay media with the appropriate concentrations of $CdCl_2$ ($0-10^{-3}$ M) were added to the wells. Following 48 h of exposure, 12 mM stock (1.2 mM final concentration) MTT (Molecular Probes) was added to each well and the plates were returned to the incubator (37°C and 5% CO_2) for 4 h followed by the addition of detergent for another 3-4 h. The amount of insoluble formazan was determined by measuring the absorbance at 565 nm using the BioTek plate reader. Data were then expressed as the means ± SEM of n=6 in duplicate.

Mitochondrial toxicity assays. HPNE and AsPC-1 cell lines were plated at a final density of 20,000-50,000 cells/well and allowed to adhere for at least 24 h prior to the assay. The initiation of toxicant exposure begins by removing the growth media and replacing with DMEM supplemented with either 25 mM

glucose or 10 mM galactose. This method of determining mitochondrial toxicity is similar to that described in the study by Sanuki *et al* (55) and Dott *et al* (56). The treatment groups consisted of cells exposed to Cd (0.1-100 μM) or control (media only) for 4 h followed by the addition of 12 mM MTT dye (1.2 mM final concentration). The absorbance was measured at 565 nm, and the data were then expressed as the means ± SEM of n=12 in duplicate.

To establish the association between cell death and the loss of ATP, we used a luciferin-based detection system (Mitochondrial ToxGlo™; Promega) (57). The principle of the mitochondrial toxicity tests is that substituting 10 mM galactose for 25 mM glucose will increase susceptibility to mitochondrial toxins (58-60). The cells were exposed to $CdCl_2$ (1 or 50 μM) for 48 h prior to the initiation of the assay. These two concentrations were selected based on 50 μM approximating 6 ppm, and 1 μM was significantly lower than our threshold of 10 μM. Following 30 min of incubation at 37°C in a CO_2 incubator, viability was determined by measuring the fluorescence (485 nm_{ex}/525 nm_{em}) emitted. The second step in the multiplex was to quantify the amount of ATP present by directly adding the luciferin-based detection system into the wells that have already been quantified for cell viability with the amount of luminescence being directly proportional to the amount of ATP present. Collectively, by comparing the cellular responses in the two datasets, a better understanding of $CdCl_2$ can be obtained as a cellular toxicant that results in mitochondrial dysfunction or non-mitochondrial related cytotoxic mechanisms.

Effect of $CdCl_2$ on mitochondrial permeability. To assess cell health, we utilized a dye that is permeable to the mitochondrial and will fluoresce red or green depending on the membrane potential of the mitochondria. A high mitochondrial membrane potential ($\Delta\psi_M$) is representative of a healthy cell, whereas a low $\Delta\psi_M$ is indicative of an unhealthy cell. The higher the ratio, the greater the polarization of the mitochondrial membrane. We utilized the JC-1 kit (Cayman Chemical Co.) for this purpose. The cells were plated at approximately 10^5 cells/well and allowed to adhere for 24 h. Exposure was initiated by removing the growth media from each well and adding the treatment media with $CdCl_2$ (1 μM), or the vehicle (water). The cells were returned to the incubator for 48 h at 37°C and 5% CO_2. Following exposure, the cells were prepared according to manufacturer instructions; staining was initiated by the addition of 10 μ JC-1 dye and the cells were returned to the incubator for 20 min. The fluorescence intensity was measured using the BioTek plate reader at 535ex/595em to measure JC-1 aggregates (healthy cells; green) and then a second reading using 485ex/535em which measures JC-1 monomers (apoptotic/unhealthy cells; red).

Statistical analysis: All data were analyzed using GraphPad Prism software (version 7.04). Data from initial $CdCl_2$ exposure assays were analyzed by one-way ANOVA followed by Dunnett's post hoc comparison to control values. In the oxidative stress and LDH assays, data were analyzed by two-way ANOVA (2x3 or cell line x concentration) using Sidak's or Tukey's test for multiple comparisons, respectively. All data are expressed as the means ± SEM of n=4 (ATP assay), n=6 (MTT LC_{50} assay), n=8 (DCFH oxidative stress assay) or n=12

(mitochondrial toxicity assay) performed in either duplicate or triplicate. In the LC_{50} and mitochondrial toxicity assays, the data were first fit by non-linear single-site nonlinear regression inhibition curve (single-site) to determine the IC_{50} value for $CdCl_2$ in each media. A ≥3-fold leftward shift in LC_{50} or IC_{50} values in the galactose group was indicative of mitochondrial toxicity. When curve-fitting, we utilized the D'Agostino and Pearson Test for Normality (omnibus *K2*) to assure that the curves followed a Gaussian distribution. One-way analysis of variance was performed with Dunnett's test for post hoc comparison to control LC_{50} values. For individual comparisons of LC_{50} values, a Student's t-test was used to compared cell lines and treatments. For analyzing the mitochondrial toxicity data using dual-labeling, we utilized a three-way ANOVA (2x2x2; cell type, concentration, media) design with Sidak's test for post hoc multiple comparisons. Data are presented first as the result of the ANOVA where appropriate (F-value) representing whether there was a significant association within the group, such as a concentration effect. Post-hoc multiple comparisons were then performed, usually comparing to the control value; however, the value obtained from the post-hoc comparison is labeled in the figures by a symbol. A significance level was set at P<0.05.

