This is a post-print version of the paper by Egbowon et al., which was published online in Toxicology In Vitro in December 2015 and in hard copy in April 2016. The journal version of this paper and supplementary video material can be accessed via the following link:

http://dx.doi.org/10.1016/j.tiv.2015.12.017

Sub-lethal Concentrations of $CdCl_2$ Disrupt Cell Migration and Cytoskeletal Proteins in Cultured Mouse TM4 Sertoli Cells

Biola F. Egbowon,* Wayne Harris, Gordon Arnott, Chris Lloyd Mills and Alan J. Hargreaves*

School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS

* Joint corresponding authors

Email: biola.egbowon@ntu.ac.uk (BFE) or alan.hargreaves@ntu.ac.uk (AJH)

Tel: +441158488061

Fax: +441158483384

Abstract

The aims of this study were to examine the effects of $CdCl_2$ on the viability, migration and cytoskeleton of cultured mouse TM4 Sertoli cells. Time- and concentration-dependent changes were exhibited by the cells but 1 µM $CdCl_2$ was sub-cytotoxic at all time-points. Exposure to 1 and 12 µM $CdCl_2$ for 4 h resulted in disruption of the leading edge, as determined by chemical staining. Cell migration was inhibited by both 1 and 12 µM $CdCl_2$ in a scratch assay monitored by live cell imaging, although exposure to the higher concentration was associated with cell death. Western blotting and immunofluorescence staining indicated that $CdCl_2$ caused a concentration dependent reduction in actin and tubulin levels. Exposure to Cd^{2+} also resulted in significant changes in the levels and/or phosphorylation status of the microtubule and microfilament destabilising proteins cofilin and stathmin, suggesting disruption of cytoskeletal dynamics. Given that 1-12 µM Cd^{2+} is attainable in vivo, our findings are consistent with the possibility that Cd^{2+} induced impairment of testicular development and reproductive health may involve a combination of reduced Sertoli cell migration and impaired Sertoli cell viability depending on the timing, level and duration of exposure.

Key words: Cadmium chloride; Sertoli cells; reproductive toxicity; cytoskeleton; cell migration; testicular development.

Abbreviations: AIDA, Advanced Image Data Analyser; BSA, bovine serum albumin; $CdCl_2$, cadmium chloride; CFSE, carboxyfluorescein diacetate succinimidyl ester; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; H and E, haematoxylin and eosin; IC_{50}, inhibiting concentration (50 %); LDH, lactate dehydrogenase; MFs, microfilaments; MTs, microtubules; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; TBS, Tris-buffered saline; 5-FU, 5-fluorouracil

1. INTRODUCTION

Heavy metals are environmental pollutants of great concern because of their persistent occurrence, arising from increasing industrialisation and other anthropogenic activities (Borrego et al., 2002). A primary concern regarding exposure to heavy metals centres on their deleterious effects on human health including metabolic, neurodegenerative, reproductive and developmental disorders (Godt et al., 2006; Bernard, 2008). Exposure to metal compounds including arsenic, cadmium and mercury has long been known to cause damage to mammalian testes (Parizek, 1957; Marettov et al., 2015), potentially contributing to the decline in male reproductive health that has occurred over the last 60 years (Huyghe et al., 2003; Bray et al., 2006). Cadmium is of particular concern due to its increasing environmental levels, caused by pollution from a variety of sources (IPCS, 1992; Jarup et al., 1998; WHO, 2007, ATSDR, 2011; Six and Smolders 2014; Van Assche et al., 2014; CCC, 2014). To date, cadmium has no known biological function in mammals and prolonged exposure to it has been associated with developmental and functional changes within tissues including testicular tissue (Marettov et al., 2015; Prozialeck et al., 2006; Siu et al., 2009; Sarkar et al., 2013).

The mammalian testis consists of germ cells and somatic cells (Svingen and Koopman, 2013). The somatic cells comprise two major lineages, known as Sertoli cells and Leydig cells. Sertoli cells play key role in testicular development and functions (Mruk and Cheng, 2003). The early stage of gonadal development is associated with the migration of supporting cells from the coelomic epithelium of the early embryo, which contribute to the population of Sertoli cell precursors (Wilhelm et al., 2007). Furthermore, changes in cell shape, formation of adhesion and cytoplasmic protrusion in somatic gonadal precursors (SGPs) enhances ensheathment of primordial germ cells (PGCs) and the formation of compacted gonads in the developing embryo (Martineau et al., 1997). Hence, the migration and proliferation of Sertoli

cells is therefore an essential part of testis development (Mruk and Cheng, 2003; Martineau et al., 1997).

In this respect, the cytoskeleton and its regulatory proteins play important roles in cell proliferation, cell shape and motility (Artvinli, 1987; Vogl et al., 1993; Vogl et al., 2008). For instance, cell migration is dependent on the actin network and its dynamics, which are regulated by a number of proteins such as cofilin (Pollard and Borisy, 2003; Dos Remedios et al., 2002). Actin regulator protein enables (ena) plays an important role in the spacio temporal organisation of somatic gonadal precursors (SGPs) in the formation of the compacted gonad (Bear et al., 2000; Sano et al., 2012). These cellular processes are important targets for environmental disrupting compounds such as heavy metals (Waisberg et al., 2003; Rani et al., 2013). Several *in vivo* and *in vitro* studies have reported cadmium toxicity in mammalian testes and their cellular components (Siu et al., 2009; Jin et al., 2004; Xiao et al., 2014).

Reduced Sertoli cell number in sheep foetuses was observed in two *in vivo* studies following sewage sludge exposure during early stages of development (Rhind et al., 2005; Egbowon, 2010). The cause of this reduction was associated with suppressed levels of testosterone, suggesting that testosterone may play a key role in Sertoli cell proliferation (Johnston et al., 2003), and raising the possibility that treatment-induced suppression of testosterone levels could have contributed to the reduction in Sertoli cell number. However, it is possible that exposure to environmental pollutants may have direct effects on the population of the migrating and proliferating cells occupying the gonadal ridge of the early embryo. *In vivo* studies on testicular cells are numerous; however, effective cell lines can greatly facilitate research on testicular development and functions by providing a readily available supply of cells with consistent and predictable properties.

There are several Sertoli cell lines, some of which were created to retain properties from the parent cell type required for specific studies (Robert, 2004; Guttenbach et al., 2001). For example, TM4 cells are an established cell line of Sertoli cell origin, derived from the normal testes of a prepubertal 11 – 13 day old BALB/c mouse (Mather et al., 1982; Mather, 1980). Many studies in which this cell line was used as a model of Sertoli cell function have shown that it maintains many of the characteristics of immature and differentiated native Sertoli cells (Guttenbach et al., 2001; Catalano et al., 2003; Shaban et al., 1995).

The current study has used the TM4 cell line to evaluate the toxicity of cadmium on Sertoli cell migration with respect to the effects on cell survival, cell morphology, cytoskeletal organization and the underlying molecular events associated with cell migration.

2. MATERIALS AND METHODS

2.1 Cell Culture

The mouse Sertoli cell line TM4 (ATCC number: CRL-1715) was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in growth medium, consisting of Dulbecco's modified Eagle's medium (DMEM) containing Ham's F-12 in a 1:1 mix (DMEM/HAMs F-12) with 15 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 2.5 mM glutamine, 0.5 mM sodium pyruvate and 1.2 g/L sodium bicarbonate (Bio-Whittaker, Lonza, UK), supplemented with 5 % v/v horse serum (HS) and 2.5 % v/v fetal bovine serum (FBS) (Sigma Aldrich Co. Ltd., Poole, UK), in a humidified atmosphere containing 5 % CO_2/95 % air at 37^{o}C. All experiments were performed using plastic tissue culture flasks and dishes or microplates (Sarstedt, Leicester, UK). Cell culture growth medium was changed twice weekly and cells were sub-cultured before reaching confluence.

2.2 Measurement of Cell Metabolism by Methyl Blue Tetrazolium Reduction Assay

Cell viability was monitored via the reduction of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) by cellular dehydrogenases. Cells were plated in Corning 24-well plates at 25,000 cells/ml in 0.5 ml growth medium and left for 24 h to recover. Growth medium was carefully aspirated from the wells and replaced with fresh medium containing a range of concentrations (up to 25 μM) of $CdCl_2$ for 3 exposure times (4 h, 24 h, and 48 h). A volume of 50 μl of MTT (5 mg/ml in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) was added to each well 1 h prior to the end of the experimental incubation time and cells incubated for a further 60 min at 37ºC. Growth medium was then carefully aspirated, 0.5 ml DMSO added per well and the plates were gently agitated to dissolve the reduced formazan product. The absorbance of the solubilised reduced MTT was then measured in a standard microtitre plate reader at a wavelength of 570 nm. Where appropriate, indicative concentrations causing 50% inhibition of MTT reduction compared to the control (IC_{50}) were estimated from individual concentration response curves from at least 4 independent experiments and are expressed as mean ± SEM.

