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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, Alan J. Hargreaves *

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

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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.

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 a 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, 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

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.

* Corresponding authors.
E-mail addresses: biola.egbowon@ntu.ac.uk (B.F. Egbowon), alan.hargreaves@ntu.ac.uk (A.J. Hargreaves).

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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 organisation 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 foetal bovine serum (FBS) (Sigma Aldrich Co. Ltd., Poole, UK), in a humidified atmosphere containing 5% CO_2/95% air at 37 °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 37 °C. 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™ 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 °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 s