Results

Cell viability: Effect of exposure to Cd and low serum concentration: There is a need to perform assays in reduced serum. Fetal bovine serum contains multiple factors that have been known to scavenge free radicals and afford protection to the cells when exposed to a toxic insult (61-63). These assays were performed to assess changes in cell viability in the presence of reducing concentrations of serum (fetal bovine serum), as well as a change in basal media from the standard optimized growth media to MEM with minimal supplementation. In the HPNE cells (Fig. 1A), no marked differences (P=0.61) were observed in either viability between the serum groups or between the growth media and corresponding MEM. In the AsPC-1 cells, there was a robust increase ($F_{5,41}$=27.34; P<0.0001) in viability in the presence of reduced serum (Fig. 1B). From 0 to 5% the AsPC-1 cells exhibited a 20-25% greater viability compared to the growth media. It was noteworthy that reduced serum improved the viability of AsPC-1 cells. Previous studies have suggested that reducing the serum concentration will reduce viability and cellular proliferation in a variety of cell lines (64,65). This study, at least to the best of our knowledge, is the first to examine the effects of a reduced serum concentration on AsPC-1 viability and growth.

Initial experiments examined the effects of $CdCl_2$ on the viability of HPNE and AsPC-1 cells by exposing the cells to $CdCl_2$ at final concentrations of 0, 2, 6 and 14 ppm (Table I). These concentrations were selected based on data from human studies showing 14 ppm $CdCl_2$ in the core of a pancreatic tumor, with 6 ppm on the tumor margin and 2 ppm associated with 'healthy' tissue distant from the tumor center (53). As evidenced by the data presented in Table I, we observed only minimal changes in viability or cell number in cells exposed to $CdCl_2$. In the HPNE cells, we observed an effect of treatment, (approximately 5%) in both cell number and viability compared to the control values ($F_{4,15}$=37.59; P<0.0001). Post hoc comparisons revealed no differences between the treatment and control groups. In the
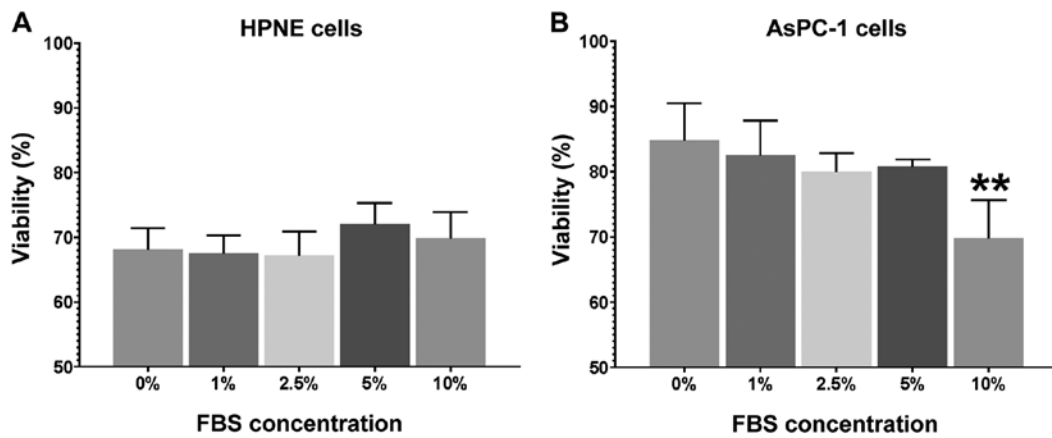
Figure 1. Effects of reduced serum (fetal bovine serum) concentration on the viability of (A) HPNE cells; and (B) AsPC-1 cells. Reducing the fetal bovine serum concentration from 10 to 0% did not markedly alter HPNE viability compared to the growth media control. In the AsPC-1 cells, there was a generalized increase in cell viability from 20-25%, apart from the 10% FBS group. The data presented represent n=4 performed in duplicate and are expressed as the means ± SEM. Data were analyzed by one-way ANOVA followed by Sidak post hoc test for the comparisons of the treatment (fetal bovine serum) to the growth media cell viability. **P<0.01 compared to growth media control values.
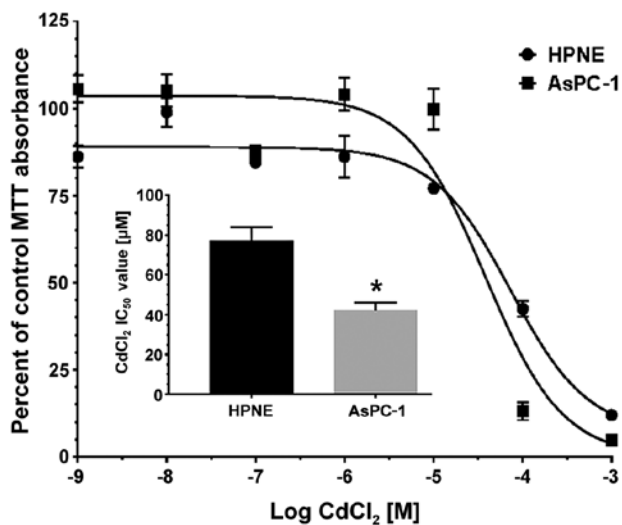

Figure 2. Determination of LC_{50} values for $CdCl_2$ in HPNE and AsPC-1 cells. Cells were plated in clear 96-well plate at approximately 10^{-5} cells/ml and allowed to adhere for 24 h. before the addition of assay media containing $CdCl_2$ (1 to 1 mM). Cells were returned to the incubator for 48 h. MTT assay was performed as described in the Materials and methods and the data were best fit to a single-site model using nonlinear iterative curve fitting (GraphPad Prism). Individual LC_{50} values were calculated for each curve (inset) and AsPC-1 cells exhibited a lower LC_{50} value compared to HPNE LC_{50} values (P<0.01). Data are expressed as the means ± SEM of 6 assays performed in duplicate (n=6). *P<0.01 compared to the HPNE LC_{50} value. LC_{50}, lethal concentration 50%; $CdCl_2$, cadmium chloride.