2.3 Measurement of Membrane Leakage by Lactate Dehydrogenase Release Assay

Cell viability was also monitored in Sertoli cells by measuring lactate dehydrogenase (LDH) release. Cells were plated in 96-well flat bottom plates, at 25,000 cells/ml in 0.2 ml growth medium and left for 24 h to recover. Growth medium was carefully aspirated from the wells and replaced with fresh medium containing a range of concentrations (up to 25 μM) of $CdCl_2$ for 3 exposure times (4 h, 24 h, and 48 h). Cell viability assays were performed by measuring the amount of LDH released into the medium, which was detected colorimetrically using the CytoTox 96® Non-Radioactive Cytotoxicity LDH assay kit (Promega, Southampton, UK).

2.4 Measurement of Viable Cell Counts by Trypan Blue Exclusion Assay

Viable cell counts were determined using an automated cell counter (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). This was achieved by using the TC20^{TM} Trypan Blue exclusion assay procedure (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). Briefly, cells were plated in T25 culture flasks at 25,000 cells/ml in 10 ml growth medium and left for 24 h for recovery. They were then treated with (1 µM and 12 µM) or without $CdCl_2$ for 4, 24 and 48 h. After the incubation period, the cell monolayers were detached with cell scrapers, pelleted by centrifugation and washed twice by centrifugation with PBS. Each cell pellet was then resuspended in 2 ml serum free medium prior to the assessment of cell viability.

2.5 Analysis of Cell Morphology

Morphological effects of $CdCl_2$ were determined on Sertoli cells stained either with Coomassie blue or haematoxylin and eosin (H and E) dyes after 4 h exposure.

2.5.1 Coomassie Blue Staining

Cells were plated in 24-well plates at 25,000 cells/ml in 0.5 ml growth medium per well and left for 24 h to recover, after which they were exposed to lethal and sub-lethal concentrations of the test compounds for 4 h. Cells were then fixed with 90 % v/v methanol for 10 min at minus 20 °C and subsequently stained with Coomassie Blue, (0.1 % w/v Coomassie Brilliant Blue R-250, 50 % v/v methanol and 10 % v/v glacial acetic acid) which was added to each well at 300 µl per well for 5 min. The staining solution was then removed, the monolayers rinsed 3 times with distilled water and left overnight to air-dry.

2.5.2 H and E staining

Alternatively, 0.5 ml of cell suspension were plated on poly-L-lysine coated coverslips at 25,000 cell/ml in 24-well plates and then incubated for 24 h for recovery. The medium was

discarded and the cells were re-incubated in the absence and presence of $CdCl_2$ (1 µM and 12 µM) for 4 h. Cells were fixed with 90 % v/v methanol for 10 min at -20^{o}C and washed three times with PBS, after which the cell monolayers were rinsed in water. This was then placed in single strength Gill's Haematoxylin (haematoxylin 0.6 % w/v, aluminium sulphate 0.42 % w/v, citric acid 0.14 %, w/v sodium iodate 0.06 % w/v, ethylene glycol 26.9 % v/v: Scientific Laboratories Supplies, Nottingham UK) for 90 seconds and washed in tap water. For cell blueing, coverslips were immersed in 1 % (w/v) lithium carbonate and then washed in distilled water. For cytoplasm staining, the coverslips were immersed in 1 % w/v eosin for 10 seconds after which they were washed in distilled water. This was then followed by placing the coverslips in an ascending ethanol series (i.e. 3 min each in 50 v/v % ethanol, 70 % v/v ethanol and absolute ethanol). The cover slips were placed in xylene for 5 minutes and allowed to air dry for 10 seconds. They were mounted on glass slides using XAM mounting medium (Merck Darmstadt, Germany).

2.6 Image Processing

The slides were examined with the aid of fluorescence microscope (Olympus BX51TF) using bright field optics at a final magnification of x 200 and images were randomly captured from nine fields of view per slide for each treatment. The captured images were processed by using Adobe Photoshop C56 (64 Bit) to quantify the proportions of Sertoli cells with a discernible leading edge and rounded or compact cell bodies. The morphological feature of a crescent shape rosette-like continuous cytoplasmic extension was identified as a discernible leading edge. The total number of parameters counted was averaged for each set of experiments and the values were expressed as total count/100 cells ± SEM for 4 independent experiments.

2.7 Indirect Immunofluorescence Staining

For immunofluorescence staining, cells were plated in 8-well µ-slide (ibiTreat) chamber slides (Thistle Scientific, Glasgow, UK) at 25,000 cells/ml in 0.2 ml growth medium and left for 24 h to recover, after which they were exposed to 1 µM and 12 µM $CdCl_2$ for 4 h. Cells were then fixed with pre-warmed formalin at 37 ˚C for 10 min and washed three times with PBS. Permeabilization was performed by incubating the cells for 15 min at room temperature with 0.05 % (v/v) Tween-20 in PBS after which cells were washed 3 times for 5 min with PBS. Non-specific binding was prevented by blocking with 3 % (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were then incubated overnight at 4ºC in humidified chamber with primary antibodies to cytoskeletal proteins, as indicated in Table 1. After washing 3 times for 5 min with PBS, the cells were incubated for 2 h at 37˚C in a humidified chamber with Alexa Fluor 488 rabbit anti mouse IgG (Life Technologies Ltd, Paisley, UK) diluted 1:500 in blocking buffer, after which a second series of PBS washes was performed. Fluorescence images were acquired using a Leica laser-scanning confocal microscope (Leica SP5 II).

2.8 Sertoli Cell Migration Assays

In order to monitor the effects of $CdCl_2$ on cell migration, a monolayer 'scratch' assay was performed to mimic the wound healing process in migrating cells such as fibroblasts. In a conventional wound healing process, it has been suggested that the generated wound closes by migration and proliferation of the cells (Menon et al., 2009; Mertens-Walker et al., 2010). Therefore cell proliferation was stopped prior to $CdCl_2$ treatment. In brief, cells were seeded in black 96-well microplates at 25,000 cells/ml in 0.2 ml growth medium and incubated at 37ºC. When the cell monolayer reached 90 % confluence, growth medium was replaced with fresh growth medium containing 5 µg/ml 5-fluorouracil (5-FU), which was not toxic to the cells as determined by MTT assay after 24 h. The monolayer was rinsed with serum free growth medium and the cells were incubated with 10 µM carboxyfluorescein diacetate

Antibody	Type	Source	Dilution: WB/IF
Anti-actin (AC40)	Mouse IgG	Sigma-Aldrich, UK	1:1000 (1:100)
Anti-tubulin (B512)	Mouse IgG	Sigma-Aldrich, UK	1:500 (1:100)
Anti-intermediate filament antigen	Mouse IgG	ATCC	1:200 (1:100)
Anti-cofilin	Rabbit IgG	abcam Biochemicals	1:1000
Anti-phospho cofilin	Rabbit IgG	abcam Biochemicals	1:1000
Anti-oncoprotein 18	Goat IgG	Santa Cruz Biotechnology	1:500
Anti-phospho oncoprotein 18	Rabbit IgG	Santa Cruz Biotechnology	1:500

Table 1 Antibodies used for western blotting and indirect immunofluorescence analysis. WB = Western blotting; IF = Indirect immunofluorescence.

succinimidyl ester (CFSE) dye for green fluorescence. This was followed by scratching of monolayers with a p20 pipette tip to create an extended and definite scratch in the centre of the well. The detached cells were removed by washing with serum free growth medium, which was then replaced with fresh growth medium with or without 1 µM or 12 µM $CdCl_2$ and incubated for a further 24 h. Fluorescence images of live cells of the scratch closure areas were monitored and acquired using a HC PL APO 10×/0.40 objective lens on a Leica-TCS-SP5 II laser scanning confocal microscope (Leica Microsystems), equipped with a humidified, temperature and CO_2 controlled live cell chamber. For imaging, CFSE was excited with a 488 nm laser-line for typically between 100-500 ms/image with laser intensity set to 25 % maximum. Confocal image z-stacks of living cells were recorded with a frame size of 512 x 512 pixels, a pixel size of 100 nm and a z-step size of 400 nm every 30 min.

