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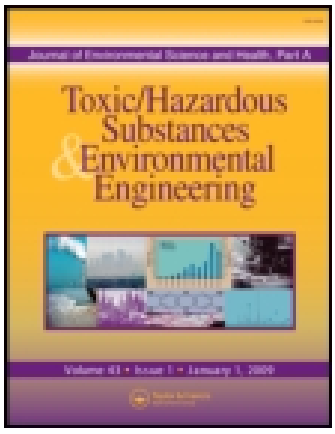

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Endocrine disruptive effects of cadmium on steroidogenesis: Human adrenocortical carcinoma cell line NCI-H295R as a cellular model for reproductive toxicity testing

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Endocrine disruptive effects of cadmium on steroidogenesis: Human adrenocortical carcinoma cell line NCI-H295R as a cellular model for reproductive toxicity testing

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Cadmium (Cd) is a known endocrine disruptor with the ability to affect the production of hormones involved in the regulation of reproductive processes. In this study human adrenocortical carcinoma cell line NCI-H295R was used as an *in vitro* biological model to study the effect of cadmium ($CdCl_2$) on steroidogenesis. The cell cultures were exposed to different concentrations of $CdCl_2$ (1.90, 3.90, 7.80, 15.60, 31.20 and 62.50 μM) and compared to control (medium without $CdCl_2$). Cell viability was measured by the metabolic activity (MTT) assay for estimation of mitochondria structural integrity. Quantification of sexual steroid production directly from aliquots of the medium was performed by enzyme linked immunosorbent assay (ELISA). Following 48 h culture of the cells in the presence of $CdCl_2$ a concentration-dependent depletion in progesterone production was observed at the lower concentrations of $CdCl_2$. The lowest amount of progesterone was significantly detected in groups with the higher doses ($\geq$ 31.20 μM) of $CdCl_2$, which elicited significant ($P < 0.01$) cytotoxic action, too. Cadmium decreased testosterone release in the whole applied range even at the lower concentration of $CdCl_2$. The release of 17β-estradiol decreased as well, but the decline was less pronounced compared to decrease of progesterone and testosterone. The cytotoxic effect was significantly ($P < 0.01$) detected at all concentrations of $CdCl_2$ (1.90–62.50 μM) used in the study. However, the cell viability remained relatively high (>75%) up to 7.80 μM of $CdCl_2$ and significantly ($P < 0.01$) decreased at 15.60 μM and higher concentrations of $CdCl_2$. These results suggest that cadmium has endocrine disruptive effects on sexual steroid synthesis even at very low concentrations.

Keywords: Cadmium chloride, cell viability, endocrine disruption, NCI-H295R cell line steroid hormones.

Introduction

Cadmium (Cd; atomic number 48; relative atomic mass 112.40) is a toxic metal that belongs to group IIB in the periodic table. It occurs in nature at low concentrations, mainly in association with the sulfide ores of zinc (Zn), lead (Pb), and copper (Cu). However, due to the widespread nature of its occurrence, it is presented in measurable amounts in almost everything that we eat, drink, and breathe.[1] This transition metal has been reviewed by the International Register of Potentially Toxic Chemicals of the United Nations Environment Program, and included on the list of chemical substances considered to be potentially dangerous at the global level.[2]

Exposure to Cd occurs as a result of atmospheric emission during Cd production and processing, from combustion of fossil energy sources, waste and sludge, phosphate fertilizers, and deposition of waste and slag at disposal sites.[3] Cigarette smoking is also a high source of Cd exposure.[4] Meat, fish, and fruits generally contain up to 50 μg Cd kg^{-1} on fresh weight basis, whereas vegetables, potatoes, and grain products may contain up to 150 μg Cd/kg fresh weight.[3] Cadmium is an industrial and environmental contaminant unique among metals because of its non-biodegradable nature, long environmental persistence, extremely protracted biological half-life, low rate of excretion from the body and predominant storage in soft tissue (primarily liver and kidney).[5, 6] Cadmium has been identified as a human carcinogen by the International Agency for Research on Cancer[7] and the National Toxicology Program.[8, 9] However, the precise mechanism of carcinogenesis caused by Cd remains unknown.[10]