AsPC-1 cells, exposure to $CdCl_2$ (treatment effect) resulted in a significant change in both viability ($F_{4,15}$=66.69; P<0.0001) and in cell number ($F_{4,15}$=10.91; P=0.0002). Dunnett's post hoc analysis was used to compare changes in the treatment groups to the control values. Only exposure to the highest concentration of $CdCl_2$, 14 ppm, elicited significant reductions in both cellular viability (51%) and cell number (54%). Collectively, these data suggest that the control HPNE cells are relatively resistant following changes in serum concentration, as well as $CdCl_2$ exposure. We observed minimal changes in both cell viability, as well as in cell number which would be indicative of proliferation/growth changes. The AsPC-1 cells, on the other

hand, were more sensitive to the changes in serum concentration and exposure to $CdCl_2$. Generally, only the highest concentration of $CdCl_2$ elicited significant reductions in both cell viability and number. Subsequent experiments assessed $CdCl_2$-mediated changes in oxidative stress and mitochondrial toxicity as a secondary means for $CdCl_2$ exposure resulting in cellular damage.

To determine the LC_{50} value of $CdCl_2$ in the HPNE and AsPC-1 cells, each cell line was incubated with 7 concentrations of $CdCl_2$ (1 to 1 mM) for 48 h, and cell viability was determined by MTT staining (n=6). Curves for both cell lines were best fit to a single-site model (Fig. 2). Individual response curves were analyzed to assess the LC_{50} for $CdCl_2$ in each assay run. LC_{50} data were compared for the HPNE (77.3±6.8 μM) and AsPC-1 (42.1±4.0 μM) cells by Student's t-test and we observed a significant (P=0.0013) 45.5% reduction in LC_{50} values in the AsPC-1 group (Fig. 2, inset). We also compared our means at each concentration to their corresponding control values to determine the threshold at which we observed a significant decrease in cell viability. This threshold provides a value where $CdCl_2$ effects are significantly different from the control and compliments our LC_{50} values. Using a one-way ANOVA with Dunnett's test to compare treatment groups to their corresponding control, we were able to identify the threshold at which the concentration yielded a significant reduction in cell number. The viability of both the HPNE ($F_{6,35}$=92.27; P<0.0001) and AsPC-1 ($F_{6,35}$=115; P<0.0001) cells was significantly affected by the increasing concentrations of $CdCl_2$. The maximum concentration where we observed no reduction in viability was 10 μM in both cell lines. Therefore, although there was a slight rightward shift in LC_{50} values in the AsPC-1 group, the threshold for viability reduction was unaltered between the cell lines.

Cd-mediated induction of oxidative stress. The precise role of $CdCl_2$ in the generation of free radicals has been a topic of considerable discussion. It is clear that transition metals have been implicated in the generation of free radicals (66,67). There is also evidence to suggest that oxidative stress and associated

Table I. Effects of exposure to $CdCl_2$ for 48 h on the live cell number and viability of HPNE and AsPC-1 cells.

			CdCl$_2$ concentration		
	Growth media	Low-serum MEM	2 ppm	6 ppm	14 ppm
HPNE					
Live cell no. (RFU)	3,711±100	3,591±71	3,478±70	3,585±52	3,518±87
Viability (%)	84.2±0.3	81.9±0.2	80.7±0.4	80.6±0.2	78.6±0.5
AsPC-1					
Live cell no. (RFU)	15,501±1,518	15,247±1,206	12,318±231	12,048±1,033	7,054±678[a]
Viability (%)	62.4±2.3	71.1±1.1	58.9±1.4	56.9±6.6	30.7±4.1[a]

[a]P<0.0001 compared to the growth media. Data are expressed as the means ± SEM, n=4. CdCl$_2$, cadmium chloride.
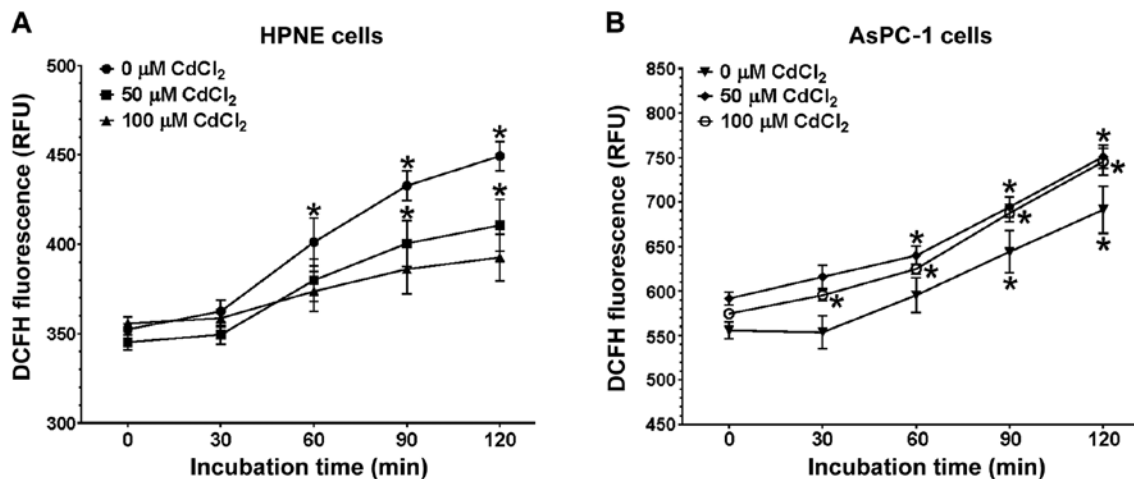

