Invited paper

ENDOCRINE DISRUPTION BY HEAVY METALS ON STEROIDOGENESIS IN MODEL SYSTEMS

Lukáč N.[*1], *Forgacs Z.*[2], *Kňažická Z.*[1], *Lukáčová J.*[1], *Tvrdá E.*[1], *Kročková J.* [1], *Massányi P.*[1]

[1]Department of Animal Physiology, Slovak University of Agriculture, Tr. A. Hlinku 2, Nitra, 94976, Slovak Republic
[2]Deparement of Molecular and Cell Biology, National Institute of Chemical Safety, P. O. Box 36, 1450 Budapest, Hungary
*Corresponding author: Norbert.Lukac@uniag.sk

Abstract

In this study human adrenocortical carcinoma cell line NCI-H295R was used as an *in vitro* biological model to study the effect of heavy metals on steroidogenesis. The cell cultures were exposed to different concentrations of cadmium (1.90; 3.90; 7.80; 15.60; 31.20; 62.50 μM of $CdCl_2$), mercury (1.0; 5.0; 25; 50; 100 μM of $HgCl_2$), nickel (3.90; 7.80; 15.60; 31.20; 62.50; 125; 250; 500 μM of $NiCl_2$) and compared to control. 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). Cadmium decreased the release of progesterone and testosterone already 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 at 15.60 μM and higher concentrations of $CdCl_2$. Concentration-dependent depression in testosterone production was detected particularly for higher concentration of $HgCl_2$. Progesterone production was also decreased, but at the lower concentrations (1.0 and 5.0 μM) of $HgCl_2$ this decline was lower compared to depression of testosterone. The cell viability significantly decreased at 25 μM and higher concentration of $HgCl_2$. Results of the our study indicate dose dependent decreases in both sexual steroid hormones by NCI-H295R cell culture following a 48 h *in vitro* $NiCl_2$ exposure. The lowest concentration of progesterone was significantly (P<0.01) detected in groups with the higher doses (≥ 500 μM) of $NiCl_2$, which elicited significant cytotoxic effect. The testosterone production was decreased as well, but this decline was more pronounced compared to depression of progesterone. These results suggest that heavy metals have detrimental effects on steroid hormone synthesis even at very low concentrations and consecutively on reproductive physiology.

Key words: *cell viability, endocrine disruption, heavy metals, NCI-H295R cell line, steroid hormones*

Introduction

Currently, there is increased evidence that various chemicals introduced to the environment have the potential to disrupt the endocrine system, 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 key enzymes involved in steroidogenesis (Sanderson, 2006). Heavy metals such as cadmium, mercury and nickel are also reported to have an endocrine disruptive potential (Zhu et al., 2000). Cadmium (Cd) 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) (Massanyi et al., 2002). As a well-known endocrine disrupting chemical, Cd is not only a regulator of hypothalamus and pituitary hormone secretion (Lafuente et al., 2003), but also disrupts steroidogenesis including the syntheses of androgen, progesterone and estrogen, leading to suppression of reproductive functions (Takuguchi and Yoshihara, 2006). Mercury (Hg) is one of the oldest toxicants known and is considered to be a risk factor of the environment and food chain (Tazisong and Senwo, 2009). Exposure to a high concentration of Hg causes an increase in reproductive problems (Schuurs, 1998), which can be reflected in the process of steroidogenesis. The endocrine disruptive effects of Hg have recently become one of the major public concerns. There in sufficient evidence from animal studies supporting the disruptive effects of Hg on the functions of the thyroid, adrenal gland, ovary and testis, although several factors make it difficult to extrapolate the animal data to humans (Zhu et al., 2000). Nickel (Ni) is considered to be an essential micronutrient (Eisler, 1998) that is only required in very small amounts (Das, 2009). Nickel serves as a cofactor or a structural component of several metalloenzymes (Przybyla et al., 1992). There is sufficient evidence that nickel ions (Ni^{2+}) have potent toxic effects on the reproductive system (Das and Dasgupta, 2000). Nickel salts are capable of morphological changes such as degeneration of testicular germinal epithelium (Pandey et al., 1999), testicular sarcomas and functional disorders such as inhibition of spermatogenesis (Yokio et al., 2003) or disruption of steroidogenesis (Krockova et al., 2011).