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Although Cd is not essential for growth and development in mammals, it generally follows the metabolic pathways of essential elements as Zn and Cu.[11, 12] Cadmium has a strong preferential affinity for the liver and the kidney over a wide range of exposure levels. In general, about 50% of the total body burden is found in these two organs.[13] Cadmium causes tissue damage in humans, animals and many toxicological studies have found the functional and structural changes in the kidneys, liver, lungs, bones, ovaries and fetal effects.[14–17] Reproductive organs, such as the testis and placenta, are sensitive to the toxic effects of Cd.[10] Its action may be either direct, affecting the gonads and accessory organs, or indirect via interference with the hypothalamus-pituitary-gonadal axis.[18]

Increasing evidence demonstrated that environmental exposure to Cd is associated with male infertility and the poor semen quality.[19, 20] Several studies showed that Cd induces apoptosis in testicular germ cells.[21, 22] As a well-known endocrine disrupting chemical, Cd not only regulates hypothalamus and pituitary hormone secretion,[23, 24] but also disrupts steroidogenesis including the syntheses of androgen, progesterone and estrogen, leading to suppression of reproductive functions.[10, 25] This metal inhibits the expression of testicular steroidogenic acute regulatory (StAR) protein, which is responsible for the rate-limiting step in steroidogenesis.[25, 26] In addition, Cd exposure down-regulates the level of cytochrome P450 cholesterol side-chain cleavage ($P450_{scc}$) enzyme, cytochrome P450 17β-hydroxysteroid dehydrogenase ($P450_{17\beta}$), 17β-hydroxysteroid dehydrogenase (17β-HSD) and other steroidogenic enzymes.[25, 27]

Sexual steroid hormones are key factors involved in the regulation of reproduction in vertebrates and are also involved in numerous other processes that are related to development and growth. Thus, chemicals that can disrupt the production of steroid hormones may be directly linked to adverse outcomes for these processes.[28] The cell lines are an ideal biological object to study the direct effects of different chemical and physical factors on steroidogenesis. Therefore, in the present study the human adrenocortical carcinoma cell line NCI-H295R was used as a model system for detection of the effect of $CdCl_2$ on the production of sexual steroid hormones *in vitro.* This cell line was derived from H295 cells, which were established from a primary hormonally active adrenocortical carcinoma.[29, 30]

The NCI-H295R cells represent unique *in vitro* model system having the ability to produce all steroid hormones found in the adult adrenal cortex and the gonads, allowing testing the effects of corticosteroid synthesis and the production of sexual steroid hormones.[29] Another advantage of the H295R cell bioassay is that it can be used to evaluate the enzymatic activities of steroidogenic genes.[31, 32] The H295R Steroidogenesis Assay has been included in the Tier1 Screening Battery of United Staes Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP). The test guideline of the H295R Steroidogenesis Assay (TG 456) was validated in Organization for Economic Cooperation and Development (OECD).[33]

The objective of our study was to determine the effects of cadmium ($CdCl_2$) on the steroidogenesis of human adrenocortical carcinoma cell line (NCI-H295R). Specifically, we examined the dose-dependent changes of $CdCl_2$ as endocrine disruptor in relation to release of progesterone, testosterone and 17β-estradiol by adrenocortical carcinoma cells *in vitro*.

Materials and methods

Cell culture

The human adrenocortical carcinoma cell line (NCI-H295R) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in a Good Laboratory Practice (GLP) certified laboratory (National Institute of Chemical Safety, Budapest; OGYI/31762-9/2010) according to previously established and validated protocols.[31–34] After initiation of the NCI-H295R culture from the original ATCC batch cells were cultured for five passages and these cells were split and frozen down in liquid nitrogen (−196°C). The cells for the experiments were cultured for a minimum of five additional passages using new NCI-H295R batches from frozen stocks prior to initiation of the exposure studies.