The representative images are maximum intensity Z-projections of 10 slices of 1 micron thickness at 4, 12 and 24 h. The captured images were processed using Adobe Photoshop C56

(64 Bit) to quantify the invading cells in the scratch area at each time point, as previously described (Camp et al., 2010; Stewart et al., 2012; Ling et al., 2013). Data were obtained from at least four separate experiments.

2.9 Gel Electrophoresis and Western Blotting Analysis

Sertoli cell lysates were prepared to determine changes in protein levels. In brief, cells were plated in T75 culture flasks at 25,000 cells/ml in 40 ml growth medium and left for 24 h to recover, after which they were exposed to 1 µM and 12 µM $CdCl_2$ for two exposure times (4 and 24 h). The growth medium was removed and monolayers rinsed gently with ice-cold PBS. Cells were then detached in ice-cold 0.02 % (w/v) ethylenediaminetetraacetic acid (EDTA) in Tris-buffered saline (TBS: 10 mM Tris, 140 mM NaCl, pH 7.4) using a cell scraper. Cells were pelleted by centrifugation and the pellets were lysed in pre-heated 0.5 % (w/v) sodium dodecyl sulphate (SDS) (w/v) in TBS and then incubated at 100 °C for 5 min. Protein concentration of cell extracts was determined by the bicinchoninic acid assay using BSA as the standard (Walker, 1996).

After protein assay, lysates were boiled in electrophoresis sample buffer (62.5 mM TRIS, 2 % w/v SDS, 10 % v/v glycerol, 0.002 % w/v bromophenol blue, 5 % v/v β-mercaptoethanol, pH 6.8) for 5 min (Laemmli, 1970). Approximately 10 µg of protein from each sample from at least 4 independent experiments were separated by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) in a 10 % w/v polyacrylamide resolving gel overlaid with a 4 % w/v polyacrylamide stacking gel (Laemmli, 1970), after which they were electrophoretically transferred onto nitrocellulose membrane filters as previously described (Towbin et al., 1989). Membranes were blocked for 1 h at room temperature with 3 % w/v BSA in TBS, and then probed overnight at 4°C with a variety of primary antibodies, diluted as indicated in Table 1. Membranes were then washed thoroughly with 0.05 % v/v Tween20

in TBS (typically six 10-min washes) and incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat immunoglobulin G diluted 1:1000 in 3 % w/v BSA in TBS, as appropriate. After extensive washing with 0.05 % w/v Tween20 in TBS, antibody binding was revealed with the enhanced chemiluminescence western blotting detection reagent ECL plus (GE Healthcare, Hatfield UK). Digital images were captured using a G:BOX imager (Syngene, Cambridge, UK), and band intensity was quantified using Advanced Image Data Analyser (AIDA) software (Raytest GmbH, Straubenhardt, Germany).

2.10 Statistical Analysis

Graph Pad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analysis. The significance of differences between more than 2 sets of data were determined by one-way analysis of variance (ANOVA) and comparison between means was analysed by the Bonferroni Multiple Comparison Post-hoc Test. Differences were deemed to be significant when $p<0.05$ and are highlighted with asterisks. All data are presented as mean $\pm$ SEM.

3. RESULTS

3.1 Effects of $CdCl_2$ on Cell Viability in Sertoli Cells

The effects of $CdCl_2$ on cell viability were assessed by MTT reduction assays, lactate dehydrogenase (LDH) release into the growth medium and Trypan Blue exclusion assay (Figure 1). Exposure of Sertoli cells to different $CdCl_2$ concentrations (1 - 25 µM) for up to 48 h resulted in a dose and time dependent fall in the level of MTT reduction.

Cells exposed to concentrations from 6 - 25 µM exhibited statistically significant decreases in MTT reduction after 24 h exposure (approximately 25 – 50 % inhibition; $p<0.05$) (Figure 1). Increasing incubation time to 48 h resulted in increased toxicity being observed between 3 - 25 µM (approximately 50 - 80 % inhibition of control values; $p<0.05$). However, $CdCl_2$ did not affect MTT reduction by Sertoli cells after 4 h of incubation at any of the concentrations tested and 1 µM $CdCl_2$ had no effect on MTT reduction at any of the time points. The toxic effects of $CdCl_2$ on Sertoli cells at different exposure times were further compared in terms of IC_{50} value at each time point, which is defined as the concentration required to inhibit the level of MTT reduction by 50 % compared to the non-exposed control. The IC_{50} value was not reached at 4 h exposure but was estimated to be 12 µM and 4 µM after 24 and 48 h exposure, respectively.

In agreement with this data, the results from LDH release assays showed that exposure of Sertoli cells to different concentrations (1-25 µM) of $CdCl_2$ resulted in a dose and time-dependent increase in LDH leakage. Concentrations from 6 - 25 µM exhibited statistically significant ($p<0.05$) cytotoxic effects on Sertoli cells following 24 h exposure, and increasing incubation time to 48 h resulted in increased LDH release being observed between 3 - 25 µM at 48 h (Figure 1). As was the case for MTT reduction assay, 1 µM $CdCl_2$ had no significant cytotoxic effects at any time point. The results from Trypan Blue exclusion assays showed that cell proliferation (total viable cell count) was slightly but not significantly increased by 1 µM $CdCl_2$ exposure up to 24 h. However, cell proliferation was significantly reduced by exposure to 12 µM $CdCl_2$ from 24 to 48 h (Figure 1).

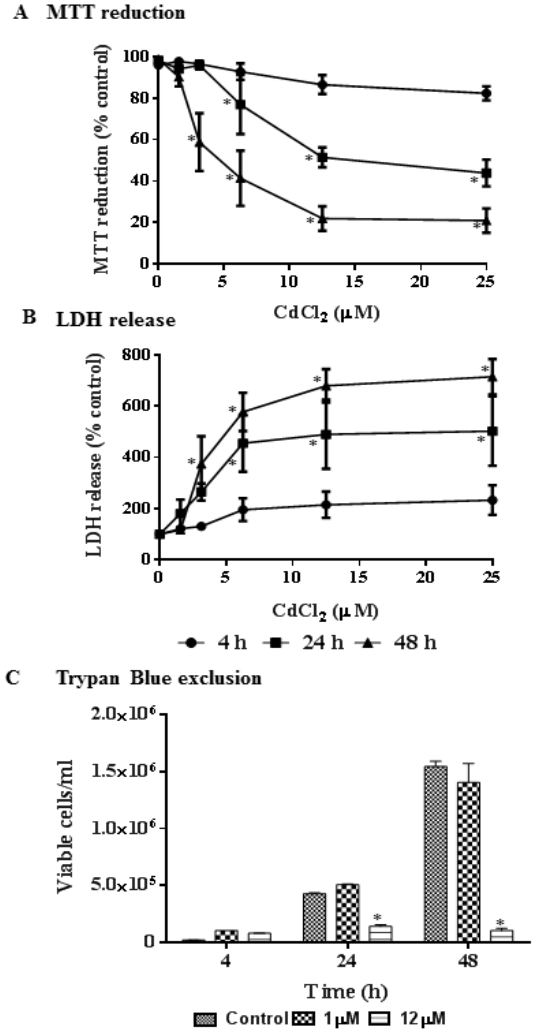


Figure 1: Effects of $CdCl_2$ on cultured Sertoli cell viability measured by MTT reduction, LDH release and Trypan Blue exclusion assay. Cells were incubated in the absence (0) or presence of 1-25 µM $CdCl_2$ at different time points, and cell viability assessed by (A) MTT reduction, (B) LDH release and (C) Trypan Blue exclusion assays as described in Materials and Methods. Data in A and B represent means of four independent experiments ± SEM and the results are presented as percentage relative to the corresponding control (=100 %) and data for the latter represent means of four independent experiments ± SEM. Asterisks * indicate $p<0.05$.

3.2 Effects of $CdCl_2$ on Sertoli Cell Morphology

In order to assess changes in the morphology of Sertoli cells after $CdCl_2$ treatment, cells were incubated for 4 h in the absence and presence of 1 µM or 12 µM $CdCl_2$. Cells were fixed and stained with either Coomassie blue or H & E dyes (Figure 2). By using light microscopy, the morphological features that were studied included 1) leading edges (cytoplasmic protrusion) of migrating cells.