In the present study we investigated the effects of heavy metals on steroidogenesis in the human adrenocortical carcinoma cell line (NCI-H295R), which serves as a model system for screening endocrine-disruptive chemicals (Ding et al., 2007). This cell line was derived from H295 cells, which were established from a primary hormonally active adrenocortical carcinoma (Rainey et al., 2004). The NCI-H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells (Staels et al., 1993) and represent an unique *in vitro* model system which has the ability to produce all of the steroid hormones found in the adult adrenal cortex and gonads, allowing to test the effects on both corticosteroid synthesis together with the production of sexual steroid hormones (Gazdar et al., 1990) as well as permit the measurement of hormone production. The objective of our study was to determine the effects of heavy metals (Cd, Hg and Ni) on steroidogenesis of human adrenocortical carcinoma cell line (NCI-H295R). Specifically, we examined the dose-dependent changes of heavy metals as endocrine disruptors in relation to release of progesterone and testosterone by adrenocortical carcinoma cells *in vitro*.

Material 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 (Hilscherova et al., 2004; OECD, 2011). The adrenocortical carcinoma cells were grown (37ºC, with a 5% CO_2 atmosphere) 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) (1:1) supplemented with 1.20 g/L $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA), 5.00 mL/L of ITS+Premix (BD Biosciences, San Jose, CA, USA) and 12.50 mL/L of BD Nu-Serum (BD Biosciences, 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. The cell suspensions were plated (with final volume of 1.00 mL/well) into sterile plastic 24-well plates (TPP, Grainer, Germany) for estimation of steroid hormones. For cytotoxicity evaluation the cells (100 μL/well) were seeded into 96-well plates (MTP, Grainer, Germany). The seeded plates were incubated at 37ºC with a 5% CO_2 atmosphere for 24 h to allow the cells to attach to the wells (Knazicka et al., 2013).

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; 62.50 μM cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA), 1.0; 5.0; 25; 50; 100 μM mercury chloride ($HgCl_2$; Sigma-Aldrich, St. Louis, MO, USA) and 3.90; 7.80; 15.60; 31.20; 62.50; 125; 250 or 500 μM nickel chloride ($NiCl_2$; Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were maintained for 48 h.

Cytotoxicity evaluation

The viability of the cells exposed to heavy metals was evaluated by the metabolic activity (MTT) assay (Mosmann, 1983). 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 heavy metals ($HgCl_2$, $CdCl_2$ and $NiCl_2$) exposure, the cells were stained with MTT (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.20 mg/mL. After 2 h incubation (37ºC, 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 optical density 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. optical density of formazan from cells not exposed to heavy metals).

Hormone measurement

Enzyme linked immunosorbent assay (ELISA) was used for the quantification of steroid hormones (progesterone, testosterone) 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, 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, and the intra- and inter-assay coefficients of variation were ≤

4.00% and ≤ 9.30%, respectively. 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.

Statistical analysis

Obtained data were statistically analyzed by the PC program GraphPad Prism 6.00 (GraphPad Software Incorporated, San Diego, California, 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 * $P<0.05$; ** $P<0.01$ and *** $P<0.001$.

Results and discussion

Release of steroid hormones by adrenocortical carcinoma cells after $CdCl_2$ exposure

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. 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 and testosterone in the whole applied range even at a very low concentration (1.90 μM) of $CdCl_2$ (Table 1), 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 $CdCl_2$ (Figure 1). These results are in agreement with a previous report by Tchounwou et al. (2001) indicating the high degree of $CdCl_2$ toxicity to human liver carcinoma cells (HepG2).