Figure 3. Oxidative stress in (A) HPNE and (B) AsPC-1 cells following exposure to CdCl$_2$. Cells were grown and plated as described in the methods section. After loading with 100 μM DCFH, cells were washed twice with warm PBS and baseline fluorescence was measured (time 0). Treatment groups of (0 μM, PBS-only), and 50 or 100 μM CdCl$_2$ were added to the appropriate treatment wells. DCF fluorescence was quantified at 30, 60, 90 and 120 min using a BioTek plate reader set at 485 nm$_{(ex)}$/530 nm$_{(em)}$. The RFU values expressed were corrected for background (35-48 RFU) for each group. There was a significant effect of treatment-concentration in both HPNE ($F_{2,105}$=10.45; P<0.0001) and AsPC-1 ($F_{2,105}$=16.45; P<0.0001) cells. Data are expressed as the means ± SEM of 8 assays (n=8) performed in duplicate. CdCl$_2$, cadmium chloride. *P<0.01 compared to the corresponding 0-time-point.

inflammation are underlying causes of cancer (68,69). The role of CdCl$_2$ is not as clear as with other metals such as iron, lead, mercury. In this study, the ability of 50 or 100 μM CdCl$_2$ to increase oxidative stress in the HPNE and AsPC-1 cells (Fig. 3A and B, respectively) was assessed by measuring DCF fluorescence. Prior to exposure to CdCl$_2$, basal DCF fluorescence was measured to yield a baseline to which subsequent treatment values would be compared. The basal levels of DCF fluorescence were 57.7% higher in the AsPC-1 cells compared to the HPNE values (555.8 and 352.4 RFU respectively; P<0.0001) at the initial 0-time-point. When comparing the means between the AsPC-1 and HPNE cells at 60 min of incubation, the trend was similar with the AsPC-1 cells, demonstrating a 48.4% increase in oxidative stress (595.2 vs. 401.2 RFU respectively; P<0.0001). Data were analyzed using a three-way ANOVA (cell type x time x treatment) and there was a clear association regarding cell type ($F_{1,56}$=531.2; P<0.0001) and time ($F_{4,105}$=162.6; P<0.0001) responses to CdCl$_2$. In both cell lines, increasing the time of exposure resulted in an increased fluorescence. The trend for these increases was similar between

the HPNE ($F_{4,105}$=26.75; P<0.0001) and AsPC-1 ($F_{4,105}$=59.71; P<0.0001) cells with no significant association between time and CdCl$_2$ concentration in either the HPNE ($F_{8,105}$=1.766; P=0.09) or AsPC-1 ($F_{8,105}$=0.2748; P=0.92) cells.

Mitochondrial toxicity elicited by exposure to Cd. The next series of experiments examined the effects of increasing concentrations of CdCl$_2$ on cellular toxicity in the HPNE (Fig. 4A) and AsPC-1 (Fig. 4B) cells. Following 4 h of exposure to CdCl$_2$, cellular viability was measured by MTT assay. This assay examined the shift in IC$_{50}$ value for CdCl$_2$ following exposure to the metal in the presence of 25 mM glucose or 10 mM galactose-containing media. A rightward shift in IC$_{50}$ for the galactose curve of 2.5-3.0-fold would be indicative of a mitochondrial toxin (55,56). All curves where fit to a single-site model with a r^2 values of 0.72, 0.73, 0.75 and 0.86 for HPNE-glucose, HPNE-galactose, AsPC-1-glucose and AsPC-1-galactose, respectively. After assessing the normality values, all curves passed as fitting normal Gaussian distribution (P-values of 0.07, 0.56, 0.17 and 0.92, respectively).
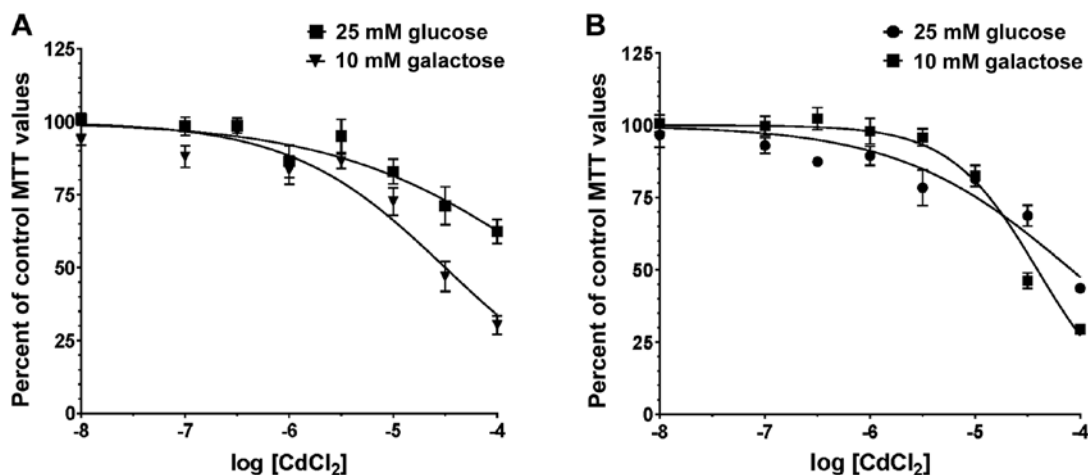
Figure 4. Mitochondrial toxicity following exposure to various concentrations of $CdCl_2$ in the presence of 25 mM glucose or 10 mM galactose. Cells were maintained in regular growth media until assayed. Cells were exposed to 0.01-100 μM $CdCl_2$ for 4 h and the number of viable cells was estimated by MTT assay in the (A) HPNE and (B) AsPC-1 cells. Note the slight leftward shift in the curves for the 10 mM galactose group indicating a greater sensitivity to $CdCl_2$ toxicity. Data are expressed as the means ± SEM of 12 assays (n=12) performed in duplicate and represent a composite of all assays analyzed by nonlinear curve fitting - single site inhibition analysis. $CdCl_2$, cadmium chloride.