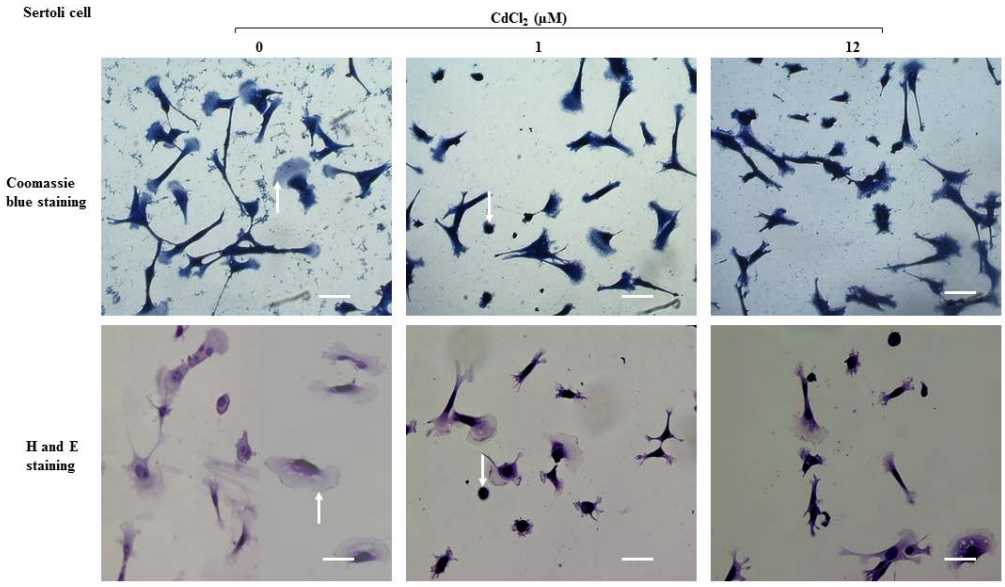


Figure 2

Figure 2: Effects of $CdCl_2$ on the morphology of cultured Sertoli cells. Sertoli cell monolayers were incubated for 4 h in the absence (0) and presence (1 µM or 12 µM) of $CdCl_2$. They were then fixed in 90 % v/v methanol before staining with Coomassie blue or haematoxylin and eosin dyes (H and E) and analysed as described in Materials and Methods. Indicated are typical examples of leading edges (upward arrows) and round cells (downward arrow). Scale bar = 100 µm.

This represents formation of a continuous smooth curved rosette shaped feature that is equal to or wider than the width of cell body 2) rounded or compact cell shape in other cells. The proportion of Sertoli cells exhibiting a clearly discernible leading edge was significantly reduced in the presence of 1 µM and 12 µM $CdCl_2$ (Figure 3). The formation of rosette like nature of leading edge was disrupted and became shorter and truncated with the higher concentration (Figure 2). The population of rounded or compact cells increased significantly following exposure to 1 µM concentrations of $CdCl_2$ (Figure 3). As microfilament (MF) assembly at the leading edge is known to play an important role in cell migration, we next investigated whether $CdCl_2$ treatment affected the Sertoli cell cytoskeleton, by indirect immunofluorescence staining.

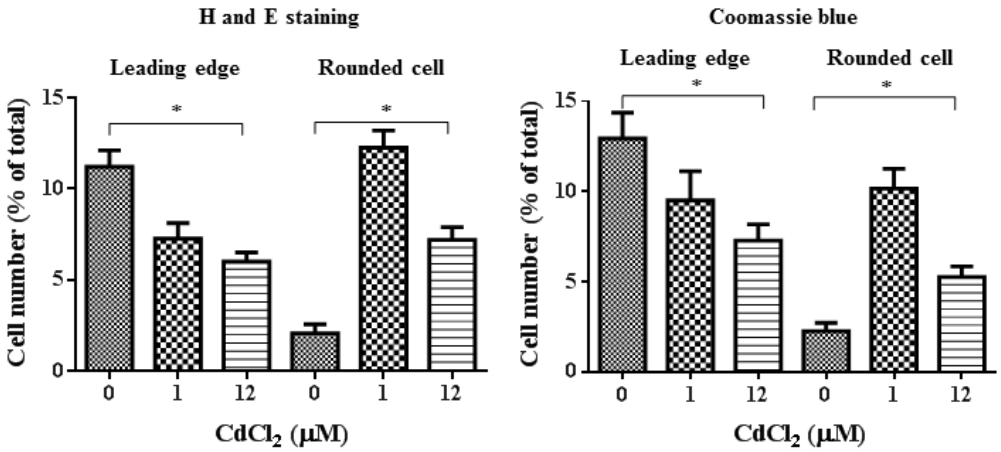
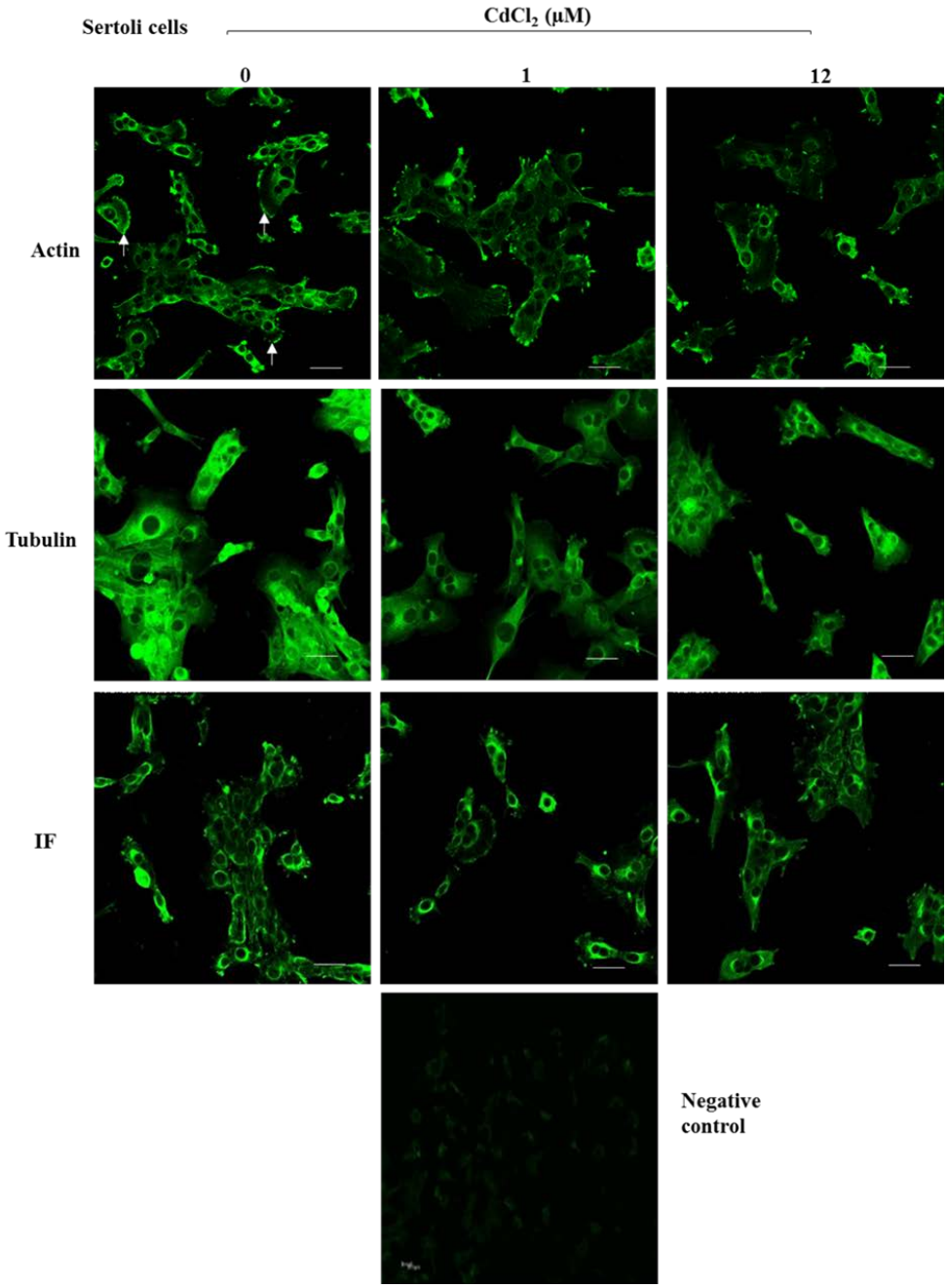


Figure 3: Quantification of morphological changes in Sertoli cells after $CdCl_2$ treatment. Shown are the morphological data from both Coomassie blue (CBB) and H and E stained Sertoli cells. Histogram data represent mean count of each parameter per 100 cells for each treatment from four independent experiments ± SEM. Asterisk indicates a significant change ($p<0.05$) compared to the untreated control.