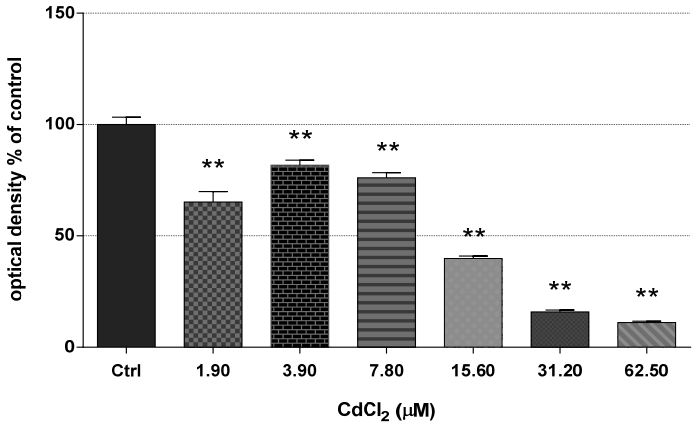

Figure 1. *The viability of NCI-H295R cells in culture after 48 h of $CdCl_2$ exposure*

Legend: Cytotoxicity was assessed using the MTT assay following $CdCl_2$ exposure. Bar values represent the arithmetic mean (±S.D.) optical density 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).

Cadmium disrupts steroid biosynthesis in a variety of cells (Paksy et al., 1992). The studies conducted using cultured 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 (Jolibois et al., 1999), cytochrome $P450_{SCC}$, which coverts pregnenolone to progesterone (Kawai et al., 2002). 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 significantly detected in groups with the highest doses (≥ 31.20 μM) of $CdCl_2$. The authors Paksy et al. (1992 and 1997) reported a decrease of progesterone production by human granulosa cells after Cd treatment *in vitro*. Cadmium did not cause a significant alteration in progesterone accumulation during 4 h incubation period, however, progesterone production decreased after 24 h. The testosterone production was decreased as well, but this decline was more evident at 7.80 μM of $CdCl_2$ in comparison to the decrease in progesterone release. Laskey and Phelps (1991) 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. Testicular cells were incubated with metal cations (1.0 to 5000 μM) for 3 h absence and presence of maximally stimulating concentrations of human chorionic gonadotropin (hCG), dibutyl cyclic adenosine monophosphate (db-cAMP), 20α-hydroxycholesterol (HCHOL) or pregnenolone (PREG). The results showed no change in Leydig cell viability with any metal cation treatment during 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 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.

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

Groups	**Control**	**1.90**	**3.90**	**7.80**	**15.60**	**31.20**	**62.50**
	Ctrl	**F**	**E**	**D**	**C**	**B**	**A**
$CdCl_2$ (μM)							
Progesterone (ng/mL)							
x	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
Testosterone (ng/mL)							
x	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

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

Release of steroid hormones by adrenocortical carcinoma cells after $HgCl_2$ exposure

The results of our present study indicate dose-dependent decreases in both progesterone and testosterone release by NCI-H295R cell line in culture following a 48 h *in vitro* $HgCl_2$ exposure detected at low concentration (Table 2), which does not elicit significant (P>0.05) cytotoxic action. The viability of cells significantly decreased at 25 μM and higher concentration of $HgCl_2$ (Figure 2). However, at 25 μM $HgCl_2$ exposure the cell viability remained relatively high (> 80%). The measurement of cell viability and *in vitro* sexual steroid production proved to be sensitive for assessing a direct action of environmental chemical factors. Our presented data showed that progesterone was decreased, but at the lower concentrations (1.0 and 5.0 μM) of $HgCl_2$ this decline was less pronounced comparing to depression of testosterone. Burton and Meikle (1980) found that mitochondrial conversion of cholesterol to pregnenolone was inhibited in testicular tissue following mercury (MeHg) exposure and this could inhibit steroidogenesis. Veltman and Maines (1986) studied the synthesis of corticosterone in rats following exposure to $HgCl_2$, and determined that there was an increase in specific cytochrome P450 enzymes (CYPs) in the mitochondrial fraction of the adrenal glands, which in turn caused an increase in side-chain cleavage of cholesterol and a seven fold increase in the rate of production of pregnenolone.