The cells were grown in 75 cm^2 plastic cell culture flasks (TPP Techno Plastic Products AG, Switzerland) in an incubator under standard conditions (37°C with a 5% CO_2 atmosphere). Subsequently, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.20 g/L $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA), 5.00 mL/L of ITS+Premix (BD Bioscience, San Jose, CA, USA) and 12.50 mL L^{-1} of BD Nu-Serum (BD Bioscience, San Jose, CA, USA).

The medium was changed 2–3 times per week and cells were detached from flasks for sub-culturing using sterile 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA). Cell density was determined using a hemocytometer and adjusted with culture medium to a final concentration of 300 000 cells mL^{-1}. The cell suspensions were plated (with final volume of 1.00 mL $well^{-1}$) into sterile plastic 24-well plates (TPP, Grainer, Germany) for estimation of steroid hormones. For cytotoxicity evaluation the cells (100 μL $well^{-1}$) were seeded into 96-well plates (MTP, Grainer, Germany). The seeded plates were incubated at 37°C with a 5% CO_2 atmosphere for 24 h to allow the cells to attach to the wells.[35]

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In vitro *exposure*

After 24 h attachment period the cell culture medium was removed from the plates and replaced with a new medium supplemented with 1.90; 3.90; 7.80; 15.60; 31.20 and 62.50 μM cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were set in 96-well plates (MTP, Grainer, Germany) after 48 h of $CdCl_2$ exposure. The experimental groups (exposed to different concentrations of $CdCl_2$) with control (Ctrl) (medium without $CdCl_2$) were compared.

Cytotoxicity evaluation

The viability of the cells exposed to $CdCl_2$ was evaluated by the metabolic activity (MTT) assay.[36] This colorimetric assay measures the conversion of a yellow tetrazolium salt [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan was measured spectrophotometrically. Following the termination of $CdCl_2$ exposure, the cells were stained with MTT (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.20 mg mL^{-1}. After 2 h incubation (37°C, with a 5% CO_2 atmosphere), the cells and the formazan crystals were dissolved in 150 μL of acidified (0.08 M HCl) isopropanol (CentralChem, Bratislava, Slovak Republic). The absorbance was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate reader (Anthos MultiRead 400, Austria). The data were expressed in percentage of the control (i.e., absorbance of formazan from cells not exposed to $CdCl_2$).

Release of hormones

At the end of 48 h of $CdCl_2$ exposure, the aliquots of the culture medium were removed from the 24-well cell culture plates and after centrifugation the supernatant was collected and frozen at −80°C until steroid hormones measurements. Enzyme linked immunosorbent assay (ELISA) was used for the quantification of testosterone, progesterone and 17β-estradiol directly from the aliquots of the medium. The ELISA kits were purchased from Dialab GmbH (Wiener Neudorf, Austria). According to the manufacturer's data the sensitivity of testosterone assay was 0.075 ng mL^{-1}, and the intra- and inter-assay coefficients of variation were 4.60% and 7.50%, respectively.

Cross-reactivity with 5α-dihydroxytestosterone was 16.00%. The sensitivity of progesterone assay was 0.05 ng mL^{-1}, and the intra- and inter-assay coefficients of variation were ≤ 4.00% and ≤ 9.30%, respectively. The intra- and inter-assay coefficients of variation for the 17β-estradiol assay were ≤ 9.00% and ≤ 10.00%, and the sensitivity was 8.68 pg/mL. The absorbance was determined at a wavelength 450 nm using an Anthos MultiRead 400 (Anthos MultiRead 400, Austria) microplate reader and the data were evaluated by WinRead 2.30 computer software. Values were expressed in percentage of the untreated controls (control groups solved as 100%).

Statistical analysis

Obtained data were statistically analyzed by the PC program GraphPad Prism 6.00 (GraphPad Software Incorporated, San Diego, CA, USA). Descriptive statistical characteristics (arithmetic mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. The level of significance was set at $^{***}P < 0.001$; $^{**}P < 0.01$ and $^{*}P < 0.05$.