3.3 Effect of $CdCl_2$ on the Organisation of Cytoskeletal Components in Cultured Sertoli Cells

The morphological changes induced by 4 h exposure to $CdCl_2$ in Sertoli cells were further examined by indirect immunofluorescence microscopy of fixed cell monolayers using monoclonal antibodies against the cytoskeletal proteins actin, tubulin and intermediate filaments (Figure 4). Reduced staining intensity and possible disorganization of actin and tubulin networks appeared to be induced by $CdCl_2$ treatments. For instance, actin staining at the leading edge in $CdCl_2$ treated cells appeared to be relatively weak compared to the control (Upward arrows in Figure 4). By contrast, the staining showed no major effect on the cellular distribution of intermediate filament networks with $CdCl_2$ treatment. The possibility that the morphological changes consistent with cytoskeletal disruption by cadmium exposure reflect an effect on cytoskeletal protein levels or filament dynamics was next investigated.



Figures 4: Effects of $CdCl_2$ on cytoskeletal networks in cultured Sertoli cells. Shown are monolayers of Sertoli cells cultured for 4 h in the absence (0) or presence (1µM or 12 µM) of $CdCl_2$, before being fixed, permeabilised and incubated with anti-actin, anti-tubulin and anti-IF antibodies. This was followed by incubation with Alexa Fluor 488 rabbit anti mouse IgG, as described in Materials and Methods. Scale bar = 100 µm. The intensity of actin and tubulin staining was reduced with $CdCl_2$ treatments. Indicated are the typical leading edges (upward arrows) of $CdCl_2$ treated and untreated Sertoli cells. Actin staining at the leading edges was reduced and/or disrupted with $CdCl_2$ treatments.

3.4 Effects of $CdCl_2$ on the Levels and Phosphorylation Status of Cytoskeletal Proteins in Sertoli Cells

In order to examine the effects of $CdCl_2$ on cytoskeletal proteins and their regulatory proteins in Sertoli cells, Western blotting analysis was used to determine the levels of actin, total cofilin and phosphorylated cofilin (p-cofilin) following exposure to 1 µM and 12 µM $CdCl_2$. A comparative panel of probed blots is shown in figure 5. The levels of reactivity of anti-actin with cell lysates showed a consistent dose dependent decrease at all-time points (Figure 6).

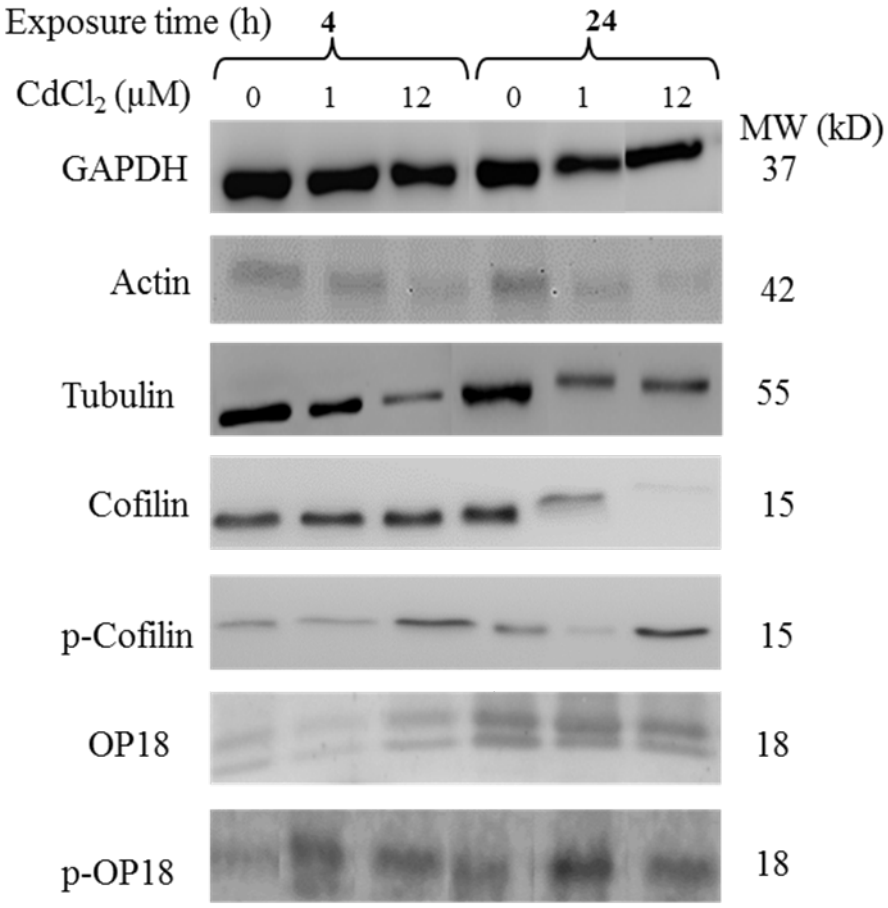


Figure 5: Cytoskeletal protein detection on Western blots of Sertoli cell lysates. Cells were incubated without (0) or with1 µM and 12 µM $CdCl_2$ at the time points indicated. They were then lysed and protein extracts analysed by SDS-PAGE and Western blotting, as described in Materials and Methods. Shown are images of representative blots (from 4 independent experiments) probed with antibodies to actin, tubulin, cofilin, p-cofilin, OP18 and p-OP18, as indicated.