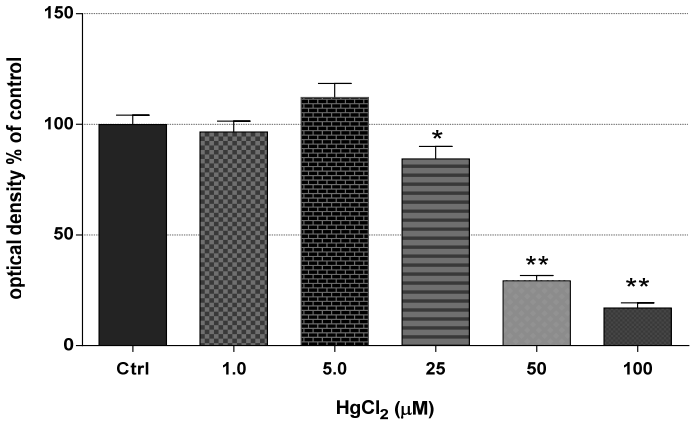

Figure 2. *The viability of NCI-H295R cells in culture after 48 h of $HgCl_2$ exposure*

Legend: Cytotoxicity was assessed using the MTT assay following $HgCl_2$ exposure. Bar values represent the arithmetic mean (±S.D.) optical density in % of (untreated) controls. The number of replicate wells was 20-32 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).

The testosterone seems to be more vulnerable than progesterone to $HgCl_2$ 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 (McVey et al., 2008). Vachhrajani and Chowdhury (1990) examined the testicular steroidogenesis after an intraperitoneal administration of $HgCl_2$ (5.0; 10 μg/kg) and methylmercury (50; 100 μg/kg of MeHg) for 90 days. Both ($HgCl_2$, MeHg) inhibited the activity of 3β-hydroxysteroid dehydrogenase in the rat, leading to a significant decrease in serum testosterone levels and an induced cellular disintegration of Leydig cells.

Table 2. *Effect of 48h $HgCl_2$ exposure on release of sexual steroid hormones (ng/mL) by adrenocortical carcinoma (NCI-H295R) cells*

Groups	**Control**	**1.0**	**5.0**	**25**	**50**	**100**
	Ctrl	**E**	**D**	**C**	**B**	**A**
			$HgCl_2$ (μM)			
Progesterone (ng/mL)						
x	18.53	11.79*	17.31	1.43**	0.007**	0.01**
Minimum	12.14	7.92	11.88	1.09	0.00	0.00
Maximum	23.58	15.21	22.24	2.02	0.02	0.02
S.D.	4.80	2.99	4.37	0.41	0.009	0.01
CV (%)	25.89	25.40	25.26	28.65	127.66	115.47
%	100.00	63.63	93.42	7.72	0.38	0.05
Testosterone (ng/mL)						
x	12.21	4.85**	5.51**	1.06**	0.36**	0.88**
Minimum	6.69	2.59	2.84	0.89	0.10	0.05
Maximum	18.50	8.29	8.24	1.38	0.78	2.05
S.D.	4.90	2.45	2.59	0.22	0.30	0.92
CV (%)	40.16	50.51	47.04	20.27	83.40	104.53
%	100.00	39.72	45.13	8.68	3.00	7.20

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

Release of steroid hormones by adrenocortical carcinoma cells after $NiCl_2$ exposure

Following 48 h culture of the cells in the presence of $NiCl_2$ a concentration-dependent depletion of steroid hormones release was observed in the whole applied range of concentrations (1.90 to 500 μM), including the lower concentrations of $NiCl_2$. The lowest amount of progesterone was significantly (P□0.01) detected in groups with the higher doses (≥ 500 μM) of $NiCl_2$ (Table 3), which elicited significant cytotoxic effect. However, the cell viability remained relatively high up to 62.50 μM of $NiCl_2$ (Figure 3). The testosterone production was decreased as well, but this decline was more pronounced compared to depression of progesterone. These results clearly confirm previous reports by Forgacs et al. (2011) and Ocztos et al. (2011), who observed the effect of Ni^{2+}, Hg^{2+} and Cd^{2+} on the progesterone and testosterone production of H295R cells.