Results

Cell viability

The cytotoxic effect of $CdCl_2$ was significant ($P < 0.01$) for all concentrations. However, the cell viability remained relatively high (>75%) up to 7.80 μM of $CdCl_2$ and significantly ($P < 0.01$) decreased from 15.60 μM and higher concentration of $CdCl_2$ (Fig. 1).

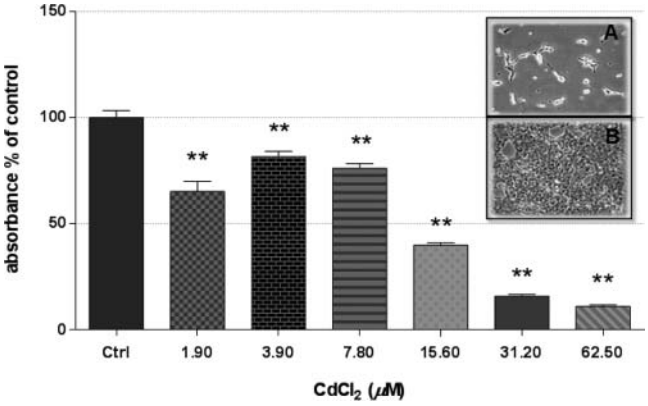

Fig. 1. The viability of NCI-H295R cells in culture after 48 h of $CdCl_2$ exposure (A – low-density adrenocortical carcinoma cell monolayers; B – high density adrenocortical carcinoma cell monolayers). Cytotoxicity was assessed using the MTT assay following $CdCl_2$ exposure. Bar values represent the arithmetic mean (± S.D.) absorbance in % of (untreated) controls. The number of replicate wells was 21-32 at each point. A decline in optical density reflects a decline in cell viability. Statistical difference between the values of control and treated cells was indicated by asterisks $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (One-way ANOVA with Dunnett's multiple comparison test).

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Release of progesterone by adrenocortical carcinoma (NCI-H295R) cells

Following 48 h culture of the cells in the presence of $CdCl_2$ a concentration-dependent depletion of progesterone release was observed even at low concentrations of $CdCl_2$. The lowest amount of progesterone was significantly detected in groups with the higher doses ($\geq$31.20 μM) of $CdCl_2$ (Table 1). The control mean release of progesterone (100%) was 26.72± 9.68 ng mL^{-1}. The percentage changes of progesterone release after $CdCl_2$ exposure are described in Figure 2.

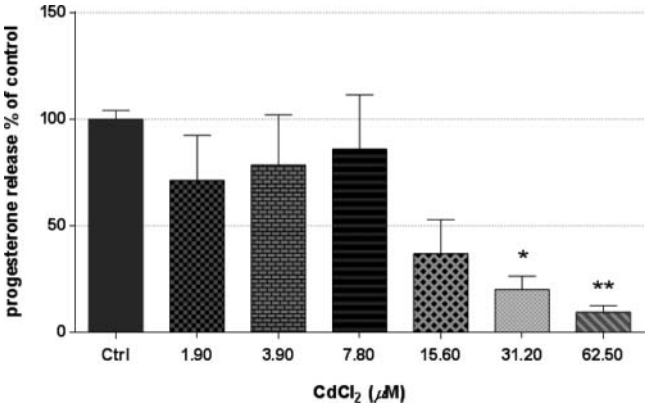

Fig. 2. Progesterone release (%) by NCI-H295R cells in culture after 48 h of $CdCl_2$ exposure. Bar values represent the arithmetic mean (± S.D.) progesterone% of (untreated) controls. The number of replicate wells was 5–6 at each point per experiment. The statistical difference between the values of control and treated cells was indicated by asterisks $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (One-way ANOVA with Dunnett's multiple comparison test).

Release of testosterone by adrenocortical carcinoma (NCI-H295R) cells

The testosterone production was decreased as well, but this decline was more evident at 7.80 μM (8.82 ± 2.26 ng mL^{-1}) of $CdCl_2$ in comparison to the decrease in progesterone release. However, only a low testosterone release was detected at 3.90 μM of $CdCl_2$ (13.31± 4.65 ng mL^{-1}), which was not significant ($P > 0.05$) when compared to the control (14.02± 3.15 ng mL^{-1}) (Table 2). The percentage changes of testosterone release after $CdCl_2$ exposure are described in Figure 3.