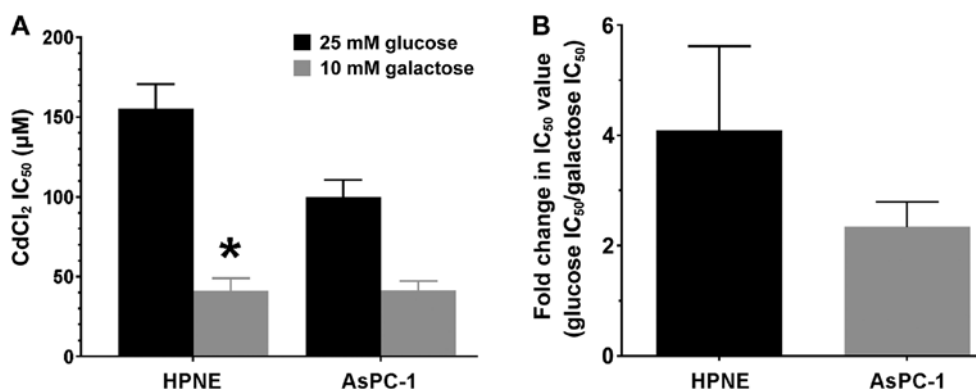Figure 5. Comparison of $CdCl_2$ IC_{50} values in HPNE and AsPC-1 cells following 4 h of exposure to $CdCl_2$. Media contained either 25 mM glucose or 10 mM galactose. Individual IC_{50} values were obtained from the data in Fig. 4 by analyzing each curve independently and then grouped by cell type. IC_{50} values (A) are expressed as μM and the rightward fold-shift in mean IC_{50} values (B) are expressed as the means ± SEM of 12 assays (n=12) performed in duplicate. $CdCl_2$, cadmium chloride. *P<0.01 compared to HPNE IC_{50} value with 25 mM glucose.

Individual IC_{50} values were obtained for each sample using non-linear curve fitting and graphically represented as the means ± SEM for each group (Fig. 5A). There was a significant effect of media on the IC_{50} values (glucose vs. galactose; $F_{1,44}$=25.38; P<0.0001) that was observed in both cell lines. Sidak post hoc analysis revealed that only the IC_{50} value in the HPNE-galactose group was significantly (P<0.01) lower than that in the HPNE-glucose group (Fig. 5A). When examining the shift in IC_{50} values (Fig. 5B), we observed that there was almost a 2- to 4-fold rightward shift in IC_{50} values in both cell lines. The IC_{50} shift suggests that $CdCl_2$ does act as a mitochondrial toxin in the HPNE and AsPC-1 cells.

Using a dual-labeling assay kit, we measured both cell viability (Fig. 6A) and ATP release from the mitochondria, which is indicative of mitochondrial toxicity (Fig. 6B). The use of the more sensitive dual-labeling system revealed a complex response pattern to the action of $CdCl_2$ in both cell lines. The four possible outcomes from this assay are: i) No change in ATP signal + no change in membrane integrity = not a mitochondrial toxicant; ii) reduced ATP + increased membrane integrity = primary necrosis; iii) reduced ATP + no change in

membrane integrity = mitochondrial toxin; and iv) very low ATP at all concentrations + discordant change in membrane integrity = mitochondrial toxicant. Exposure to $CdCl_2$ significantly ($F_{1,24}$=214.5; P<0.0001) affected membrane integrity (Fig. 6A). The cytotoxicity response was also dependent on the type of media, glucose vs. galactose ($F_{1,24}$=13.54; P=0.0012) and cell type, HPNE vs. AsPC-1 ($F_{1,24}$=173.4; P<0.0001). There was a significant ($F_{1,24}$=37.11; P<0.0001) association between the media and $CdCl_2$ concentration. In each group, exposure to 50 μM $CdCl_2$ resulted in a significant reduction (P<0.05) in viability compared to the corresponding 1 μM $CdCl_2$ values in each of the groups. Viability in both the 1 and 50 μM $CdCl_2$ groups in the galactose/AsPC-1 group was significantly (P<0.05) lower than the corresponding 25 mM glucose viability values. Across the $CdCl_2$ concentrations and glucose/galactose supplementation, the viability values were significantly (P<0.05) reduced in the AsPC-1 cells compared to the corresponding viability values in the HPNE cells.

When examining the levels of ATP, we observed a less robust change across he treatment groups (Fig. 6B), yet the trends were similar compared to the data observed from the
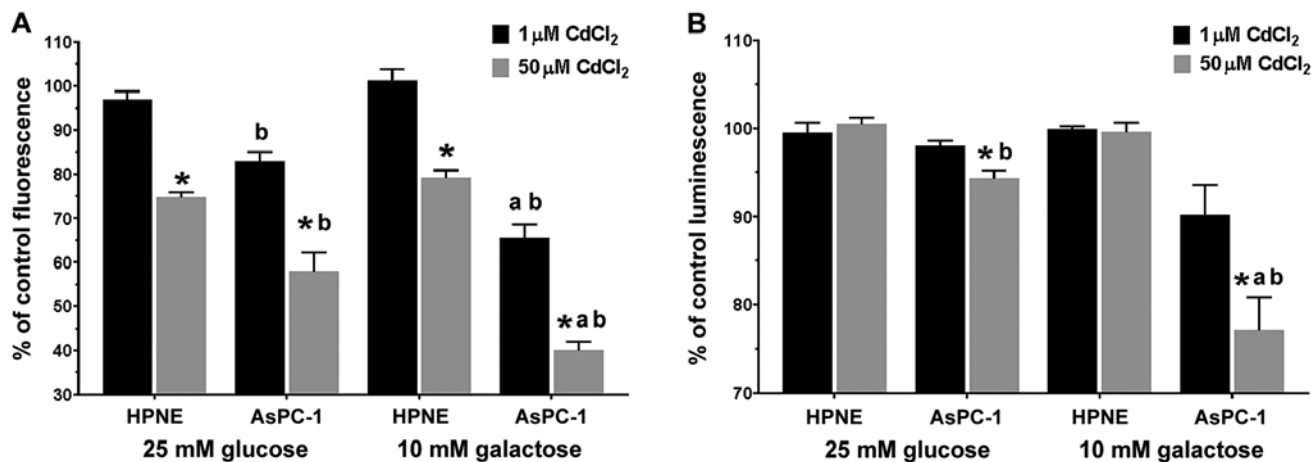
Figure 6. Determination of membrane integrity and ATP release following exposure to 1 or 50 μM CdCl$_2$ in the HPNE and AsPC-1 cells. (A) Effects of exposure to CdCl$_2$ on membrane integrity following incubation in the presence of 25 mM glucose, or 10 mM galactose. AsPC-1 cells displayed a greater sensitivity to CdCl$_2$ compared to the HPNE cell responses, and this great sensitivity was most evident when cells were incubated in the presence of 10 mM galactose. (B) Measurement of ATP release from damaged cells demonstrated a small ATP reduction in the AsPC-1 50 μM CdCl$_2$ group. Incubation in the presence of 10 mM galactose revealed that AsPC-1 cells were most sensitive to the actions of CdCl$_2$. Data represent the means ± SEM of 4 assays (n=4) performed in duplicate (^{a}P<0.05 compared to the corresponding 25 mM glucose values; ^{b}P<0.05 compared to the corresponding HPNE group values; *P<0.05 compared to the corresponding 1 μM CdCl$_2$ values). CdCl$_2$, cadmium chloride.