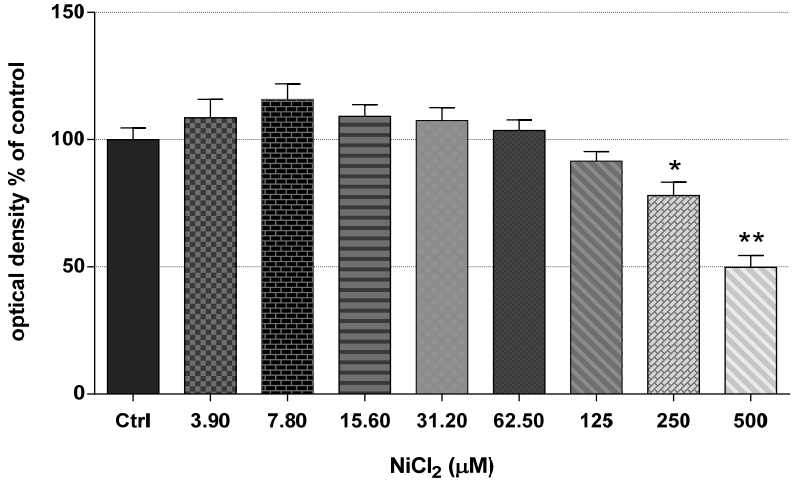

Figure 3. *The viability of NCI-H295R cells in culture after 48 h of $NiCl_2$ exposure*

Legend: Cytotoxicity was assessed using the MTT assay following $NiCl_2$ exposure. Bar values represent the arithmetic mean (±S.D.) optical density in % of (untreated) controls. The number of replicate wells was 22-32 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).

Table 3. *Effect of 48 h $NiCl_2$ exposure on release of sexual steroid hormones (ng/mL) by adrenocortical carcinoma (NCI-H295R) cells*

Groups	**Control**	**3.90**	**7.80**	**15.60**	**31.20**	**62.50**	**125**	**250**	**500**
	Ctrl	**H**	**G**	**F**	**E**	**D**	**C**	**B**	**A**
$NiCl_2$ (μM)									
Progesterone (ng/mL)									
x	21.05	19.56**	11.07**	10.59**	11.70**	9.93**	7.33**	6.18**	5.79**
minimum	15.24	15.64	6.24	6.84	8.27	6.88	5.27	4.25	3.55
maximum	28.25	24.12	14.25	15.26	14.85	14.49	10.58	8.18	7.58
S.D.	4.40	4.00	3.26	3.06	2.65	3.00	1.99	1.62	1.35
CV (%)	20.91	20.46	29.48	28.96	22.96	30.24	27.15	26.13	23.41
%	100.00	68.39	52.59	50.28	55.50	47.19	34.83	29.37	27.48
Testosterone (ng/mL)									
x	10.75	4.42**	3.18**	1.98**	4.96**	1.46**	1.22**	2.18**	1.86**
minimum	6.54	2.12	1.84	0.48	3.02	0.27	0.25	0.88	0.57
maximum	16.44	7.28	5.87	3.54	7.12	2.71	2.33	3.19	3.21
S.D.	3.45	2.02	1.47	1.18	1.71	0.90	0.74	0.89	1.07
CV (%)	32.12	45.66	46.33	59.52	34.45	61.85	60.43	41.05	57.51
%	100.00	41.10	29.55	18.41	46.14	13.53	11.30	20.23	17.26

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

Krockova et al. (2011) examined the effects of $NiCl_2$ on the testosterone secretion, cell viability and apoptosis in mouse Leydig cells *in vitro*. Their study demonstrated that $NiCl_2$ decreased the testosterone production already at a low dose (15.67 μM) and subsequently confirmed structural and functional alterations in the Leydig cells. Ng and Liu (1990) reported that $NiCl_2$ had no deleterious effects on cell viability and hormone-induced

steroidogenesis in the adrenal gland and Leydig cells when tested up to a concentration of 100 μM.

Conclusions

Data obtained from this *in vitro* study indicate that the release of sexual steroid hormones by adrenocortical carcinoma cells can be associated with the dose of heavy metals administration. Testosterone release seemed more vulnerable than progesterone to heavy metals exposure. Probably the effect of enzymatic action of 17β-hydroxysteroid dehydrogenase is more expressive, which results in decreased release of testosterone in comparison with progesterone and thereby the effect of enzymatic action of 3β-hydroxysteriod dehydrogenase. In conclusion, the present study suggests the endocrine disruptive and reproductive toxicological effects of these selected heavy metals.