Release of 17β-estradiol by adrenocortical carcinoma (NCI-H295R) cells

The 17β-estradiol production also decreased (Fig. 4), but this decline was less pronounced comparing the decrease in release of progesterone and testosterone. The highest release of 17β-estradiol by adrenocortical carcinoma (NCI-H295R) cells was recorded in control (1.09± 0.31 pg mL^{-1}). The results are shown in Table 3.

Table 1. Effect of 48 h $CdCl_2$ exposure on release of progesterone (ng/mL) by adrenocortical carcinoma (NCI-H295R) cells.

	$CdCl_2$ (μM)						
Groups	*Control Ctrl*	*1.90 F*	*3.90 E*	*7.80 D*	*15.60 C*	*31.20 B*	*62.50 A*
x (ng/mL)	26.72	19.03	20.97	22.93	9.85	5.34*	2.49**
Minimum	15.24	11.77	10.89	8.15	4.88	2.44	1.25
Maximum	42.88	34.24	35.07	32.53	12.25	6.88	3.88
S.D.	9.68	13.84	9.17	10.60	2.70	1.58	0.90
CV (%)	36.24	43.05	43.71	46.25	27.37	29.55	36.15
%	100.00	71.20	78.47	85.80	36.86	19.99	9.31

x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

Currently, there is increased evidence that various chemicals introduced to the environment have the potential to disrupt the endocrine system,[37] which may result in adverse effects on differentiation, growth and development. It is possible for certain environmental contaminants (including metals) to cause or contribute to a hormonal disruption and interfere with functions of the key enzymes involved in steroidogenesis.[38] According to several research studies, Cd can affect multiple points of the steroidogenesis pathway, inhibiting enzymes important for hormone synthesis.[10, 39, 40] Recently, the effects of Cd on steroidogenesis have been described, but results vary depending on the experimental model, time-duration of exposure and the dose used. Therefore, the general objective of this study was to provide information of the impact of Cd on steroidogenesis.

Our results suggest a direct toxic action of Cd on the steroid-producing cells and subsequent changes in hormonal release. Cadmium decreased the release of progesterone, testosterone and 17β-estradiol in the whole applied range even at a very low concentration (1.90 μM) of $CdCl_2$, while the cell viability remained relatively high (> 75%) up to 7.80 μM of $CdCl_2$ and significantly ($P < 0.01$) decreased from 15.60 μM and higher concentrations of $CdCl_2$. These results clearly confirm previous reports by Forgacs et al.[41] and Ocztos et al.,[42] who observed the effect of Ni^{2+}, Hg^{2+} and Cd^{2+} on the progesterone and testosterone production of H295R cells. Similar results were also obtained in our previous study with mercury.[35]

Cadmium disrupts steroid biosynthesis in a variety of cells.[18] The recent studies conducted using cultured

Table 2. Effect of 48 h $CdCl_2$ exposure on release of testosterone (ng/mL) by adrenocortical carcinoma (NCI-H295R) cells.

	$CdCl_2$ (μM)						
Groups	*Control Ctrl*	*1.90 F*	*3.90 E*	*7.80 D*	*15.60 C*	*31.20 B*	*62.50 A*
x (ng/mL)	14.02	9.56	13.31	8.82*	4.10**	2.65**	2.21**
Minimum	9.22	4.03	8.28	4.97	2.13	1.08	1.02
Maximum	18.50	14.66	22.24	12.28	7.12	4.88	3.88
S.D.	3.15	4.26	4.65	2.26	1.78	1.37	0.87
CV (%)	22.49	44.51	34.96	25.66	43.37	51.77	39.36
%	100.00	68.13	94.83	46.57	19.84	18.88	15.74