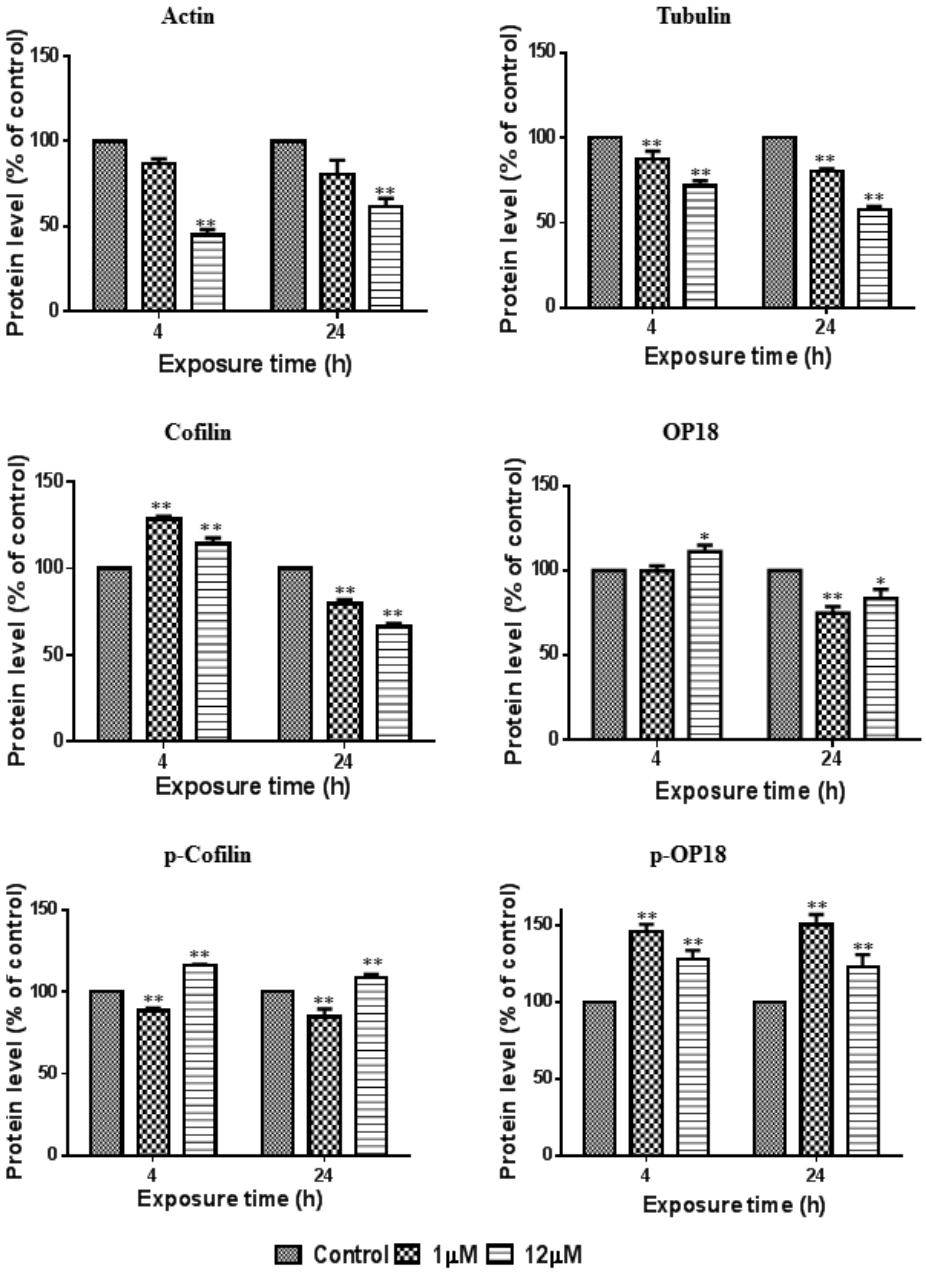


Figure 6: Densitometric analysis of probed Western blots of Sertoli cell lysates. Reactive bands on images of Western blots were quantified using AIDA software as described in Materials and Methods. Data represent means from four separate independent experiments and results are presented as percentage of control (normalised to GAPDH - data not shown) ± SEM. Asterisks indicate changes that were significant (*$p<0.05$, **$p<0.01$) compared to the corresponding non treated control.

The role of cofilin in the regulation of MF dynamics and in relation to the modulation of cell migration is well established (Sumi et al., 1999; Gerthoffer, 2007; Zhang et al., 2011). When cofilin binds to polymerised actin it can destabilise MFs and its interaction with actin is

blocked when phosphorylated by specific protein kinases, such as LIM (Lin11/Isl-1/Mec3) kinase (LIMK1), which target members of the ADF/cofilin family of actin binding and filament severing proteins (Sumi et al., 1999). Western blots were therefore probed with antibodies available to total cofilin and LIMK phosphorylated cofilin, to study the effects of Cd2+ exposure on the phosphorylation status of cofilin. Using this approach, a biphasic effect was observed in the case of reactivity with anti-total cofilin in cells exposed to both concentrations of $CdCl_2$, with significant increases at 4 h followed by decreases at 24 h exposure relative to the corresponding controls (Figure 6). Anti-p-cofilin reactivity with whole cell lysates was reduced on exposure to 1 µM $CdCl_2$ at 4 h and 24 h. By contrast, in the presence of 12 µM $CdCl_2$, reactivity of anti-p-cofilin was increased relative to the control at all-time points (Figure 6).

To analyse possible effects of the $CdCl_2$ treatments on the expression of other cytoskeletal proteins, the reactivity of antibodies to MT proteins such as tubulin was also determined by Western blotting analysis (Figure 5). The results showed that anti-tubulin reactivity was significantly reduced in a time and dose dependent manner in response to $CdCl_2$ treatments.

Since MTs play important roles not only in mitosis but also in cell motility (Cole and Lippincott, 1995; Larsson et al., 1999), it was of further interest to determine whether $CdCl_2$ was capable of interfering with regulation of MT dynamics by affecting the levels and phosphorylation status of regulatory proteins such as oncoprotein18 (OP18)/stathmin, which can destabilise MTs (Belmont and Mitchison, 1996; Andersen, 2000; Cassimeris, 2002). Continuous exposure of Sertoli cells to 1 µM $CdCl_2$ significantly reduced reactivity of anti-OP18 after 24 h exposure but there was a slight but not statistically significant transient increase at 4 h and decline at 24 h in the presence of 12 µM $CdCl_2$ (Figure 6). However, both concentrations of $CdCl_2$ induced a significant increase in the reactivity of anti-p-OP18 under all conditions tested.

Since exposure of Sertoli cells to $CdCl_2$ results in disruption of structures resembling the leading edge, disruption of cytoskeletal networks and their regulatory proteins, the possibility that exposure to this agent may reflect an effect on the regulation of cell migration was investigated

3.5 Inhibition of Sertoli Cell Migration by $CdCl_2$ Detected by Live Cell Imaging

In order to determine whether the morphological and cytoskeletal changes observed in Cd^{2+} exposed Sertoli cells might reflect altered ability of cells to migrate, scratch assays were performed in the absence and presence of 1 µM and 12 µM $CdCl_2$ (Figure 7). Since in a

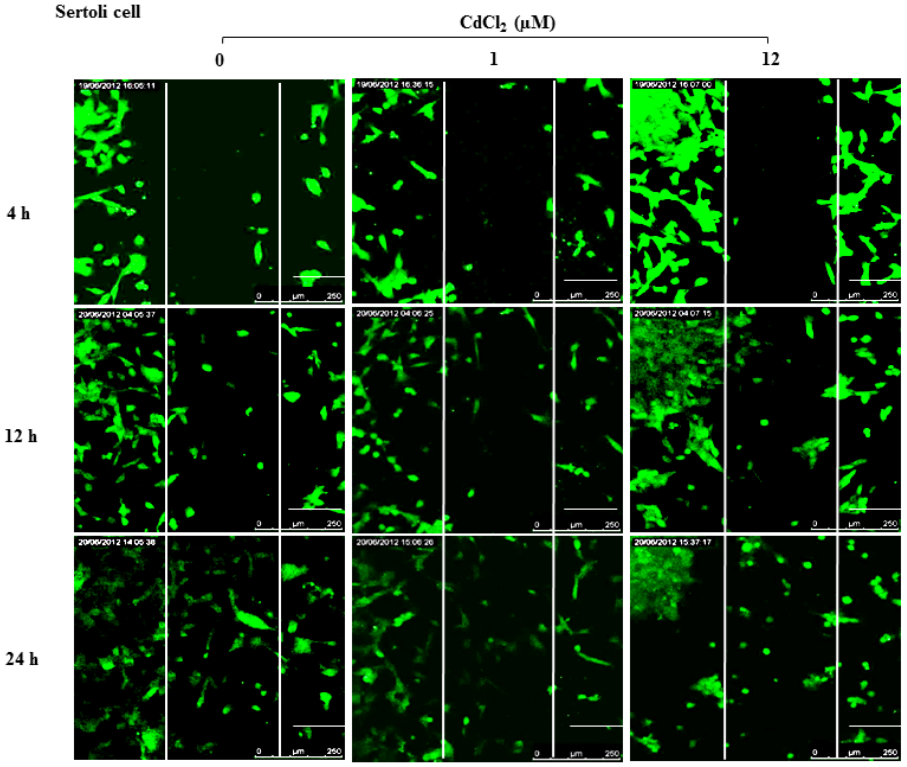


Figure 7: Typical images showing the effects of $CdCl_2$ on Sertoli cell migration. After 5-FU treatment and CFSE labelling of the cells, a scratch application was made and live cell migration was monitored in the absence and presence of $CdCl_2$ by confocal fluorescence microscopy as described in Materials and Methods. Shown are typical images of CFSE labelled Sertoli cells at 4, 12 and 24 h after scratching. Scale bar represents 100 µm. Vertical white lines delineate areas in which cell migration was assessed.

conventional wound healing process, the generated wound closes by migration and proliferation of the cells, it was necessary to block cell proliferation prior to the scratch assay in this study. For this, Sertoli cells were treated for 24 h prior to $CdCl_2$ treatment with 5-FU.