Acknowledgments

The research leading to these results has received funding from the European Community under project no 26220220180: Building Research Centre „AgroBioTech".

References

1. Burton GV and Meikle AW 1980. Acute and chronic methyl mercury poisoning impairs rat adrenal and testicular function. J. Toxicol. Environ. Health. 6, 3, 597–606.
2. Das KK 2009. A comprehensive review on nickel (II) and chromium (VI) toxicities – possible antioxidant (*Allium Sativum Linn*) defences. Al. Ameen J. Med. Sci. 2, 43-50.
3. Das KK and Dasgupta S 2000. Effect of nickel on testicular nucleic acid concentrations of rats on protein restriction. Biol. Trace. Elem. Res. 73, 175-180.
4. Ding L, Murphy MB, He Y, Xu Y, Yeung LWY, Wang J, Zhou B, Lam PKS, Wu RSS and Giesy JP 2007. Effects of brominated flame retardants and brominated dioxins on steroidogenesis in H295R human adrenocortical carcinoma cell line. Environ. Toxicol. Chem. 26, 764-772.
5. Eisler R 1998. Nickel hazards to fish, wildlife, and invertebrates: A synoptic review. Biological Science Report USGS/BRD/BSR-1998-0001. Contaminant Hazard Reviews Report 34, 98 pp.
6. Forgacs Z, Szivos-Racz M and Ocztos G 2011. Study the effects of hormone disruptors on H295R cell line. XVIII. Primer Prevencios Forum, Budapest, May 19, 2011. Magyar Onkológia, 2011, 55, 3, 220–221, from http://huon.hu/2011/55/3/0213/0213a.pdf.
7. Gazdar AF, Oie HK, Shackleton CH, Chen TR, Triche TJ, Myers CE, Chrousos GP, Brennan MF, Stein CA and La Rocca RV 1990. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Research 50, 5488-5496.
8. Hilscherova K, Jones PD, Gracia T, Newsted JL, Zhang X, Sanderson JT, Yu RMK, Wu RSS and Giesy JP 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 1, 78–89.
9. Jolibois LSJr, Burow ME, Swan KF, George WJ, Anderson MB and Henson MC 1999. Effects of cadmium cell viability, trophoblastic development, and expression of low density lipoprotein receptor transcripts in cultured human placental cells. Reprod. Toxicol. 13, 473-480.

10. Kawai M, Swan KF, Green AE, Edwards DE, Anderson MB and Henson MC 2002. Placental endocrine disruption induced by cadmium: effects on P450 cholesterol side-chain cleavage and 3beta-hydroxysteroid dehydrogenase enzymes in cultured human trophoblasts. Biol. Reprod. 67, 178-183.

11. Knazicka Z, Lukac N, Forgacs Z, Tvrda E, Lukacova J, Slivkova J, Binkowski Ł and Massanyi P 2013. Effects of mercury on the steroidogenesis of human adrenocarcinoma (NCI-H295R) cell line. J. Environ. Sci. Health. Pt. A. 48, 348-353.

12. Krockova J, Massanyi P, Sirotkin AV, Pivko J, Makarevic AV, Lukac N, Capcarova M, Toman R and Polakova Z 2011. Nickel induced structural and functional alterations in mouse Leydig cells *in vitro*. J. Tr. Elem. Med. Biol. 25, 1, 15-18.

13. Lafuente A, Cano P and Esquifino AI 2003. Are cadmium effects on plasma gonadotropins, prolactin, ACTH, GH and TSH levels, dose-dependent? Biometals 16, 243–250.

14. Laskey JW and Phelps PV 1991. Effect of cadmium and other metal cations on *in vitro* Leydig cell testosterone production. Toxicol. Appl. Pharmacol. 108, 296-306.

15. Massanyi P, Bardos L and Toman R 2002. Effects of cadmium on the testicular structure and the level of retinoids and β-carotene in testis in mice after an experimental administration. Polish Journal of Natural Sciences 10, 133-139.