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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human placental trophoblastic cells suggest that Cd reduces progesterone synthesis by inhibiting the gene expression of the low-density lipoprotein (LDL) receptor, which controls the internalization of cholesterol into steroidogenic cells,[43] cytochrome $P450_{SCC}$, which coverts pregnenolone to progesterone.[44] On the other hand, Cd administered to female rats during estrus and diestrus resulted in increased serum progesterone level[39, 45, 46] and stimulated progesterone synthesis in both cultured porcine granulosa cells[47] and JAR choriocarcinoma cells, a malignant trophoblast cell line.[48]

The results of our present study indicate dose-dependent decrease in progesterone release by NCI-H295R cell line in culture following a 48-h *in vitro* $CdCl_2$ exposure. The lowest amount of progesterone was detected in groups with the higher doses ($\geq$ 31.20 μM) of $CdCl_2$, which elicited significant cytotoxic action, too. Massanyi et al.[49] suggested that degeneration and luteinisation of granulosa cells also cause the progesterone production. Basal progesterone production of cadmium-treated cells remained unchanged (20 ng mL^{-1}) or was enhanced; this supports the theory of Cd - calcium competition.[50]

Henson and Chedrese[51] discussed the dual effects of Cd on progesterone synthesis in their review. They suggested that low concentrations of Cd stimulate $P450_{SCC}$ gene transcription resulting in enhancement of the steroidogenic pathway, whereas high concentrations of Cd inhibit $P450_{SCC}$ activity resulting in the suppression of progesterone synthesis. Results of the study by Smida et al.[52] support the concept that depending on the concentration, Cd^{2+} can exert dual effects on steroidogenesis.

When used in concentrations greater than 0.6–3.0 μM, $CdCl_2$ stimulated transcription of the $P450_{SCC}$ gene and the steroidogenic pathway in stable porcine granulosa cell line JC-410. At high (5.0 μM) concentration $CdCl_2$ inhibited $P450_{SCC}$ gene promoter activity and progesterone synthesis and it has cytotoxic effects that produce changes in cell morphology and cell death. Dual effects of Cd^{2+} have been described in other experimental models as well.[53, 54] Generally, there is a lack of data describing the effect of Cd on granulosa cells[51, 52] mostly monitoring the biochemical aspects of toxicity. The effects of $CdCl_2$ observed in the present study ranged at concentrations between 1.90 and 62.50 μM. Based on our results, it is reasonable to think that even exposure to low concentrations of $CdCl_2$ may be sufficient to affect the steroidogenic pathway.

The production of testosterone may be disrupted by Cd without inducing a loss of testosterone-producing cells by

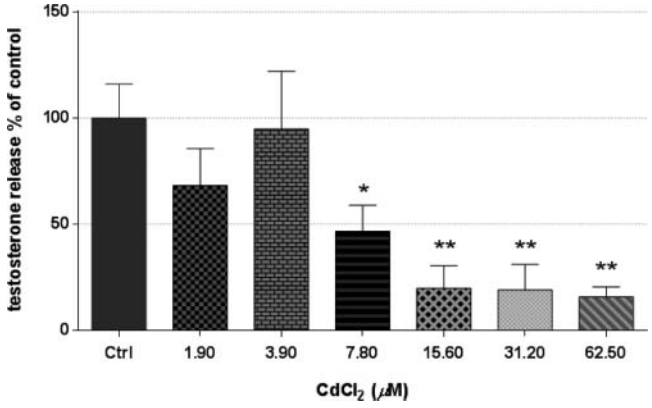

Fig. 3. Testosterone release (%) of NCI-H295R cells in culture after 48 h of $CdCl_2$ exposure. Bar values represent the arithmetic mean (± S.D.) testosterone % of (untreated) controls. The number of replicate wells was 6–12 at each point per experiment. The statistical difference between the values of control and treated cells was indicated by asterisks *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (One-way ANOVA with Dunnett's multiple comparison test).