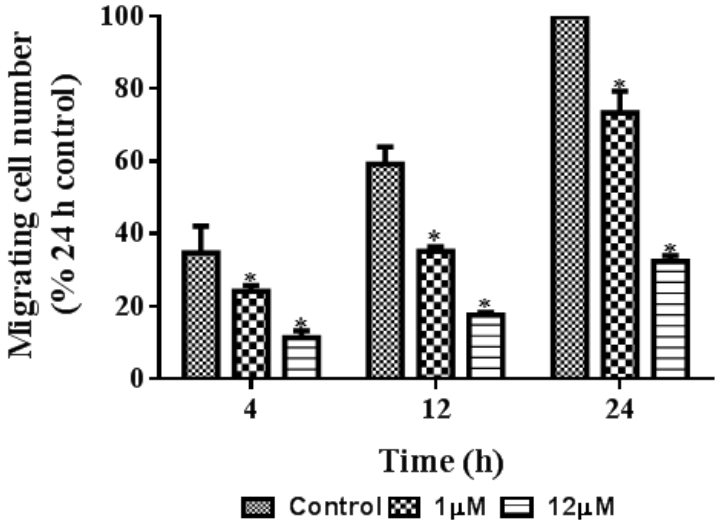


Figure 8: Morphological analysis of the effects of $CdCl_2$ on Sertoli cell migration. Cell migration into the scratch area was assessed as described in Materials and Methods. Data represent mean values for each treatment from four separate independent experiments ± SEM and results are presented as percentage of control at 24 h. Asterisk indicates changes that were significant compared to the non Cd^{2+}-treated control ($p<0.05$). Cell migration was reduced with $CdCl_2$ treatments in Sertoli cells though cell death is likely to have contributed to the effect at 12 µM.

In non Cd^{2+} treated cells, the population of migrating cells in the scratch area progressively increased after scratch application and the scratch region was 90 % closed after 24 h. The data shown in figure 8 indicate a concentration dependent inhibitory effect of $CdCl_2$ on the invasion of the scratch area by Sertoli cells compared to the non $CdCl_2$ treated control. Movies with time lapse images are available in supporting information files online (Figure S1).

4. DISCUSSION

Data obtained from a series of viability assays in the present study have demonstrated that Cd^{2+} is cytotoxic to TM4 Mouse Sertoli cells in a time and concentration-dependent manner, but that 1 µM $CdCl_2$ is sub cytotoxic at all of the time points studied. Using MTT reduction as the main end point, Jiang et al. (2009) suggested that $CdCl_2$ had a biphasic effect on the

proliferation of human embryo lung fibroblasts inducing an increase at low concentrations (i.e. 1 µM) and a decrease at higher concentrations (i.e. 10 µM).

Cd^{2+} concentrations higher than 1 µM inhibit DNA synthesis but as low as 100 pM has been found to stimulate DNA synthesis and cell proliferation significantly in three mammalian cell types, namely L6J1 rat myoblasts, LLC-PK1 porcine renal epithelial cells, and a primary rat chondrocyte culture (Zglinicki et al., 1992; Misra et al., 2002). The data from the present study demonstrate that cell proliferation is slightly but not significantly increased by exposure to 1 µM $CdCl_2$ up to 24 h, but that the level of cell proliferation is significantly reduced by exposure to 12 µM $CdCl_2$ at later time points.

Thus, accumulated evidence suggests that, under certain conditions, $CdCl_2$ exposure can induce cell proliferation or reduce cell viability, consistent with the possibility that transient changes in cell proliferation may occur at lower $CdCl_2$ concentrations. Inconsistencies with the data obtained in the present study for 1 µM $CdCl_2$, may be due to differences in cell type and culture conditions. The data obtained in the present study with 12 µM $CdCl_2$, however, are consistent with previous reports that cadmium concentrations ranging from 10 µM to 40 µM reduce Sertoli cell viability and proliferation in piglets (Zhang et al., 2010).

The IC_{50} values for Sertoli cells exposed for 24 h and 48 h to $CdCl_2$ in the current study were 12 µM and 4 µM respectively, as determined by MTT reduction assay, indicating a progressive increase in toxicity over time. A number of previous studies have demonstrated cytotoxic effects of cadmium in various animal and cell models; such data are summarised in Table 2. Compared to the data shown for other cell lines, the relatively low IC_{50} value for $CdCl_2$ toxicity towards Sertoli cells in culture indicates a relatively high sensitivity of this cell type to the cytotoxic effects of $CdCl_2$. This is consistent with the fact that testicular tissue is a

Chemical form	Cell line	AT	EC_{50}/IC_{50} (µM)	ET (h)	Reference
$CdCl_2$	HTC (hepatoma cells)	MTT	100	24	Fotakis and Timbrell, 2006
$CdCl_2$	HepG2 (hepatoma cells)	MTT	15	24	Fotakis and Timbrell, 2006
$Cd(CH_3CO_2)_2$	REF (rat embryo fibroblasts	CA	1.5	24	Lin et al., 1995
$CdCl_2$	WRL (human fetal liver)	TB	4.7	24	Bucio et al., 1995
$CdCl_2$	CHO (hamster ovary cells)	NR	8.3	24	Garcia-Femandez et al., 2002
$Cd(CH_3CO_2)_2$	HFW (human skin fibroblasts)	CA	25	24	Lin et al., 1995
$CdCl_2$	1407 (human intestinal epithelium)	NR	53	48	Keogh et al., 1994
$CdCl_2$	LLC-PK1(Porcine renal epithelium)	NR	40	24	Olabarrieta et al., 2001
$CdCl_2$	C6 (rat glioma cells)	NR	1	48	Wätjen et al., 2002
$CdCl_2$	C6 (rat glioma cells)	NR	0.7	24	Wätjen et al., 2002
$CdCl_2$	E367 (rat neuroblastoma cells)	NR	10	48	Wätjen et al., 2002
$CdCl_2$	A549(human lung adenocarcinoma cells	NR	160	48	Wätjen et al., 2002
$CdCl_2$	Primary rat mid-brain neuron-glia	MTT	2.5	24	Yang et al., 2007

Table 2 Published EC_{50}/IC_{50} values for Cd^{2+} towards cultured mammalian cells. Shown are µM effective or inhibitory concentrations (EC_{50}/IC_{50}) after exposure to cadmium in different *in vitro* cell cultures. AT = Assay type, ET = Exposure time, MTT = Methylblue tetrazolium test, CA = Clonogenic assay, TB = Trypan Blue, NR = Neutral Red.

known target of Cd^{2+} toxicity *in vivo* and strongly supports the view that the TM4 cell line represents a useful model for mechanistic studies of cadmium toxicity in testicular cells (Jin et al., 2004; Zhang et al., 2010).

The morphological changes induced by 4 h exposure to $CdCl_2$ were consistent with the possibility that exposure to this agent might affect the formation of the leading edge in migrating cells and that sub-lethal concentrations of this heavy metal might be capable of disrupting cell migration in Sertoli cell cultures. The appearance of increasing numbers of compact or rounded cells with $CdCl_2$ treatment could be indicative of increased rounding up of cells prior to cell division or cell death, and/or disruption of cytoskeletal networks.

Changes to the morphology of other cell types and cell organelles could also be involved in the cytotoxicity of $CdCl_2$. For instance, disruption of axons and dendrites has been noted in neuronal cells (Lopez et al., 2003). Sub-lethal concentrations of $CdCl_2$ disrupted mitochondria and endoplasmic reticulum in human foetal hepatic cells and buffalo green monkey (BGM) renal cell lines, as observed by transmission electron microscopy (Bucio et al., 1995; Romero et al., 2003). The rounding up of cells as a result of exposure to sub-lethal concentrations of $CdCl_2$ was also observed in sponge cells (Cebrian and Uriz, 2007), BGM cell lines (Romero et al., 2003), human lens epithelial cells (Song and Koh, 2012), and cortical neurons (Lopez et al., 2003), suggesting that it is likely to be a common effect of Cd^{2+} in a wide range of cell types.

The morphological changes in $CdCl_2$ treated Sertoli cells observed in the current study suggested that exposure to this heavy metal compound involved cytoskeletal disruption. Analysis of images of fixed cell monolayers stained by indirect immunofluorescence using anti-actin antibodies confirmed that there was a major change in the distribution of MFs. The staining pattern suggested that the amount of polymerised actin in the cytoplasm was reduced by $CdCl_2$ treatments. Exposure of human Sertoli cells to $CdCl_2$ at doses considered non-cytotoxic (0.5 - 20 μM) caused disorganisation of actin microfilaments and induced relocation of adhesion proteins including N-cadherin and β-catenin at the cell-cell interface (Xiao et al., 2014). The perturbation was shown to be mediated via changes in F-actin

organization in which microfilaments became truncated and fragmented, retracting from areas near the cell-cell interface. Consequently, cell adhesion protein complexes failed to anchor onto the actin based cytoskeleton. Of particular interest was the fact that comparison of the staining intensity of this antibody with control cells suggested that the amount of actin polymer at the tip of the leading edge was reduced in Cd^{2+} treated cells. As lamellipodia formation is dependent on MF assembly at the leading edge (Pollard and Borisy, 2003), this finding suggests that disruption of MF assembly and/or dynamics might underlie the reduced formation of the leading edge, which could reflect a potential impact on cell migration and cytoplasmic protrusion in premature Sertoli cells.