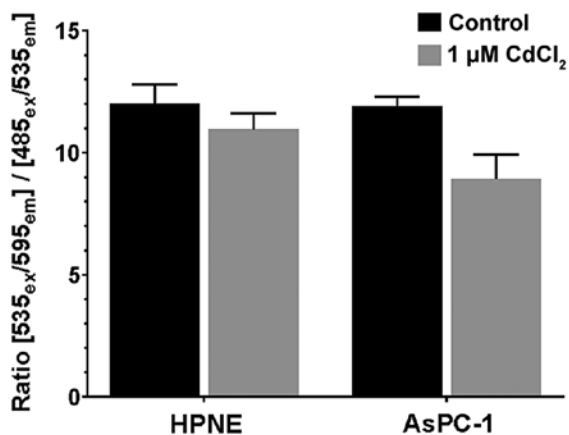
Figure 7. Measurement of mitochondrial permeability using JC-1 dye following exposure to 1 μM CdCl$_2$ in HPNE and AsPC-1 cells. Fluorescence intensity was measured at 535ex/595em to measure JC-1 aggregates (healthy cells; green) and then a second reading using 485ex/535em which measures JC-1 monomers (apoptotic/unhealthy cells; red). The ratio of green/red represents a relative number of cells which are 'healthy'. There was a significant effect of treatment ($F_{1,16}$=7.349; P=0.0154) with reductions in the ratio following exposure to 1 μM CdCl$_2$ in both groups. Individual comparisons to control values did not reveal any difference in the fluorescent ratio in either cell line. Data represent the means ± SEM of 5 assays (n=5) performed in duplicate. CdCl$_2$, cadmium chloride.

cytotoxicity experiments. Exposure to CdCl$_2$ significantly ($F_{1,24}$=55.58; P<0.0001) affected membrane integrity (Fig. 6A). The cytotoxicity response was also dependent on the type of media, glucose vs. galactose ($F_{1,24}$=22.67; P<0.0001) and cell type, HPNE vs. AsPC-1 ($F_{1,24}$=9.10; P=0.006). There were two interactions that were significantly associated and these were media type x CdCl$_2$ concentration ($F_{1,24}$=21.03; P=0.0001) and cell type x CdCl$_2$ concentration ($F_{1,24}$=10.53; P=0.0034), suggesting that the CdCl$_2$ concentration had a significant impact on both membrane integrity and ATP quantity, depending on the media (glucose vs. galactose) type or cell type (HPNE vs. AsPC-1). The AsPC-1 cells exhibited a

slightly higher sensitivity to the effects of CdCl$_2$, as indicated in Fig. 6B. The sensitivity of the AsPC-1 cells was particularly visible at the higher concentration of 50 μM of CdCl$_2$, with the reduction in ATP was more pronounced in the 10 mM galactose group. If there is a change in membrane integrity associated with the changes in ATP, then that would be indicative of a necrotic toxin, leading to a reduction in ATP. We observed two associated interactions, with both the media and the cell type being significantly associated with the concentration of CdCl$_2$.

An additional measure of mitochondrial function is the measure of mitochondrial permeability (referred to as $\Delta\psi_M$). We used the JC-1 dye due to its ability to permeate the mitochondria, and to either form aggregates (healthy cells) or monomers (unhealthy cells) which fluoresce at different wavelengths. We observed that CdCl$_2$ (1 μM) had a significant effect on the health of the cells ($F_{1,16}$=7.35; P=0.0184), slightly reducing the ratio of health/unhealthy cells in both cell lines (Fig. 7). Neither treatment group differed significantly from its corresponding control value.