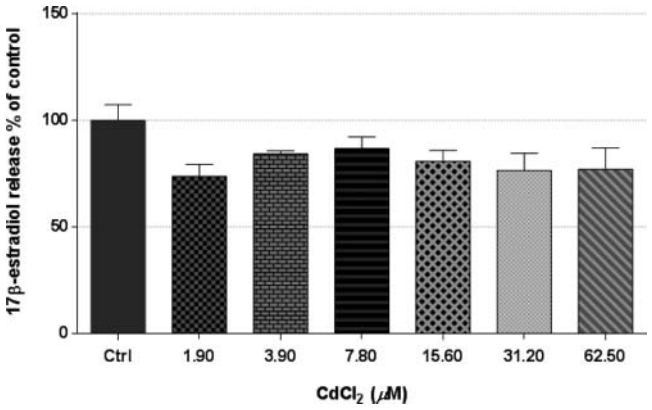

Fig. 4. 17β-estradiol release (%) by NCI-H295R cells in culture after 48 h of $CdCl_2$ exposure. Bar values represent the arithmetic mean (± S.D.) 17β-estradiol % of (untreated) controls. The number of replicate wells was 7–12 at each point per experiment. No statistical difference between the values of control and treated cells was not recorded ($P > 0.05$) (One-way ANOVA with Dunnett's multiple comparison test).

Table 3. Effect of 48 h $CdCl_2$ exposure on release of 17β-estradiol (pg/mL) by adrenocortical carcinoma (NCI-H295R) cells.

	$CdCl_2$ (μM)						
Groups	*Control Ctrl*	*1.90 F*	*3.90 E*	*7.80 D*	*15.60 C*	*31.20 B*	*62.50 A*
x (pg/mL)	1.09	0.80	0.91	0.94	0.88	0.83	0.84
Minimum	0.53	0.57	0.88	0.73	0.70	0.69	0.58
Maximum	1.68	1.05	0.97	1.05	1.09	0.97	1.08
S.D.	0.31	0.16	0.03	0.13	0.14	0.15	0.22
CV (%)	28.28	20.19	3.54	14.03	15.43	18.13	25.87
%	100.00	73.69	84.23	86.75	80.77	76.44	77.06

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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necrosis.[10] Laskey and Phelps[55] examined effect of Cd^{2+} and other metal cations (Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+}) on *in vitro* Leydig cell testosterone production. The results showed no change in Leydig cell viability with any metal cation treatment during the 3 h incubation. Dose-response depletion in both hCG- and db-cAMP-stimulated testosterone production were noted with Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+} treatment. Surprisingly, Cd^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}, which caused a depletion in hCG- and db-cAMP-stimulated testosterone production, caused significant increases in HCHOL- and PREG-stimulated testosterone production over untreated and similarly stimulated cultures.

This indicated that these cations may act at multiple sites within the Leydig cell. Zeng et al.[56] reported that serum testosterone levels were significantly increased by chronic oral Cd exposure (50, 100 and 200 ppm) for 3 months. Although the mechanism underlying the Cd-induced increase in the serum testosterone levels is unclear. The authors consider that chronic oral Cd exposure might have induced endocrine homeostasis disruption through a mechanism different from that associated with other routes of Cd administration, e.g., subcutaneous or intraperitoneal administration used in most studies.

Our presented data showed that testosterone seem to be more vulnerable than progesterone and 17β-estradiol to cadmium exposure suggesting multiple sites of action of this metal in steroidogenesis. Disorders of the testosterone synthesis could result in a reduction of the activity of the key enzymes implied in the biosynthesis of testosterone.

Reduction in testosterone hormone was manifested by lowered follicle stimulating hormone and luteinizing hormone plasma levels,[57–59] apart from Cd-induced decrease in total testosterone hydroxylase activity,[60] which resulted in a dramatic decrease in testosterone hormone levels. In turn, this decline may be responsible for significant decrease in the hCG-stimulated serum testosterone levels,[61] interference of Cd with cAMP in testis and depression of protein kinase.[62] Massanyi et al.[49] examined the effects of $CdCl_2$ on ultrastructure and steroidogenesis in cultured porcine ovarian granulosa cells *in vitro*. Evaluation of steroidogenesis indicated that $CdCl_2$ induces an increase in progesterone production and a decrease in 17β-estradiol production, which are required at further stages of reproduction.