Cadmium treatment has been found to disrupt actin filaments in cultured MDCK cells (Mills and Ferm, 1989), and LLC-PK cells, causing a breakdown in tight junctions of the latter (Prozialeck and Niewenhuis, 1991). Studies of transepithelial electrical resistance in a cellular model of the blood-testis barrier, suggested that Cd^{2+} also disrupted Sertoli cell tight junctions *in vitro* (Janecki et al., 1992). This may at least in part involve cytoskeletal damage, as disruption of tight junction-associated MFs in rat Sertoli cells was linked to Cd^{2+} toxicity (Hew et al., 1993). The apparent lack of effect on overall distribution of IFs observed by indirect immunofluorescence in the current work suggests that the organisation of the MF network is more sensitive than the IF arrays to disruption by $CdCl_2$ in Sertoli cells.

Since the observation that exposure to 1 µM $CdCl_2$ impaired the formation of the leading edge and MF distribution at the leading edge suggested possible interference with cell movement, we then examined the effects of $CdCl_2$ on Sertoli cell migration. The results from live cell imaging of scratched monolayers indicated that both concentrations of $CdCl_2$ had an inhibitory effect on Sertoli cell migration compared to non Cd^{2+} treated controls. Since actin

is directly involved in cell movement, we then determined the effect of $CdCl_2$ on the level of actin by Western blotting. Analysis of densitometric data suggested that $CdCl_2$ induced a concentration dependent reduction in the levels of anti-actin reactivity, suggesting that total actin levels were depleted during the course of cell migration experiments.

The observed effects on cell migration in the current study are in agreement with the ability of similar (0.5-1 µM) sub-cytotoxic levels $CdCl_2$ to interfere with trophoblast cell migration by disrupting the actin cytoskeleton (Alvarez and Chakraborty, 2011). The current study has shown that exposure to 1 µM $CdCl_2$ has no effect on Sertoli cell proliferation or viability but inhibits Sertoli cell migration, and that this is consistent with disruption of actin filaments. However, the data from cytotoxicity assays following 24 h exposure suggest that cell death may contribute to the impaired migration observed at the higher Cd^{2+} concentration.

Analysis of anti-cofilin and anti-p-cofilin probed blots suggested a significant increase and decrease in reactivity, respectively, after 4 h exposure to 1 µM $CdCl_2$ followed by a slight but significant decrease in the levels of both after 24 h. These effects are consistent with a transient increase in the availability of cofilin and a reduction in its phosphorylation state in the early stages of the cell migration assay in the presence of 1 µM $CdCl_2$ compared to the control. Given the known role of cofilin in the regulation of MF dynamics, increased levels of active cofilin could be indicative of increased actin turnover, resulting in shorter actin filaments and thus shorter or less stable protrusions (Zhang et al., 2011). Our data therefore suggest that the inhibition of cell migration by 1 µM $CdCl_2$ involves disruption of MF dynamics through interference with the expression pattern and phosphorylation state of cofilin.

The data obtained from Western blots of Sertoli cells exposed to 12 µM $CdCl_2$ suggest that, although 4 h treatment lead to a rise in the levels of cofilin compared to the control, there was a similar increase in the level of p-cofilin, followed by a fall in the levels of total cofilin but not its phosphorylation state after 24 h. Western blotting analysis also suggested that enhanced cofilin phosphorylation occurs as a late event with 1 µM $CdCl_2$ but as an early event with the higher concentration. Although any change in the levels of total or p-cofilin could potentially disrupt the normal regulation of MF dynamics, the observed differences in these parameters in lysates from cultures exposed to the two concentrations of $CdCl_2$, suggest that they are likely to reflect a different impact on MF dynamics and organisation.

In vitro studies on $CdCl_2$ induced changes to cofilin are scarce; nevertheless, a number of studies on heat shock have described overexpression of cofilin being compensated by increased cofilin phosphorylation, which would reduce its binding to actin (Yang et al., 1998; Aizawa et al., 2001), an effect which was only observed with 12 µM $CdCl_2$ in the present study. Other reports are documented in different cell lines especially in relation to cell migration studies. In lymphocytes, overexpression of HSP70 prevented the heat-induced phosphorylation of cofilin by reducing the extent of aggregation of the temperature dependent cofilin phosphatase slingshot. As a consequence, this led to improved cellular distribution of cofilin and hence increased chemotaxis (Simard et al., 2011; Zhang et al., 2011).

The current study also shows an effect on the distribution of MTs, which appear to be depleted as shown by indirect immunofluorescence after 4 h exposure to $CdCl_2$,. Therefore, the possibility that this toxin might affect MT dynamics at this and later time points was also investigated at a molecular level by western blotting analysis. This part of the study focused on the MT core protein tubulin and the MT binding protein OP18. A number of reports have associated OP18 with sequestering of tubulin heterodimers or promotion of MT catastrophes

in the regulation of MT dynamics (Larsson et al., 1997; Howell et al., 1999; Larsson et al., 1999). These activities are dependent on the availability of specific sequences at the C and N terminal region of OP18, which determine the interaction between OP18 and tubulin (Howell et al., 1999). Other studies have reported that phosphorylation of OP18 is cell cycle regulated by cyclin-dependent kinases (Deacon et al., 1999).

Densitometric analysis of Western blots probed with antibodies to tubulin indicated that there was a dose dependent decrease in the levels of tubulin at a the two time points, though levels did not fall below approximately 70 % of control values in probed lysates from 1 µM $CdCl_2$ treated cells. Further analysis of blots probed with antibodies to OP18, indicated that continuous exposure to 1 µM $CdCl_2$ led to slight reductions in the levels of total OP18 after 24 h, whereas 12 µM $CdCl_2$ treatment caused a transient rise at 4 h followed by reduced levels compared to the control at 24 h. By contrast, the data were consistent with significant increases in OP18 phosphorylation for both $CdCl_2$ treatments at 4 and 24 h.

These findings suggest a consistent increase in the phosphorylation state of OP18 at the early time point for both concentrations of $CdCl_2$, which could result in increased MT stability as a result of reduced binding of OP18 to tubulin. This effect would be accentuated at 24 h, where the total levels of OP18 but not the levels of phospho-OP18 drop significantly.

With respect to the clinical relevance of the data presented in the current work, the levels of $CdCl_2$ used in the current study are consistent with those of Cd^{2+} detected in various human reproductive tissues including testes (0.56 µg/g dry weight: 1.3 µM), epididymis (0.97 µg/g dry weight: 2.2 µM), prostate glands (0.63 µg/g dry weight:1.4 µM) and seminal vesicle (0.71 µg/g dry weight:1.6 µM) (Oldereid et al., 1993). Seminal plasma cadmium concentrations in infertile males range from of 0.28 µg/l (2.5 nM) to 1.57 mg/l (14 µM) was associated with abnormal sperm number and motility in infertile men, while up to

approximately 0.9 ng/mg dry weight (2 µM) was detected in testicular tissue from infertile men with varicoceles, suggesting that much larger amounts accumulate in tissues than body fluids (Benoff et al., 2005; Akinloye et al., 2005; Benoff et al., 2009). Similar concentrations of $CdCl_2$ were also observed in other mammals such as rats, mice, and bank voles. For example, increased testicular cadmium concentration of 0.64 µg/g dry weight (1.4 µM), consistent with testicular dysfunctions such as decreased epididymal sperm concentration was observed in rats fed with cadmium-polluted radish bulbs for 12 weeks (Haouem et al., 2008). Cadmium contaminated diet resulted in increased testicular cadmium concentrations of 2.14 nmol/g (4.8 µM) and 4.00 nmol/g (8.9 µM) in 1 month and 5 month old bank voles, respectively, after six weeks (Bonda et al., 2004). This was associated with testicular injuries such as haemorrhage in the interstitium, necrosis and apoptosis in seminiferous tubule epithelium of the young bank voles.

In conclusion, this study has demonstrated for the first time that the inhibition of TM4 Sertoli cell migration by a clinically relevant sub-lethal concentration of $CdCl_2$ is associated with altered distribution and dynamics of the MF network. Results also suggest that such exposure may affect stability of the MT network. This study thus provides novel insights into the potential mechanism of toxicity of cadmium on male reproductive health.

Funding/Acknowledgments

BFE was a self-funded PhD student.

Notes

The authors declare no conflict of interest.

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