Discussion

On the periodic table, some of the most toxic metals reside in the d-block or transition metal block. Cd is part of this group; however, Cd is somewhat different in that it is a fifth-period element, unlike chromium, manganese, iron, and cobalt which are fourth-period elements. In addition, Cd is at the end of the transitional metal fifth-period row which makes it a 'post-translational element'. Therefore, there are similarities between Cd and other metals that are known toxicants, carcinogens, and free radical generators, such as mercury, iron, chromium, cobalt, nickel and manganese, although additional studies are required to improve our understanding of the cellular and intracellular actions of Cd. In this study, we aimed to work towards that end. Initial experiments examined the effects of CdCl$_2$ on viability and cell number in media containing decreasing concentrations of fetal bovine serum. We did this to confirm the lack of serum

effect for short exposure times. Other studies have reported that increasing the serum concentration up to 10% can affect the response of different cell types to toxicants (64,70). In this study, we reported that following exposure conducted in a low-serum environment, neither the growth or viability of neither cell line was significantly affected. The lack of effect aids in further *in vitro* studies and we can control the influence and impact that serum has on toxicity studies as a possible protectant. The lack of cytotoxicity observed following the exposure of HPNE cells to $CdCl_2$ is not surprising since, control cells should have better defenses to protect against $CdCl_2$-mediated damage. In addition, the 48-h exposure period may not be long enough for overt cytotoxic damage to be observed. Assay condition parameters are a consideration for further studies to avoid obscuring potential necrotic or apoptotic changes. Subsequent assays used a reduced serum to remove as many confounds as possible from additional toxicology testing. One variable we did not address in our studies is the possibility that low-level $CdCl_2$ exposure may alter cellular responses, such as apoptosis.

The ability of elemental Cd to bioaccumulate in humans and concentrate in organs, such as the liver and kidneys can increase Cd-mediated toxicity over time (71). Exposure to $CdCl_2$ can result in apoptotic, as well as necrotic changes (72-74). The results of this study indicated that exposure to only the higher concentration of $CdCl_2$ resulted in a reduction in cell viability and cell number. This reduction was only observed in the AsPC-1 cells. This study intended to establish 'threshold' levels of exposure (concentration and duration) for use in subsequent mitochondrial toxicity experiments. Since we were attempting to find a threshold concentration for toxicity, it was not unexpected that we observed a lack of robust changes in cell viability, proliferation or cell number after a 48-h exposure. Our understanding of Cd as an environmental toxicant is expanding, and its role in the development of cancer has become widely accepted (24,75-77). Health organizations have classified Cd as a carcinogen (76). However, the exact mechanism(s) through which Cd exerts these carcinogenic effects remain largely unknown. Questions have been put forth as to whether it is a 'direct vs. indirect' effect, or potentially an 'upstream vs. downstream' effect. Further studies are required to describe the intracellular effects of Cd. In this study, we chose to target the mitochondria as an intracellular organelle that needs to function normally to aid the cell in providing the necessary energy requirements. When the mitochondria fail to function normally, tumor formation can follow (46).

The determination of a $CdCl_2$ LC_{50} value in cell cultures is hard to find. It has previously been shown that the LC_{50} for $CdCl_2$ is approximately 70-80 μM in various aquatic organisms (77). Other researchers have indicated that the lethality of $CdCl_2$ is dependent on both the concentration and the duration of the exposure (78). In hepatic and pituitary cultures, the reported LC_{50} values for $CdCl_2$ range from 40-50 μM (78,79). In this study, our findings of 40-70 μM fall within the LC_{50} range that has been reported elsewhere. The concentrations selected in the present study should represent concentrations that are: i) Below; ii) above; and iii) at the LC_{50} value. Caution was needed not to use concentrations that were too high which would prove lethal to a large percentage number of cells. That would skew our findings and conclusions since we would primarily be examining a subpopulation of cells that was resilient enough to

guard against $CdCl_2$ toxicity. Yet a concentration that was too low would not elicit a change in cellular function, and a change in apoptotic function in particular.

The secondary aim of this study was to establish and utilize 'subtoxic' concentrations of $CdCl_2$ to assess oxidative stress elicited by exposure to $CdCl_2$. Using approximately the mid-concentration, 6 ppm or 54 μM, of $CdCl_2$, we can see that $CdCl_2$ was only weakly a pro-oxidant, leading to a 15-20% increase in free radicals over 120 min of exposure. Measuring DCF fluorescence relies on the direct generation of free radicals by $CdCl_2$. *In vivo* studies have shown that the administration of $CdCl_2$ does increase biomarker expression commonly associated with increased oxidative stress (80,81). Therefore, changes in the oxidative status of the cell alone cannot be sufficient alone to elicit intracellular changes leading to apoptotic changes (82). In the kidneys, researchers have postulated that Cd interferes with normal mitochondrial function, leading to increased oxidative stress and apoptosis (38-40). In hepatic cells, $CdCl_2$ exposure has been shown to decrease $\Delta\psi_M$, but also to directly alter apoptotic pathways by increasing the activity of both caspase-3 and caspase-9, as well as increasing the activity of p53 (41). In the pancreas, $CdCl_2$ exposure has been shown to result in the death of β-cells following increased oxidative stress followed by a downstream-mediated activation of apoptotic pathways regulated by mitochondria (42). Other researchers have postulated that environment or occupational exposure to Cd, and the resulting Cd-mediated death or damage to β-cells is an underlying cause of diabetes and obesity (43). It is apparent that the Cd-mediated effects in the pancreas can alter β-cell function, hormonal responses, and may lead to the development of PC.

Healthy or 'normal' cells prefer oxidative phosphorylation (generation of 38 ATPs) to derive their energy (Crabtree), but immortalized cells lines may be adapted to using high glucose, generating ATP via glycolysis (only generates 2 ATPs), which will decrease the sensitivity of the cells to mitochondrial toxins (44). In this study, we examined the effects of $CdCl_2$ on mitochondrial function and the Warburg effect, which involves the differential production of ATP in media containing glucose or galactose. Subsequently, by comparing the toxicity of a compound in the presence of glucose media, or the presence of galactose media, we can determine direct mitochondrial toxicity compared to other indirect mechanisms (55,57,58). The use of galactose-containing (10 mM) media will force the cell to increase their reliance on oxidative phosphorylation to generate ATP. The direct assessment of $CdCl_2$ as a mitochondrial toxin suggested that $CdCl_2$ is a weak mitochondrial toxicant. Comparisons of the glucose/galactose MTT curves in the presence of increasing concentrations of $CdCl_2$ revealed an almost 3-fold shift in IC_{50} values with glucose IC_{50} values > galactose IC_{50} values.