Subsequently, structural and functional alterations in the ovarian granulosa cells after $CdCl_2$ administration were confirmed. A decrease in 17β-estradiol concentrations was observed; in this respect our results are supported by those obtained by Paksy et al.[39] in human granulosa cells. Han et al.[63] showed that 10.0 mg kg^{-1} $CdCl_2$ on weight gain decreases the secretion of estradiol and progesterone in serum of the pigs during long-term exposure. They concluded that Cd might have a direct effect on the function of granulosa cells and lutein cells because of its accumulation in the ovary, and it is also possible that cadmium produces the change of estradiol and progesterone levels through combining with their receptors. Similar results were also reported from female rats.[64, 65]

In addition, Cd acts as metallohormone, which mimics the biosynthesis of estrogen.[66, 67] This metal induces proliferation,[68–70] increases the transcription and expression of estrogen regulated genes such as the progesterone receptor,[68] and activates estrogen receptor α (ERα).[69] According to Martin et al.[71] and Garcia-Morales et al.[68] Cd exhibited estrogen activity through an ERα in MCF-7 human breast cancer cells. Their results suggest that the effects of Cd are mediated directly by ERα and are independent of estradiol. However, additional studies are required to define the mechanism by which Cd activates steroid receptors.

The measurement of cell viability and *in vitro* sexual steroid production proved to be sensitive for assessing a direct action of environmental chemical factors. The relative cytotoxicity of a variety of Cd has been evaluated *in vitro* using various cell types, such as alveolar macrophages,[72] lymphocytes,[73] fibroblasts,[74] red blood cells,[75] ovarian cells,[76] and adrenocortical carcinoma cells.[77, 78] Some metals have adverse effects in experimental animals but not in the cell culture model. Few studies have been conducted on the effect of divalent metals on steroidogenesis of adrenocortical carcinoma cells.[78] Therefore, this study was conducted to ascertain whether Cd has a direct toxic effect on steroidogenesis of cells isolated from human adrenocortical carcinoma cell line.

The cytotoxic effect was significantly ($P < 0.01$) detected at all concentrations of $CdCl_2$ used in the study (1.90–62.50 μM). However, the cell viability remained relatively high (> 75%) up to 7.80 μM of $CdCl_2$ and significantly ($P < 0.01$) decreased from 15.60 μM and higher concentrations of $CdCl_2$. These results are in agreement with a previous report by Tchounwou et al.[79] indicating the high degree of $CdCl_2$ toxicity to human liver carcinoma cells (HepG2). Ng and Liu[80] noted that Cd (100 μM)

exerted an adverse effect on the viability of isolated rat adrenal capsular (*zona glomerulosa*), adrenal decapsular (*fasciculata et reticularis*) and Leydig cells of the testis, which was linked to a decreased corticosterone production and luteinizing hormone-stimulated testosterone production. The authors explained that $CdCl_2$ had a specific toxic mechanism on the adrenal glands as well as the Leydig cells of the testis.

Conclusions

The results of the present study contribute to the knowledge of the effect of cadmium on steroidogenesis, and should also serve to increase the level of awareness of its effect on reproductive physiology. Data obtained from this *in vitro* study indicate that the release of sexual steroid hormones by adrenocortical carcinoma cells can be associated with the doses of cadmium administration. Testosterone release seemed more vulnerable than progesterone and 17β-estradiol to cadmium exposure. Probably the effect of enzymatic action of 17β-hydroxysteroid dehydrogenase is more sensitive, which results in decreased release of testosterone in comparison with progesterone/17β-estradiol and thereby the effect of enzymatic action of 3β-hydroxysteriod dehydrogenase/aromatase.

In conclusion, the present study suggests the endocrine disruptive and reproductive toxicological effects of this metal. Cadmium toxicity may also reflect at other points of the steroidogenesis pathway. Therefore, further studies are needed to clarify the precise mechanism of action of cadmium on the sexual steroid production and their metabolites, whose production is conditioned by steroidogenic enzymes.

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