16. McVey MJ, Cooke GM, Curran IH, Chan HM, Kubow S, Lok E and Mehta R 2008. An investigation of the effects of methylmercury in rats fed different dietary fats and proteins: testicular steroidogenic enzymes and serum testosterone levels. Food Chem. Toxicol. 46, 270-279.

17. Mosmann T 1983. Rapid colorimetric assay for cellular growth and surrival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 1-2, 55–63.

18. Ng TB and Liu WK 1990. Toxic effect of heavy metals on cells isolated from the rat adrenal and testis. *In Vitro* Cell Dev. Biol. 26, 1, 24-28.

19. Ocztos G, Forgacs Z and Szivos-Racz M 2011. H295R Steroidogenesis Assay for study endocrine disruptors and reproductive toxicants. Fiatal Higi´enikusok VII. Fóruma, Esztergom, May 26–28, 2011, 55, 2, 132–133, from http://egeszsegtudomany.higienikus.hu/cikk/2011-2/2011-2.pdf.

20. Organization for Economic Cooperation and Development (OECD) 2011. H295R Steroidogenesis Assay, OECD Guideline for the Testing of Chemicals No. 456, Paris, 2011, from http://www.oecdilibrary.org/environment/test-no-456-h295r-steroidogenesis-assay 9789264122642-en.

21. Paksy K, Varga B and Lazar P 1992. Cadmium interferes with steroid biosynthesis in rat granulose and luteal cells *in vitro*. BioMetals 5, 245-250.

22. Paksy K, Rajczy K, Forgacs Z, Lazar P, Bernard A, Gati I and Kaali GS 1997. Effect of cadmium on morphology and steroidogenesis of cultured human ovarian granulosa cells. J. Appl. Toxicol. 17, 321-327.

23. Pandey R, Kumar R, Singh SP, Saxena DK and Srivastava SP 1999. Male reproductive effect of nickel sulphate in mice. Biometals 12, 339-346.

24. Przybyla AE, Robbins J, Menton N and Peck HD 1992. Structure-function relationship among the nickel-containing hydrogenases. Microbiol. Rev. 8, 109-135.

25. Rainey WE, Saner K and Schimmer BP 2004. Adrenocortical cell lines. Mol. Cell. Endocrinol. 228, 23-28.

26. Sanderson JT 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals: Review. Toxicol. Sci. 94, 3-21.

27. Schuurs AH 1998. Working with mercury: cause of infertility in women? Ned Tjidschr Tandheelkd 105, 11, 401-403.

28. Staels B, Hum DW and Miller WL 1993. Regulation of Steroidogenesis in Ncl-H295 Cells – a Cellular-Model of the Human Fetal Adrenal. Mol. Endocrinol. 7, 3, 423-433.

29. Takiguchi M and Yoshihara S 2006. New Aspects of Cadmium as Endocrine Disruptor. Environmental Sciences 13, 2, 107-116.

30. Tazisong I and Senwo Z 2009. Mercury concentration and distribution in soils impacted by long-term applied broiler litter. Bull. Environ. Contam. Toxicol. 83, 291-294.

31. Tchounwou PB, Ishaque AB and Schneider J 2001. Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2) exposed to cadmium chloride. Mol. Cell. Biochem. 222, 21-28.

32. Vachhrajani KD and Chowdhury AR 1990. Distribution of mercury and evaluation of testicular steroidogenesis in mercuric chloride and methylmercury administered rats. Ind. J. Exp. Biol. 28, 8, 746-751.

33. Veltman JC and Maines MD 1986. Alterations of heme, cytochrome P-450, and steroid metabolism by mercury in rat adrenal. Arch. Biochem. Biophys. 248, 2, 467-478.

34. Yokio K, Uthus EO and Nielsen FH 2003. Nickel deficiency diminishes sperm quantity and movement in rats. Biol. Trace. Elem. Res. 93, 2, 141-154.

35. Zhu X, Kusaka Y, Sato K and Zhang Q 2000. The endocrine disruptive effects of mercury. Environ. Health. Prev. Med. 4, 174-183.