In support of this finding was our observation that ATP levels were reduced, primarily in AsPC-1 cells, with little concomitant changes in viability, suggestive of mitochondrial toxicity (57,59). These data suggest that the $CdCl_2$ concentration has a significant impact on both membrane integrity and ATP quantity depending on media (glucose vs. galactose) type or cell type (HPNE vs. AsPC-1). When examining the data to determine whether $CdCl_2$ is a mitochondrial toxin, or whether it elicits toxicity through another mechanism, the amount of ATP

present needs to be quantified. We would have expected a reduction in ATP in the presence of $CdCl_2$ compared to control values suggesting a mitochondrial toxin. The cytotoxic response must then be examined, with no change in membrane integrity + reduction in ATP being indicative of being a mitochondrial toxin.

Following the addition of the JC-1 dye data, we observed only a small decrease in the ratio of healthy to unhealthy cells in both cell lines. A caveat of these findings is that we used 1 μM $CdCl_2$. The rationale for this concentration was that our threshold was approximately 10 μM $CdCl_2$ when we began to see a reduction in cellular viability. We decided that a 10-fold lower concentration would be sufficient to induce changes in cellular function without resulting in a loss of viability, which would make our data difficult to interpret since the mitochondrial information would be from cells that were able to survive $CdCl_2$ exposure. From our data, it can be hypothesized that $CdCl_2$ is a weak mitochondrial toxin, with adverse effects evident at the highest $CdCl_2$ concentrations of 10 μM or higher.

Although one single theory has not come to the forefront, numerous hypotheses have been presented suggesting that the switch from oxidative phosphorylation to aerobic glycolysis signals the transition from a normal cell to a tumor cell (46). Another hypothesis is that switching to aerobic glycolysis can protect the tumor cell from a sugar-induced death when glucose uptake is high, and by switching to a high production of lactate, pro-proliferative intermediates can be produced (47). Compounds, such as $CdCl_2$, that damage the mitochondria promote a shift towards aerobic glycolysis, thus leading to tumor formation. Studies that have examined toxicant (MPTP) damage to the mitochondria have reported that oxidative phosphorylation is decreased, that apoptotic pathways are altered and there is a bioenergetic shift in glucose utilization (48). Indirect genetic damage to the mitochondria that will result in epigenetic alterations have has also been demonstrated in tumor cells, as well as inherited genetic abnormalities that predispose or facilitate the progression of the Warburg effect in tumor cells (49). The apparent importance of the mitochondria and the Warburg effect has led researchers to postulate that either 'turning' oxidative phosphorylation 'back on' or shunting away from lactate production/utilization may be an effective means of controlling tumor growth (45,50). In this study, we demonstrated a rightward shift in IC_{50} values for both the HPNE and AsPC-1 cells, suggesting that exposure to $CdCl_2$ plays a role in mitochondrial dysfunction. Additional experiments supporting the finding of $CdCl_2$ as a weak mitochondrial toxicant, include the investigation of the aspects of mitochondrial toxicity, such as changes in mitochondrial respiration, transition pore permeability, and the activation of apoptotic peptides. The ability of Cd to accumulate within mammalian tissues is a variable that needs to be addressed due to the long biological half-life. This characteristic of Cd may result in toxicity when exposure concentrations are low, but exposure duration is extended. Additional studies are underway to examine the effects of $CdCl_2$ exposure on intracellular function when subtoxic concentrations of $CdCl_2$ are added to the growth media for extended periods of time (weeks). Exposure in this manner would better represent long-term chronic exposure that many individuals are subjected to in the environment.

Collectively, these data enhance our understanding of Cd-mediated intracellular toxicity. The results also lead us to additional questions. Unlike other well-studied metals, such as mercury, iron, nickel and manganese, $CdCl_2$ appears to exert multiple weak effects, such as on oxidative stress, viability, ATP loss and oxidative phosphorylation as an energy source. $CdCl_2$ does behave as a weak mitochondrial toxicant as indicated by the 2- to 4-fold shift in IC_{50} values comparing the glucose and galactose curves. Since we observed only modest changes in viability, cell number and ATP release, it can be hypothesized that changes following low concentrations of $CdCl_2$ are most likely due to a complex and cumulative set of small changes as opposed to substantial changes. The assessment of the direct vs. indirect effects of $CdCl_2$ on cellular and mitochondrial function is essential. Potential $CdCl_2$-mediated changes which occur upstream or downstream from the mitochondria will be pivotal in determining where and how $CdCl_2$ is eliciting its cellular effects. Additional studies are underway to examine the molecular alterations in intracellular organelles associated with long-term $CdCl_2$ exposure addressing our larger question regarding $CdCl_2$ as a mitochondrial toxicant.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

DRW and ABD were responsible for the conceptualization, methodology, formal data analysis and interpretation, data curation, writing/original draft preparation, writing-review and editing, project administration, and funding acquisition. DAS and AT were involved in consultation regarding the conceptualization and interpretation of the data, data curation, composition of the draft manuscript, as well as the final manuscript and post-review editing. VD was responsible for earlier clinical studies that established the pancreatic concentrations of Cd and working to quantify *in vivo* Cd concentrations. VD and AB aided in the design of the experiments and the establishment of appropriate/relevant concentrations of Cd to be used in the study. AB worked with the overall design and interpretation of the data and assisted in the writing of the final manuscript. AS worked with the design of the viability studies, performance of viability assays and the corresponding data acquisition and analysis for those assays. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests regarding this work.

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